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## WiChR, a highly potassium selective channelrhodopsin for low-light two-photon neuronal inhibition

Johannes Vierock, Enrico Peter, Christiane Grimm, Andrey Rozenberg, Alejandro G Castro Scalise, Sandra Augustin, Dimitrii Tanese, Benoît C Forget, Valentina Emiliani, Oded Bèjà, et al.

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6 **Title**

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8 **WiChR, a highly potassium selective channelrhodopsin for**  
9 **low-light two-photon neuronal inhibition.**

10 **Short Title:** Improved K<sup>+</sup>-conducting channelrhodopsins  
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47 **Abstract**

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The electric excitability of muscle, heart and brain tissue relies on the precise interplay of Na<sup>+</sup>- and K<sup>+</sup>-selective ion channels. The involved ion fluxes are controlled in optogenetic studies using light-gated channelrhodopsins (ChRs). While non-selective cation-conducting ChRs are well-established for excitation, K<sup>+</sup>-selective ChRs (KCRs) for efficient inhibition have only recently come into reach. Here, we report the molecular analysis of recently discovered KCRs from the stramenopile *Hyphochytrium catenoides* and identify a novel type of hydrophobic K<sup>+</sup>-selectivity filter. Next, we demonstrate that the KCR signature motif is conserved in related stramenopile ChRs. Among them, WiChR from *Wobblia lunata* features an unmatched 80-fold preference for K<sup>+</sup> over Na<sup>+</sup>, stable photocurrents under continuous illumination and a prolonged open state lifetime. Well expressed in neurons, WiChR allows two-photon inhibition at low irradiance and reduced tissue heating, recommending WiChR as the long-awaited efficient and versatile optogenetic inhibitor.

## 62 MAIN TEXT

63

### 64 Introduction

65

66 Light-gated ion channels are used in modern life sciences from plant physiology  
67 to systems neuroscience to control the electrical activity of cells with the temporal and  
68 spatial precision of light (1). Simultaneous illumination of individual cells within larger  
69 brain areas is reliably achieved by holographic light shaping and two photon (2P)  
70 excitation (2). The employed light switches are with few exceptions channelrhodopsins  
71 (ChRs) that absorb light via a retinal chromophore bound to the seven-transmembrane-  
72 helix protein as a retinal Schiff base. First described as the photoreceptor responsible  
73 for phototaxis in the green alga *Chlamydomonas reinhardtii* (3), ChRs have been  
74 discovered across different families of microalgae with more than 800 described  
75 representatives today (4, 5).

76 While cation-conducting channelrhodopsins (CCRs) are well established for  
77 excitation, neuronal inhibition is most frequently achieved with anion-conducting  
78 ChRs (ACRs). However, variability of internal  $\text{Cl}^-$  concentration, which is high in  
79 axonal and synaptic terminals, in cardiomyocytes and during development, may cause  
80 depolarization instead of hyperpolarization and limits the application of ACRs (6–8).  
81 Other inhibitory tools such as light-driven pumps and inhibitory  $\text{G}_{i/o}$ -coupled Opto-  
82 GPCRs also have significant limitations. Optogenetic pumps require high expression  
83 levels and continuous bright illumination due to a strict one photon/one charge ratio  
84 and can have undesired side effects (9). Opto-GPCRs require cell type-specific  
85 examination of engaged signaling cascades and might activate multiple signaling  
86 pathways with limited temporal on-off control (10).

87 An attractive alternative for optogenetic inhibition and a long-pursued tool is a  
88 potassium selective CCR that would hyperpolarize the neuronal membrane despite high  
89 extracellular  $\text{Na}^+$  concentration and mimic the major endogenous repolarization  
90 processes based on  $\text{K}^+$  efflux. Engineering a  $\text{K}^+$ -selective ChR is an ambitious goal  
91 considering major structural differences between ChRs and natural  $\text{K}^+$  channels.  
92 Whereas ChRs conduct protons and partly hydrated ions along a sequence of water  
93 filled cavities through an overall asymmetric and still structurally unknown pore within  
94 the rhodopsin monomer itself (11),  $\text{K}^+$  channels feature a highly conserved and  
95 symmetric selectivity filter with four subsequent  $\text{K}^+$  binding sites for  $\text{K}^+$  dehydration  
96 in the symmetry axis of the tetrameric channel (12). Accordingly, first-generation  $\text{K}^+$   
97 channels combined light sensitive modules such as UV-switchable azobenzene  
98 compounds, blue-light sensitive LOV-domains or blue-light activated cyclases with  
99 ligand-, viral and cAMP-gated  $\text{K}^+$  channels as single or two component systems (13–  
00 15). The need for an additional co-factor, low expression, slow kinetics, a limited  
01 dynamic range and putative side effects limited their application in neuroscience to  
02 date. Structure-guided molecular engineering of ChRs led only to minor improvements  
03 in the  $P_{\text{K}^+}/P_{\text{Na}^+}$  permeability ratio (11), whereas modifications of the extracellular  
04 release channel of the light-driven  $\text{Na}^+$ -pump KR2 of *Dokdonia eikasta* surprisingly  
05 generated an operational light-activated  $\text{K}^+$  channel with substantial potassium  
06 conductance, but only at alkaline pH (16).

07 A veritable breakthrough represents the recent discovery of two natural  
08 potassium-conducting channelrhodopsins (KCRs) in the stramenopile *Hyphochytrium*

09 *catenoides* (HcKCR1 and HcKCR2) that were K<sup>+</sup>-selective enough to inhibit action  
10 potential firing in neuronal slices (17). Both KCRs have a bacteriorhodopsin-like DTD  
11 motif in membrane helix 3, a feature shared with the related cryptophyte CCRs that  
12 were first discovered in *Guillardia theta* (18) and include the green-light activated non-  
13 selective cation channel ChRmine from *Rhodomonas lens*, the structure of which was  
14 recently solved by Cryo-EM (19, 20). Despite an extensive biophysical characterization  
15 by Govorunova and colleagues, the molecular determinants of K<sup>+</sup>-selectivity in these  
16 newly identified KCRs remain unknown.

17 Here, we inspect the potassium selectivity of both HcKCRs, quantify their  
18 remaining Na<sup>+</sup>-conductance and identify key residues for K<sup>+</sup>-conductance and selection  
19 over Na<sup>+</sup>. A search for new CCRs containing the identified K<sup>+</sup>-selectivity signature  
20 motif, revealed WiChR from *Wobblia lunata* with improved K<sup>+</sup>-selectivity, negligible  
21 photocurrent inactivation and improved light sensitivity compared to both previously  
22 characterized KCRs. Expressed in hippocampal brain slices, WiChR showed reduced  
23 depolarization at different membrane potentials and prolonged inhibition with  
24 minimally invasive illumination protocols. Highly K<sup>+</sup>-selective, well expressed in  
25 mammalian cells and sensitive to holographic two-photon excitation, we recommend  
26 WiChR as a new tool for multi-target single cell inhibition in large neuronal networks.

## 27 28 **Results**

### 29 30 **HcKCRs are K<sup>+</sup>-selective channels with substantial Na<sup>+</sup>-conductance**

31  
32 We expressed both HcKCRs in ND7/23 cells and compared photocurrents at  
33 different ionic conditions with those of ChRmine (Fig. 1B). In the presence of high  
34 intra- and extracellular K<sup>+</sup>, large photocurrents confirmed efficient potassium  
35 conductance for all three channels with even higher photocurrent densities for both  
36 HcKCRs than for ChRmine. Extracellular buffer exchange had no effect on  
37 photocurrents of ChRmine that conducts both cations equally well, but caused a  
38 significant reduction of inward currents and a strong shift in the reversal potential for  
39 both HcKCRs confirming their pronounced preference for K<sup>+</sup> over Na<sup>+</sup> (Fig. 1C-F).  
40 Further, exchange of Na<sup>+</sup> to the larger cation NMG<sup>+</sup> abolished all remaining inward  
41 currents in HcKCRs and shifted the reversal potential in all three channels indicating  
42 that in ChRmine, as well as in HcKCRs Na<sup>+</sup> ions are still conducted to a considerable  
43 extent. Moreover, at voltages close to  $E_{rev}$  extended illumination led to a directional  
44 change of the HcKCR1 photocurrents and to a transition from a K<sup>+</sup> outward current to  
45 a Na<sup>+</sup>-influx, revealing an increasing Na<sup>+</sup>-selectivity over time. The concomitant  $E_{rev}$   
46 shifted by about 10 mV and reflects a permeability ratio ( $P_{K^+}/P_{Na^+}$ ) drop from  $29 \pm 4$   
47 to  $18 \pm 2$  (Fig. S1). At the same time, HcKCR1 photocurrents inactivate by 30 to 60 %  
48 depending on the voltage and ionic conditions and recover biphasically within seconds  
49 in the dark (Fig. S2).

### 50 51 **K<sup>+</sup>-selectivity of HcKCRs relies on aromatic residues in the extracellular half pore**

52  
53 In the next set of experiments, we identified key amino acids relevant for  
54 efficient K<sup>+</sup>-conductance and selectivity. All known ChRs conduct ions along a  
55 sequence of preformed water-filled cavities between transmembrane helices 1, 2, 3 and

56 7 (11). The putative HcKCR1 pore is spanning from the extracellular D87 in  
57 transmembrane helix 2 to the intracellular D116 in helix 3 (Fig. 2A). The aspartates  
58 D105 and D229 in the center of the pore constitute the counterion complex that  
59 stabilizes the protonated retinal Schiff base charge. The extracellular half pore is lined  
60 by a set of aromatic residues including Y81, F88, W210, Y222 and an unusual  
61 tryptophan W102 at a position most frequently occupied by arginine in other microbial  
62 rhodopsins.

63 Mutations of most of the pore-lining residues had little impact on the K<sup>+</sup>-  
64 conductance and selectivity (Fig. S3+S4). Accordingly, photocurrents of the central  
65 gate mutant C77A still resembled WT currents (Fig. 2B-C), whereas mutations of F81,  
66 F88 or S70 that are located in close contact with the pore aspartates caused strong  
67 reduction of outward currents and impaired K<sup>+</sup>-selection as indicated by the diminished  
68  $\Delta E_{rev}$ . A nearly complete loss of K<sup>+</sup>-selectivity was observed for the outer pore mutant  
69 N99L and for substitutions of W102 and Y222 by non-aromatic residues such as  
70 W102Q and Y222A. For all three mutants, inward currents were large at both high  
71 extracellular Na<sup>+</sup> or K<sup>+</sup> (Fig. 2C) with little reversal potential shift between the two  
72 conditions (Fig. 2D), similar to the non-selective cation channel ChRmine (Fig. 1C+F).  
73 K<sup>+</sup>-selectivity was also compromised in D87 and D116 mutants but came with a near-  
74 complete loss of stationary photocurrents (Fig. 2E). Mutations of the counterion  
75 complex also showed reduced photocurrent amplitudes but, by contrast, largely  
76 preserved K<sup>+</sup>-selectivity. In conclusion, all four aspartates of the pore and their  
77 interaction partners S70 and Y81 are essential for channel gating and proper pore  
78 formation in KCRs, whereas W102 and Y222 constitute essential elements of a unique  
79 hydrophobic K<sup>+</sup>-selectivity filter.

80 Based on our mutational analysis, we define the quintet D87, N99, W102, D116  
81 and Y222 as the KCR signature motif that requires functional support by S70, F88 and  
82 D105 as important interaction partners for efficient K<sup>+</sup>-conductance. (Fig. 3A).

## 84 Identification of new candidate KCRs

85  
86 A systematic search revealed that KCRs from *H. catenoides* (HcKCR1 and  
87 HcKCR2) are part of a bigger clade that unites ChRs from a small set of stramenopile  
88 protists and related metatranscriptomic sequences (Fig. 3B, Fig. S6 and S7A). The four  
89 non-hyphochytridiomycete stramenopiles that encode members of this ChR group are  
90 heterotrophic flagellates from the clade of Opalozoa: placidids *Wobblia lunata* and  
91 *Placidida* sp. Caron Lab Isolate and anoecids *Cafeteria burkhardae* and *Bilabrum* sp.  
92 (the latter identified using molecular markers). Together with cryptophyte (B)CCRs  
93 (including ChRmine) and a novel clade of uncharacterized ChRs from colpodellid  
94 alveolates these proteins form a well-supported monophylum (Fig. 3B, Fig. S6A). Most  
95 stramenopile CCRs share the functionally important DTD motif of bacteriorhodopsin  
96 (D85, T89, D96) in transmembrane helix 3 with cryptophyte BCCRs – and all of the  
97 minor variations of the motif (DSD, DSE, ETD and DLD) preserve both of the  
98 carboxylates - whereas in related colpodellid proteins only the second aspartate is  
99 conserved (GGD motif) (Fig. S6B). Clustering of the stramenopile CCRs and  
00 cryptophyte (B)CCRs indicates that cation conductivity is ancestral in this lineage.

01 Among the collected stramenopile ChRs from this clade, two proteins, W1ChR1  
02 from *W. lunata* and B1ChR2 from *Bilabrum* sp. contained a nearly complete KCR



signature motif with only one conservative substitution at the position of Y222 for a phenylalanine - a substitution that preserved K<sup>+</sup>-selectivity in HcKCR1 in our mutational analysis (Fig. S4).

## Electrophysiological characterization of WlChR1 (WiChR) in cultured cells

Expressed in ND7/23 cells, WlChR1 showed excellent membrane targeting (Fig. 3C) and large photocurrents with almost no inactivation during prolonged illumination (Fig. 3D). In contrast, B1ChR2 aggregated substantially in intracellular compartments and produced photocurrents with rapid inactivation and strong inward rectification. Photocurrent recordings at different K<sup>+</sup><sub>e</sub> concentrations confirmed a high K<sup>+</sup>-selectivity for both channels. Strikingly, WlChR1 as well as B1ChR2 even showed a complete loss of inward directed currents upon exchange of extracellular K<sup>+</sup> to Na<sup>+</sup> (Fig. 3E +F). The corresponding larger  $\Delta E_{rev}$  for WlChR1 compared to HcKCR1 (Fig. 3G) is interpreted on the basis of the Goldman-Hodgkin-Katz voltage equation as a remarkable increase in relative K<sup>+</sup>-conductance from  $P_{K^+}/P_{Na^+} = 20 \pm 4$  for HcKCR1 to  $P_{K^+}/P_{Na^+} = 80 \pm 40$  for WlChR1. Improved K<sup>+</sup>-selectivity is accompanied by enlarged stationary photocurrents (Fig. 3H) - explained by the good expression and reduced photocurrent inactivation - and slow channel closure (Fig. 3I) that both result in an improved operational light sensitivity (Fig. 3J). The blue-shifted action spectrum compared to HcKCR1 favors combination with orange or red absorbing actuators and sensors and largely overlaps with HcKCR2 (Fig. 3K).

Considering WlChR1 as a highly promising tool for optogenetic inhibition, we term it WiChR for *Wobblia* inhibitory *Channel*Rhodopsin.

## 2P holographic inhibition of neurons in organotypic slices

To explore neuronal application, we expressed HcKCR1 and WiChR in organotypic slices from the mouse hippocampus and compared their inhibitory performance using holographic 2-photon patterned stimulation and a low repetition rate pulsed laser at 1030 nm (Fig. 4A). Both KCRs expressed well (Fig. 4B) and showed no signs of toxicity (Fig. S12). We injected depolarizing square currents (1 s) to induce action potential firing and illuminated in the middle of the current injection. Both HcKCR1 and WiChR completely inhibited spiking at current injections well above the respective rheobase of the neuron (Fig. 4B). While for HcKCR1 continuous illumination was needed, for WiChR a single 5 ms pulse was sufficient to achieve complete cessation of firing for the entire 500 ms (Fig. 4C). Since extended periods of inhibition are often needed during in vivo applications, pulsed illumination can reduce the local heating in the tissue compared to continuous illumination. Hence, we compared inhibition with continuous illumination to the application of 5 ms pulses at 10, 5 and 2 Hz and found that WiChR achieves complete cessation of action potential firing down to a frequency of 2 Hz while HcKCR1 needs continuous illumination for the same degree of inhibition (Fig. S13A,B). Next, we simulated the local temperature rise ( $2I$ ) for the holographic spot (12  $\mu\text{m}$  diameter, 31  $\mu\text{W}/\mu\text{m}^2$ ) and compared continuous with pulsed illumination. We found that heat is accumulating with continuous illumination, but the pulsed protocol at 10, 5 or 2 Hz allows dissipation of the heat before the next pulse (Fig. S13C). While for one holographic spot the local

50 temperature rise of 0.23 K (continuous) and 0.14 K (pulsed) is still tolerable, most  
51 applications will demand inhibition of more than one neuron in a given region. Here,  
52 our simulations show that with a 5 ms illumination pulsed at 2 Hz, peak heating stays  
53 below 1 K and no heat is accumulated over time even when placing up to 40  
54 holographic spots within a small 100 x 100  $\mu\text{m}^2$  area. On the contrary, for continuous  
55 illumination, heat is accumulated and increases linearly with the number of spots  
56 reaching almost 4 K for the same 40 spots (Fig. S13D).

57 When we injected hyperpolarizing current steps to have more negative  
58 membrane potentials, we found that illumination caused depolarization instead of  
59 further hyperpolarization for both HcKCR1 and WiChR (Fig. 4D), explained by their  
60 residual  $\text{Na}^+$ -conductance. However, reversion from depolarization to  
61 hyperpolarization occurred at a significantly more negative membrane potential of -  
62  $92 \pm 2$  mV for WiChR than for HcKCR1 ( $-80 \pm 4$  mV) (Fig. 4E, right) confirming the  
63 higher  $\text{K}^+$ -of WiChR.

64 Lastly, we used a somatic current ramp injection and increased illumination to  
65 compare the operational power densities at 1030 nm needed for effective inhibition  
66 with HcKCR1 and WiChR. (Fig. 4C) For both KCRs increasing power densities  
67 delayed the first action potential on the ramp compared to darkness, but half-maximal  
68 inhibition was observed at six times lower power densities for WiChR than for  
69 HcKCR1 (Fig. 4F,G, right). For HcKCR1 complete suppression of spiking on the ramp  
70 was achieved at  $43 \mu\text{W}/\mu\text{m}^2$  while for WiChR, this was reached for all patched neurons  
71 already at  $4.4 \mu\text{W}/\mu\text{m}^2$ .

## 72 Discussion

73 Potassium-selective channelrhodopsins are a new functional group of light-  
74 gated ion channels. In this study, we investigate their molecular mechanism and  
75 identify the amino acid motif that is required for their high potassium selectivity.  
76 Exploiting that knowledge, we uncovered two new potassium-conducting ChRs with  
77 further improved  $\text{K}^+$ -selectivity, and in the case of WiChR with excellent inhibitory  
78 performance in neurons.

79 We show that potassium selectivity of KCRs relies on an unusual pair of  
80 aromatic residues in helix 3 and 7 that occludes the extracellular pore in the dark and  
81 constitutes the  $\text{K}^+$ -selectivity filter in the open channel (W102 and Y222 in HcKCR1  
82 highlighted in Fig 5A and Fig. S10), which is unique among rhodopsins. Additionally,  
83 six further residues are required for efficient  $\text{K}^+$ -conductance. Among them, three pore-  
84 lining carboxylates (D87, D105 and D116 in HcKCR1) found at similar positions in  
85 ChRmine (20) are expected to be generally involved in channel gating, similarly as  
86 earlier discussed for the related GtCCR2 (22). Finally, three side chains of putative  
87 secondary importance (S73, F88 and L99 in HcKCR1) ensure conformational integrity  
88 of the pore and orientation and hydrophobicity of the  $\text{K}^+$ -selectivity filter.

89 Our findings stand in contrast to the highly conserved mechanism of ion  
90 conduction in conventional highly symmetric tetrameric  $\text{K}^+$ -channels, like KcsA or Kv,  
91 where  $\text{K}^+$ -ions are conducted along the tetramer interface tightly coordinated by  
92 backbone carbonyl oxygens of the selectivity filter (23). However, for KCRs, our MD  
93 simulation and mutational analysis predict a trimeric assembly with an inter-subunit  
94 cavity most likely filled by lipids similar to ChRmine (19) and an ion permeation  
95  
96



97 pathway going through the individual KCR protomers themselves. We expect that here,  
98 the passage of potassium ions might require dehydration in order to pass the  
99 hydrophobic and aromatic side chains of the K<sup>+</sup>-selectivity where Na<sup>+</sup> with higher  
00 dehydration energy and more localized charge is blocked. In the related ChRmine, the  
01 wider and presumably water-filled access funnel could allow permeation of partly  
02 hydrated K<sup>+</sup> and Na<sup>+</sup> (Fig. 5). A similar non-canonical mechanism for K<sup>+</sup>-selectivity  
03 was recently described for the lysosomal K<sup>+</sup>-channel TMEM175, that also features a  
04 hydrophobic restriction in the center of the pore as required for K<sup>+</sup>-conductance, with  
05 important differences in K<sup>+</sup>-selectivity for different channel subtypes ranging from  
06  $P_{K^+}/P_{Na^+}$  of 2 in bacteria to  $P_{K^+}/P_{Na^+}$  of 35 in humans (24). Accordingly,  $P_{K^+}/P_{Na^+}$  also  
07 varied significantly for different KCRs despite an overall conserved signature motif  
08 and we observe diverse conformations of the key residues along the pore in the different  
09 channels (Fig. S10).

10 Now, with the ability to control K<sup>+</sup> flux in neuronal optogenetic experiments,  
11 KCRs will enable inhibition of excitable cells under yet inaccessible conditions and  
12 extend the possibilities beyond previously used ACRs. While effective neuronal  
13 inhibition is possible with HcKCR1, the newly identified WiChR, will outperform  
14 HcKCRs in several applications based on three superior properties. First the K<sup>+</sup>-  
15 selectivity over Na<sup>+</sup> is more than three times higher for dark-adapted WiChR and even  
16 increases further over light-adapted HcKCR1 due to the low inactivation of WiChR.  
17 The increased K<sup>+</sup>-selectivity translates to neuronal application in our experiments and  
18 will allow effective inhibition of neurons over a broader range of resting potentials and  
19 less dependent on the Na<sup>+</sup>-gradient. Second, the high operational light sensitivity of  
20 WiChR allowed inhibition at an order of magnitude lower light doses compared to  
21 HcKCR1. On the one hand this will significantly facilitate light delivery for *in-vivo* 2P  
22 experiments and increase the accessible depth of neurons to inhibit, while on the other  
23 hand it allows the distribution of available laser power over a larger number of target  
24 cells and simultaneous inhibition of many cells. Third, the longer open state lifetime of  
25 WiChR is fundamental to the pulsed illumination protocol that we extensively  
26 investigated here and recommend for *in-vivo* application. Continuous illumination, as  
27 necessary for HcKCRs, will cause significant temperature rise in the tissue, especially  
28 considering the extended time frames and the number of cells to inhibit that can be  
29 needed for behavior experiments. On the contrary, the pulsed illumination can be  
30 applied indefinitely and for many target cells without any heat accumulation in the  
31 tissue presenting a tremendous advantage for *in-vivo* application. Lastly, we  
32 demonstrated a high activation of WiChR with targeted 2P holographic stimulation  
33 adding deeper tissue penetration, lateral single cell resolution and increased axial  
34 resolution over widefield 1P illumination, which likewise present considerable  
35 advantages for application.

36 Based on our results, we recommend WiChR as the tool of choice for targeted  
37 holographic 2P inhibition and simultaneous silencing of tens to hundreds of cells with  
38 low light intensities, short illumination times, in depth and with minimal photodamage.  
39

## 40 **Materials and Methods**

### 41 **Search for ChR genes**

42 ChR genes from the stramenopile CCR subfamily: CbuChR1 (NCBI Genbank  
43 KAA0157615.1, NCBI genome assembly GCA\_008330645.1) from *Cafeteria*  
44 *burkhardae* BVI (formerly, *Cafeteria roenbergensis* BVI), P1ChR1  
45 (MMETSP1104\_DN12643\_c6\_g1\_i1, MMETSP assembly MMETSP1104) from  
46 *Placidida* sp. Caron Lab Isolate (formerly, “*Cafeteria*” sp.) and partial sequences of  
47 B1ChR1 and B1ChR2 from *Bilabrum* sp. (found as contamination in various algal  
48 transcriptome assemblies, in particular *Chroodactylon ornatum*, see Supplementary  
49 Text) were previously reported in (4). ChR genes of *Wobblia lunata* NIES-1015  
50 (W1ChR1-4) were obtained by assembling the raw data available from NCBI SRA  
51 (SRA run DRR049555, (25)) with Trinity v. 2.13.2 (26). Search for ChR genes in these  
52 and other assemblies was performed by querying protein sequences using microbial  
53 rhodopsin HMM profiles (<https://github.com/BejaLab/RhodopsinProfiles>) with  
54 hmmsearch from HMMER v. 3.3.2 (27). ChR sequences were obtained by searching  
55 against a curated database of microbial rhodopsins using blastp from blast+ v. 2.11.0+  
56 (28). The complete list of searched stramenopile assemblies is provided in Suppl. Data  
57 S1. De novo assemblies when necessary were obtained with Trinity for transcriptomes  
58 or spades v. 3.14.1 (29) for genomes and single amplified genomes (SAGs). Genes  
59 were predicted with TransDecoder v. 5.5.0 for transcriptome assemblies and  
60 GeneMarkS v. 4.32 for genome and SAG assemblies. Additional sequences related to  
61 stramenopile CCRs were obtained from JGI freshwater and marine metatranscriptomes  
62 using blast with stramenopile CCRs as queries. Only publicly available complete  
63 sequences from JGI were used in the analyses.

64 A curated catalog of ChRs including sequences reported here is maintained at  
65 (30).

### 66 **Phylogenetic analysis**

67 Phylogeny of the stramenopile CCRs, related clades and a reference set of ChRs  
68 from other families was obtained as follows. The sequences were aligned using mafft  
69 v. 7.475 (31) in --localpair mode, trimmed with trimAl v. 1.4.1 (32) (with -gt 0.9) and  
70 the phylogeny was reconstructed with iqtree2 v. 2.1.2 (33) with 1000 ultrafast bootstrap  
71 replicates (34). The tree was outgroup-rooted.

### 72 **Structure-based alignment**

73 Representative CCRs were aligned with 3D-coffee from t\_coffee  
74 v.13.45.0.4846264 (methods sap\_pair, mustang\_pair, t\_coffee\_msa and  
75 probcons\_msa) (35). Transmembrane regions in ChRmine (PDB: 7W9W) were  
76 predicted with the PPM web server (36).

### 78 **Molecular Biology and Cell Culture**

79 Coding sequences of HcKCR1 (MZ826862), HcKCR2 (MZ826861), B1ChR2  
80 and W1ChR1 (synthesized by GeneScript) and ChRmine (gently provided by K.  
81 Deisseroth) were fused to a Kir2.1 membrane targeting sequence, a mScarlet,  
82 mCerulean or eYFP fluorophore and an ER-Export signal and cloned either into a

83 pCDNA3.1 vector behind a CMV-promotor for basic characterization in ND7/23 cells  
84 or into a pAAV backbone behind a human Synapsin promotor for virus production and  
85 expression in neurons. Site-directed mutagenesis was performed using a QuickChange  
86 Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA), according to  
87 the manufacturer's instructions. Expression in ND7/23 cells was performed as  
88 previously described (37). ND7/23 cells were cultured at 5% CO<sub>2</sub> and 37 °C in  
89 Dulbecco's minimal essential medium supplemented with 5% fetal bovine serum, 1  
90 μM all-*trans* retinal and 100 μg/ml penicillin/streptomycin (Biochrom, Berlin,  
91 Germany). Cells were seeded on poly-lysine coated coverslips at a concentration of 0.75  
92 × 10<sup>5</sup> cells/ml and transiently transfected using the FuGENE® HD Transfection  
93 Reagent (Promega, Madison, WI) 28–48 h before measurement.

#### 94 **Whole-Cell patch clamp in ND7/23 cells**

95 Patch pipettes were prepared from borosilicate glass capillaries (G150F-3;  
96 Warner Instruments, Hamden, CT) using a P-1000 micropipette puller (Sutter  
97 Instruments, Novato, CA) and were subsequently fire-polished. Pipette resistance was  
98 1.5 to 2.5 MΩ. A 140 mM NaCl agar bridge served as reference (bath) electrode. In  
99 whole-cell recordings, membrane resistance was typically > 1 GΩ, while access  
00 resistance was below 10 MΩ. Pipette capacity, series resistance, and cell capacity  
01 compensation were applied. All experiments were carried out at 23 °C. Signals were  
02 amplified and filtered at 2 kHz, digitized at 10 kHz (DigiData1400) and acquired using  
03 Clampex 10.4 Software (all from Molecular Devices, Sunnyvale, CA). Continuous  
04 monochromatic light was generated using a Polychrome V light source (TILL  
05 Photonics, Planegg, Germany) coupled into the Axiovert 100 microscope (Carl Zeiss)  
06 and delivered to the sample using a 90/10 beamsplitter (Chroma, Bellows Falls, VT).  
07 Light exposure was controlled by a VS25 and VCM-D1 shutter systems (Vincent  
08 Associates, Rochester, NY). Light intensities were adjusted either manually by  
09 different neutral density filters or by a motorized Filter-wheel for equal photon densities  
10 in action spectra. Light intensities were measured in the sample plane with a calibrated  
11 P9710 optometer (Gigahertz Optik, Türkenfeld, Germany) with 3.7 mW/mm<sup>2</sup> at 470  
12 nm and 2.7 mW/mm<sup>2</sup> at 530 nm for standard measurements. The illuminated field of  
13 the W Plan-Apochromat 40x/1.0 DIC objective was 0.066 mm<sup>2</sup> (Carl Zeiss, Jena,  
14 Germany).

15 Extracellular buffer exchange was performed manually by adding at least 5 mL  
16 of the respective buffer to the recording chamber (500 μL chamber volume) while a  
17 Ringer Bath Handler MPCU (Lorenz Messgerätebau, Katlenburg-Lindau, Germany)  
18 maintained a constant bath level. Standard bath solutions contained 110 mM NaCl,  
19 1 mM KCl, 2 mM CaCl<sub>2</sub>, 2 mM MgCl<sub>2</sub>, and 10 mM HEPES at pH<sub>e</sub> 7.2 (with glucose  
20 added up to 310 mOsm). Standard pipette solutions contained 110 mM Potassium-D-  
21 gluconate, 1 mM NaCl, 2 mM CaCl<sub>2</sub>, 2 mM MgCl<sub>2</sub>, 10 mM EGTA, and 10 mM  
22 HEPES at pH<sub>i</sub> 7.2 (glucose was added up to 290 mOsm). For ion selectivity  
23 measurements, extracellular NaCl was replaced by 110 mM KCl or 110 mM NMDGCl.

24 I(E)-curves were measured from -80 mV to +40 mV in 20 mV steps with liquid  
25 junction potential (LJP) corrected holding voltages and 500 ms illumination. Action  
26 spectra were recorded with 110 mM Na<sup>+</sup>, 10 ms illumination and reduced light  
27 intensities at either 0 mV for K<sup>+</sup>-selective channels and mutants or -60 mV for HcKCR1

28 mutants that lost K<sup>+</sup>-selectivity. Light titrations of KCR channels were recorded at 0  
29 mV with 500 ms illumination.

30 ND7/23 measurements were analyzed using the Clampfit 10.7 software  
31 (Molecular Devices, Sunnyvale, CA), Microsoft Excel and Origin 2020® (OriginLab,  
32 Northampton, MA). Photocurrent traces were baseline corrected, filtered, and reduced  
33 in size for display purposes. Photocurrents were normalized to peak photocurrents at -  
34 40 mV and 110 mM K<sup>+</sup><sub>e</sub> and action spectra were fitted using a parametric Weibull  
35 function:

$$36 \quad y = y_0 + A \cdot \left( \frac{w_2 - 1}{w_2} \right)^{\left( \frac{1-w_2}{w_2} \right)} \cdot S^{(w_2-1)} \cdot e^{\left( -S^{w_2 + \frac{w_2-1}{w_2}} \right)}$$

$$37 \quad \text{with } S = \frac{x - x_c}{w_1} + \left( \frac{w_2 - 1}{w_2} \right)^{\left( \frac{1}{w_2} \right)}$$

38 and the estimated parameters A, y<sub>0</sub>, w<sub>1</sub>, w<sub>2</sub>, x<sub>c</sub>.

39

### 40 **Confocal microscopy in ND7/23 cells**

41 Confocal images were acquired using a FluoView1000 (Olympus, Tokyo,  
42 Japan) confocal laser scanning microscope, with a UPlanSApo 60x NA 1.2 water-  
43 immersion objective (Olympus, Tokyo, Japan). mScarlet was excited with a 559 nm  
44 DPSS laser (2% transmissivity), mCerulean with a 440 nm diode laser (10%  
45 transmissivity), and eYFP was excited with an argon laser (3% transmissivity).  
46

47 Both the membrane fluorescent density (brightness/area) and the fluorescent  
48 density within the cell were calculated from equatorial slices after background  
49 subtraction. The membrane was defined as the outermost layer of the cell, and the  
50 targeting was then evaluated as the fluorescence membrane density in the membrane  
51 divided by the intracellular fluorescence density. This calculation yields a value below  
52 one if the fluorescence is higher inside the cell, and above one if the membrane is  
53 brighter.

### 54 **Preparation and electroporation of organotypic hippocampal slices**

55 All animal procedures were performed in accordance with French law and  
56 guidelines of Directive 2010/63/EU and institutional guidelines on care and use of  
57 laboratory animals. Organotypic hippocampal slices were prepared from P5/P8  
58 C56BL/6J mouse pups as described in Gee et al. – 2017 (38). In brief, dissection of  
59 hippocampi was carried out in ice cold dissection solution composed of : 248 mM  
60 sucrose, 26 mM NaHCO<sub>3</sub>, 10 mM glucose, 4 mM KCl, 5 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, 2  
61 mM kynurenic acid and 0.001% phenol red saturated with 95% O<sub>2</sub>/5% CO<sub>2</sub>. 400 μm  
62 thick transverse sections of hippocampi were sliced with a McIlwain Tissue Chopper  
63 using double edge stainless steel razor blades. Morphologically nice slices were  
64 carefully transferred using a plastic transfer pipette onto small pieces of PTFE  
65 membrane (Millipore FHLP04700) on membrane inserts (Millicell PICMORG50) in  
66 pre-warmed culture medium. The slices were cultured at 37°C and 5% CO<sub>2</sub> in culture

67 medium consisting of 80% MEM and 20% Heat-inactivated horse serum supplemented  
68 with 1 mM L-glutamine, 0.01 mg/ml Insulin, 0.00125% Ascorbic acid D-glucose, 14.5  
69 mM NaCl, 2 mM MgSO<sub>4</sub>, and 1.44 mM CaCl<sub>2</sub>; no antibiotics were added to the culture  
70 medium. Three to four slices were cultured on one insert and every 3 days the medium  
71 was partially exchanged with pre-warmed fresh medium.

72 Bulk electroporations were performed with endotoxin-free plasmid  
73 preparations (>0.9 µg/µl) using the Y-unit of the 4D-Nucleofector® (Lonza  
74 Bioscience), both HcKCR1 and WiChR were subcloned into a pAAV-backbone and  
75 expressed as fusion proteins tagged with a fluorophore under a human syn1 promoter.  
76 At DIV3-4, the hippocampal slice was placed in a well of a 24-well culture plate (Nunc)  
77 and 10 µg plasmid DNA in a volume of 25-30 µL of AD1 Solution (Lonza Bioscience)  
78 was put as a drop on the slice. After an incubation period of 20 minutes at 37 °C, pre-  
79 warmed AD1 solution was added to a final volume of 360 µL. The 24-well Dipping  
80 Electrode Array (Lonza Bioscience) was carefully placed onto the 24-well culture plate  
81 avoiding air bubbles, which would disrupt the electroporation, immediately followed  
82 by the electroporation using program CM-158 of the 4D-Nucleofector® Y Unit. The  
83 slices were used for electrophysiology recordings 6-10 days after electroporation.

#### 84 **Holographic microscope for two-photon excitation**

85 The system, capable of holographic light shaping (2), is schematically  
86 represented in Fig. S11A. It was custom-built around a commercial upright microscope  
87 (Zeiss, Axio Examiner.Z1). An efficient holographic stimulation was based on the use  
88 of a low repetition rate amplified fiber laser, providing ~300 fs pulses at 500 kHz rate  
89 with a 1030 nm wavelength (Satsuma, Amplitude Lasers, total output power = 10 W).  
90 The laser beam, after a first beam expander (f<sub>1</sub> = 50 mm; f<sub>2</sub> = 200 mm), was directed  
91 on a spatial Light Modulator (SLM) (LCOS-SLM X13138-07, Hamamatsu Photonics,  
92 1280 × 1024 pixels, 12.5 µm pixel size). Then, a couple of 4f system (f<sub>1</sub>= 750 mm and  
93 f<sub>2</sub> = 250 mm; f<sub>3</sub> = 50 mm and f<sub>4</sub> = 250 mm), projected the SLM plane to the pupil of  
94 a 20x 1.0 NA objective (Zeiss, W Plan-Apochromat 20x/1.0 DIC ). The SLM was  
95 controlled by custom-made C++ based software (39) that uses a variation of the  
96 Gerchberg and Saxton algorithm (40) in order to calculate a phase-hologram to  
97 modulate the laser beam and to generate an arbitrary illumination shape at the sample  
98 plane. For single neuron excitation, a 12 µm-diameter circular spot was generated. A  
99 physical blocker was placed between the two lenses of the first 4-f system to avoid the  
00 zero-order of diffraction reaching the sample. For alignment and calibration,  
01 holographic patterns were projected on a thin spin-coated rhodamine fluorescent layer  
02 and the induced fluorescence was visualized on a CMOS camera (Hamamatsu ORCA-  
03 5G). To reconstruct a full 3D profile of the illumination spot (as reported in Fig. S11),  
04 a second inverted microscope was built in transmission geometry and different axial  
05 sections of the spot were imaged on a second bottom camera while translating axially  
06 the upper objective (as described in (40)). A fast shuttering and power control of  
07 illumination pulses were achieved using a built-in acousto-optic modulator that was  
08 controlled by the Digitizer (DigiData 1440, Molecular Devices) to ensure  
09 synchronization with the electrophysiology recordings. The microscope was  
10 additionally capable of widefield fluorescence imaging based on a multi-led  
11 illumination system (pE-4000, CoolLed) and a DIC transmitted imaging.



12 For whole-cell patch-clamp recordings the microscope is equipped with a  
13 micromanipulator (Junior XR, Luigs and Neuman); signals were amplified and  
14 digitized using an AxoPatch 700B (Molecular Devices) and a DigiData 1440A  
15 (Molecular Devices) while data was acquired using Clampex (Molecular Devices).

## 16 **2-Photon electrophysiology recordings in organotypic slices.**

17 Recordings were performed 6-9 days after electroporation with transfected cells  
18 identified by their fluorescence. Patch pipettes were pulled from fire-polished  
19 capillaries with filament (OD 1.5 mm/ ID 0.86 mm, World Precision Instruments) using  
20 a micropipette puller (P1000, Sutter Instruments). Pipettes with a resistance in a range  
21 from 3-6 M $\Omega$  were filled with a K-gluconate based intracellular solution (in mM): 135  
22 K-Gluc, 10 HEPES, 10 Na-Phosphocreatin, 4 KCl, 4 Mg-ATP, 0.3 Na<sub>2</sub>-GTP; 290  
23 mOsm. Extracellular solution was composed of (in mM): 125 NaCl, 26 NaHCO<sub>3</sub>, 1.25  
24 NaH<sub>2</sub>PO<sub>4</sub>, 25 D-Glucose, 2.5 KCl, 1.5 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 0.5 ascorbic acid (310 mOsm),  
25 continuously bubbled with carbogen (pH 7.4) and perfused at a rate of 1.5-2.5 ml/min  
26 (room temperature). The liquid junction potential was calculated to be -17.7 mV and  
27 corrected post acquisition for all recordings; the Nernst potential for potassium is -103  
28 mV while that for sodium is +48 mV in these conditions.

29 After establishing the whole-cell configuration in voltage clamp mode, quality  
30 of the patch was checked and access resistance was continuously monitored during and  
31 after the recording (voltage clamp); if access resistance rose above 45 M $\Omega$  recordings  
32 were discarded. After switching to current clamp mode bridge balance compensation  
33 was performed with the internal circuits of the amplifier and neuronal parameters like  
34 resting potential, input resistance and rheobase were determined (Fig. S12B). During  
35 the recording constant holding currents (between -50 pA and +50 pA) were injected (if  
36 necessary) to keep the neurons at a comparable membrane potential between -75 mV  
37 and -80 mV.

38 For the 2-photon stimulation, a circular holographic spot of  $\approx 12$   $\mu\text{m}$  diameter (  
39 Fig. S12B) was generated aiming at the cell body of the patched neuron. Stimulation  
40 light powers were measured every recording day out of the microscope objective  
41 (PM160 power meter, Thorlabs), fitted within the range of interest and used to calculate  
42 the exact photostimulation intensity; a typical average spot size of 126  $\mu\text{m}^2$  was  
43 determined from multiple images of the spot fluorescence on a thin rhodamine layer  
44 and used to calculate the power densities reported in the respective figures.

45 Square current injections of 1 s duration were increased in 10 pA steps per  
46 sweep starting with hyperpolarizing injections, with a 5 s pause between sweeps. The  
47 duration of the current injection was divided into the first 250 ms (before illumination),  
48 500 ms (during illumination) and 250 ms (after illumination) for analysis. Illumination  
49 was either continuous for the whole 500 ms or a sequence of 5 ms pulses at different  
50 frequencies (30-35  $\mu\text{W}/\mu\text{m}^2$ ). The firing rates were determined during these intervals  
51 and normalized to the firing rate during the first 250 ms. The firing rates were extracted  
52 for each neuron at current injections 50 pA and 100 pA above the respective rheobase  
53 of the neuron; with the rheobase determined as the current injection were the first spike  
54 in the first or last 250 ms (no illumination) occurred. After extraction the normalized  
55 firing rates were averaged and plotted as mean  $\pm$  SD. Control recordings were  
56 performed and analyzed in the same way, but without any light application in the



57 middle 500 ms.

58 For recording the reversal from light-induced hyperpolarization to  
59 depolarization, square current injections were increased in 10 pA steps around the  
60 reversal and after 250 ms continuous illumination at saturating intensities (30-35  
61  $\mu\text{W}/\mu\text{m}^2$ ) was switched on. Light-induced  $\Delta V_{\text{mem}}$  was extracted as the difference  
62 between  $V_{\text{mem}}$  before illumination and at 150 ms into the illumination; the value for the  
63 reversal was extracted by linear interpolation on the individual recordings and then  
64 averaged over all cells.

65 For the comparison of the effectiveness of inhibition somatic ramp current  
66 injections (800 ms) with continuous illumination of rising power densities were applied  
67 (5 s waiting between sweeps). The amplitudes of the ramp were constant for each  
68 individual neuron and chosen to give 10-20 action potentials in the dark respectively;  
69 between 75 pA to 500 pA. The timing of the action potential was extracted at the peak  
70 amplitude of the spike and the delay was calculated with respect to the first action  
71 potential on the ramp injection without illumination. Afterwards, the delay was  
72 normalized between no delay (0) and maximum delay (1, no spike on ramp). Resulting  
73 values were plotted against the power densities of the respective recording day and  
74 fitted with a five parameter logistic function; then values of the individual recordings  
75 were averaged.

76 Data was analyzed using Clampfit, custom Python scripts, Easy  
77 Electrophysiology and Origin 2021.

## 78 **Temperature Simulations**

79 The temporal distribution of the temperature rise reported in Fig. S13 was  
80 calculated by solving the Fourier heat diffusion equation (41) considering the brain  
81 tissue as an infinite medium with isotropic and uniform thermal properties as described  
82 and experimentally verified before (42). For these simulations light scattering in the  
83 tissue was neglected.

84 The reported temperature rise was calculated for  $r = 0$  representing the center  
85 of the first holographic spot in the middle of the field of view. Additional spots were  
86 randomly added to the first one to maintain a relatively uniform distribution within the  
87  $100 \times 100 \mu\text{m}$  surface.

## 88 **3D structure prediction and system preparation for MD simulations**

89 The HcKCR1, WiChR and B1ChR2 3D models were generated using their full-  
90 length amino acid sequences with AlphaFold2 v.2.1.1 (43)) with the full structural  
91 dataset running on a dedicated group server. Based on the sequence similarity to  
92 ChRmine, a trimeric assembly was chosen. For ChRmine, the trimeric cryo-electron  
93 microscopy structure was used (PDB-ID 7SFK) (19). Each protomer of HcKCR1,  
94 WiChR1 and B1ChR2 was equipped with a retinal cofactor bound to K233 in HcKCR1,  
95 K251 in WiChR1 and K232 in B1ChR2. Internal hydration was predicted using  
96 Dowser++ (44). The central pores of the HcKCR1, WiChR, B1ChR2 and ChRmine  
97 trimer were closed using 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC)  
98 molecules manually placed in PyMOL v.2.5.0 (Schrödinger, LLC). Accordingly, three  
99 lipids were positioned for HcKCR1, WiChR, ChRmine and 6 for B1ChR2. All systems

00 containing the protein, internal waters and central lipids were introduced into a  
01 homogeneous DMPC bilayer membrane, respectively, surrounded by water and  
02 neutralized using 150 mM KCl using CharmmGUI (45).

### 03 **Classical MD simulations in CHARMM at constant pH**

04 MD simulations were carried out using CHARMM version c42b1 (46) and  
05 openMM version 7.0rc1 (47). The MD simulation was computed under NPT conditions  
06 using a 2 fs time step, a 303.15 K Langevin heat bath, the particle-mesh Ewald method  
07 for long-range electrostatics, and the CHARMM36 force field (48). Before and  
08 between the initial equilibration steps, the protonation of all titratable residues was  
09 adjusted based on periodical Karlsberg2<sup>+</sup> predictions (49, 50). For further production  
10 runs, combined pK<sub>a</sub>-MD simulations were computed: during classical MD simulations,  
11 pK<sub>a</sub> values were calculated periodically for 11 snapshots of each 10 ns production run  
12 using Karlsberg2+MD (50) to subsequently adapt the protonation pattern of the system  
13 pH dependently (pH 8).

### 14 **pK<sub>a</sub> calculations in Karlsberg2<sup>+</sup>**

15 In Karlsberg2<sup>+</sup>, the holoprotein structure and ions within a 4 Å cutoff were  
16 explicitly included. The rest was substituted with continuum solvation and an implicit  
17 ion concentration of 100 mM. Single-structure pK<sub>a</sub> calculations during the  
18 equilibration were performed using APBS (51) in a conformational space of three pH-  
19 adapted conformations (PACs), while the pK<sub>a</sub> calculations based on MD simulations  
20 used the extracted snapshots and created only single PACs at pH 7. All PACs were  
21 generated using Karlsberg2<sup>+</sup> in a self-consistent cycle including adjustment of  
22 protonation patterns of titratable amino acids and salt bridge opening according to  
23 either pH -10, 7, or 20.

### 24 **Statistical Analysis**

25 Throughout the paper data is shown as mean ± standard deviation; additionally  
26 individual data points are displayed. Number of replicates and statistical analysis (if  
27 performed) are mentioned in the respective figure legends.

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The authors declare that they have no competing interests.

### Data and materials availability:

Annotated transcript sequences of the stramenopile CCR genes from *Bilabrum* sp., Placidida Caron Lab Isolate and *Wobblia lunata* are provided in Suppl. Data File S3

### List of Supplementary materials:

Supplemental Text

Supplemental Fig. S1 - S13

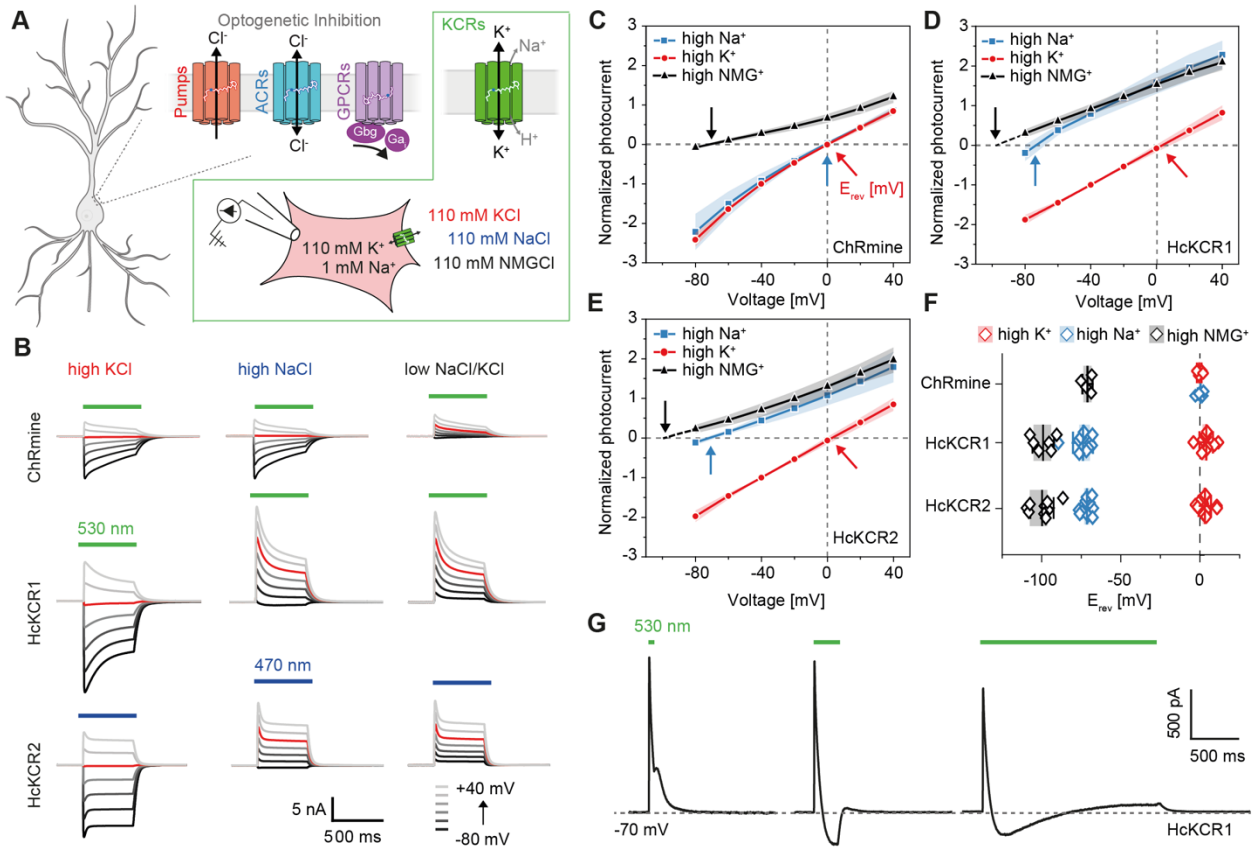
Supplemental Data File S1. Transcriptome and genome assemblies of Stramenopiles used for searching Chr genes. Excel file.

Supplemental Data File S2. Rhodopsin sequences used for phylogeny reconstruction and the resulting unrooted tree. Branch supports are ultra-fast bootstrap support values. Zip file containing fasta and newick files.

Supplemental Data File S3. Annotated transcript sequences of stramenopile CCR genes from *Bilabrum* sp., Placidida Caron Lab Isolate and *Wobblia lunata*. Zip file containing genbank flat files.

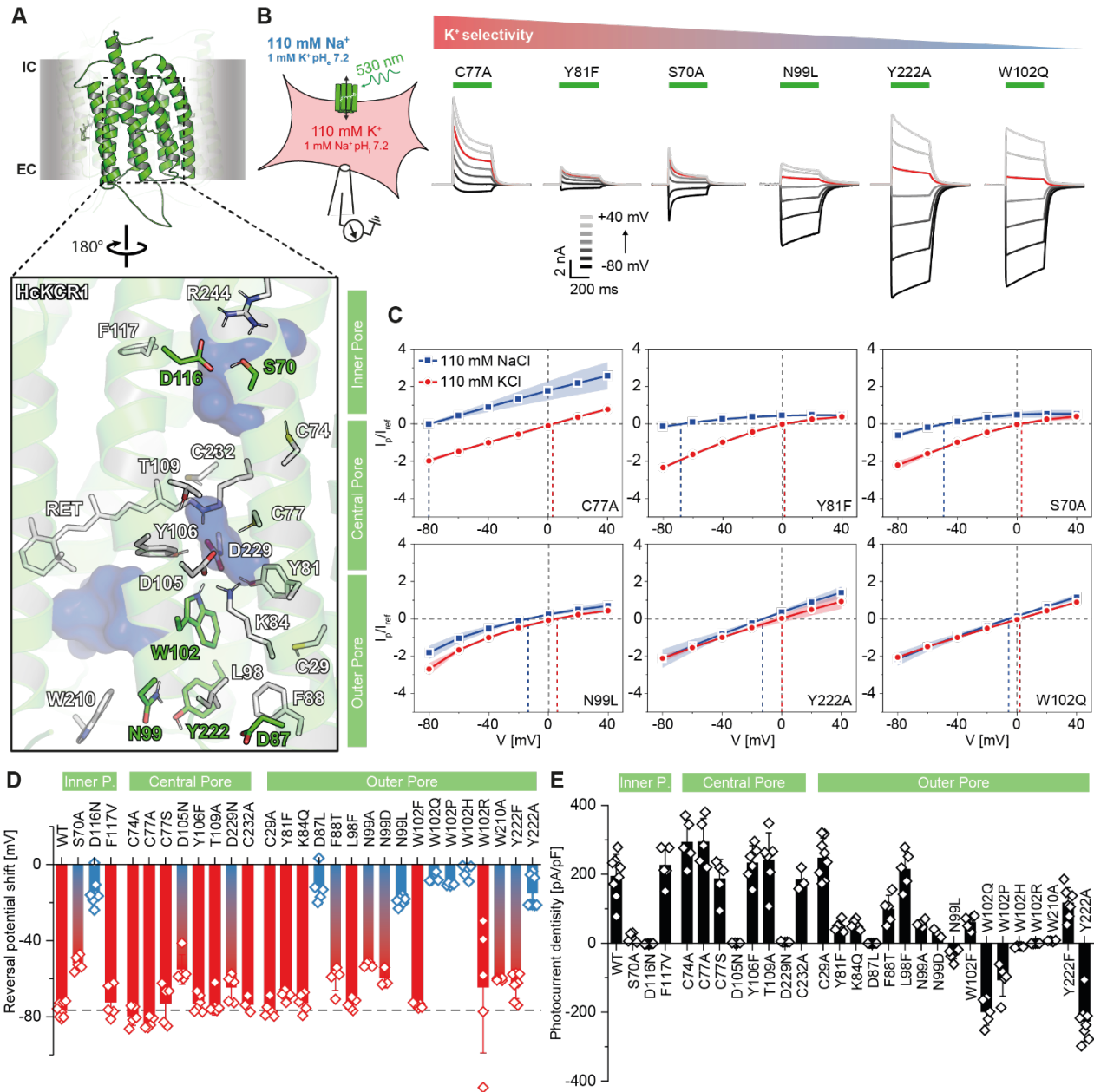


- 23 Supplemental Data File S4. Trimmed structure-based alignment of ChRmine, HcKCR1,  
24 HcKCR2, WlChR1 and B1ChR2. Clustal alignment file.
- 25 Supplemental Data File S5-S7. Equilibrated AlphaFold2 model of the HcKCR1, WiChR  
26 and B1ChR2 trimeric assemblies. PDB files.



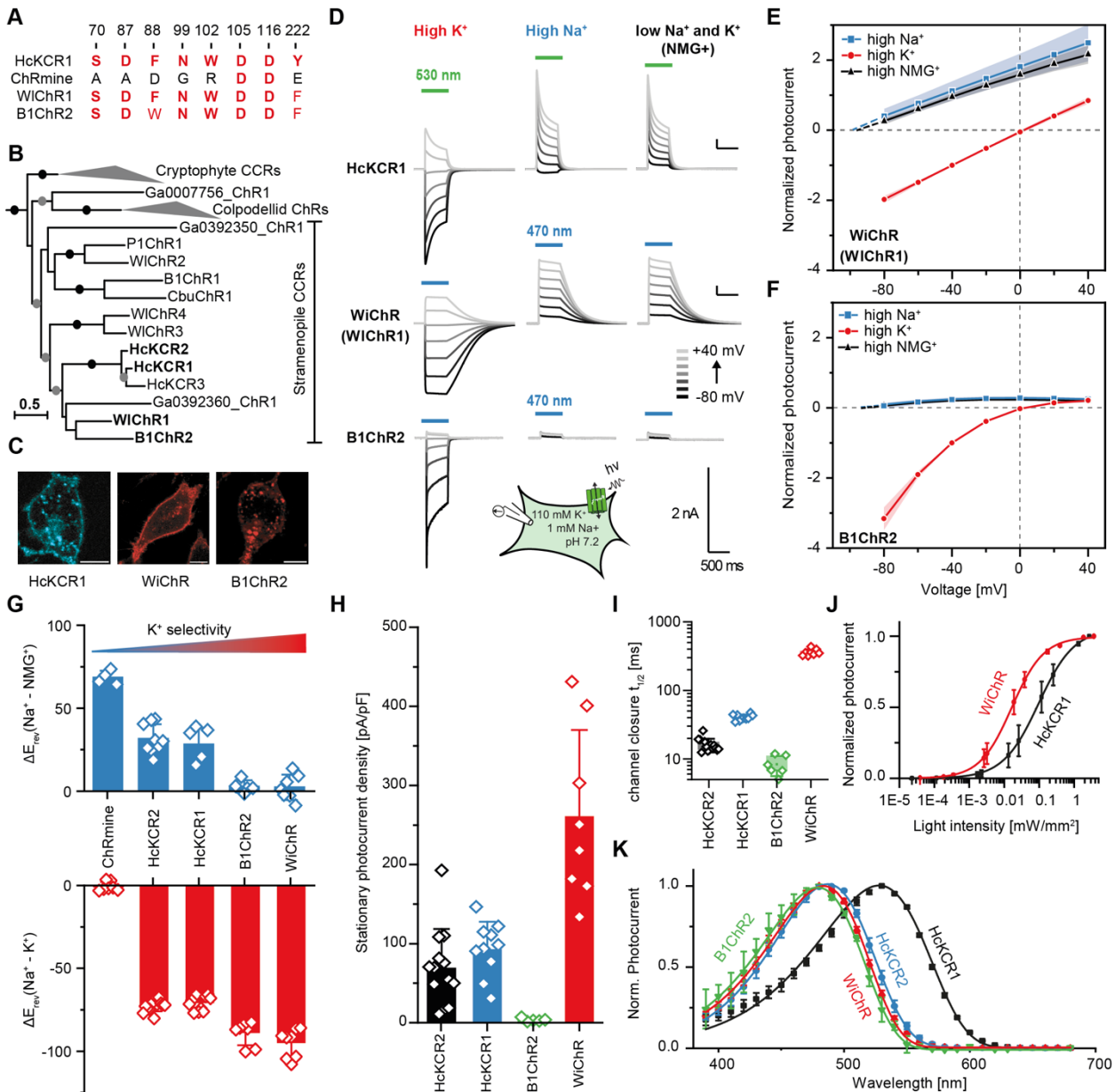
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28 **Fig. 1. K<sup>+</sup>-selectivity of HcKCRs.** (A) Established optogenetic inhibitory tools including light  
 29 driven ion pumps (red), anion conducting channelrhodopsins (blue), and G<sub>i/q</sub>-coupled opsins  
 30 (purple). (B) Photocurrents of ChRmine, HcKCR1 and HcKCR2 at 110 mM K<sup>+</sup><sub>e</sub>, Na<sup>+</sup><sub>e</sub>, or  
 31 NMG<sup>+</sup><sub>e</sub>, and 110 mM K<sup>+</sup><sub>i</sub> gluconat. (C-E) I(E) relations of the normalized peak photocurrent  
 32 amplitudes (Mean ± SD and n = 7/7/4 for ChRmine, n=10/10/6 for HcKCR1 and n=13/13/9  
 33 for HcKCR2) (F) E<sub>rev</sub> values for C - E. (G) Photocurrents of HcKCR1 at -70 mV at high Na<sup>+</sup><sub>e</sub>  
 34 upon 50 ms, 250 ms and 2 s illumination.



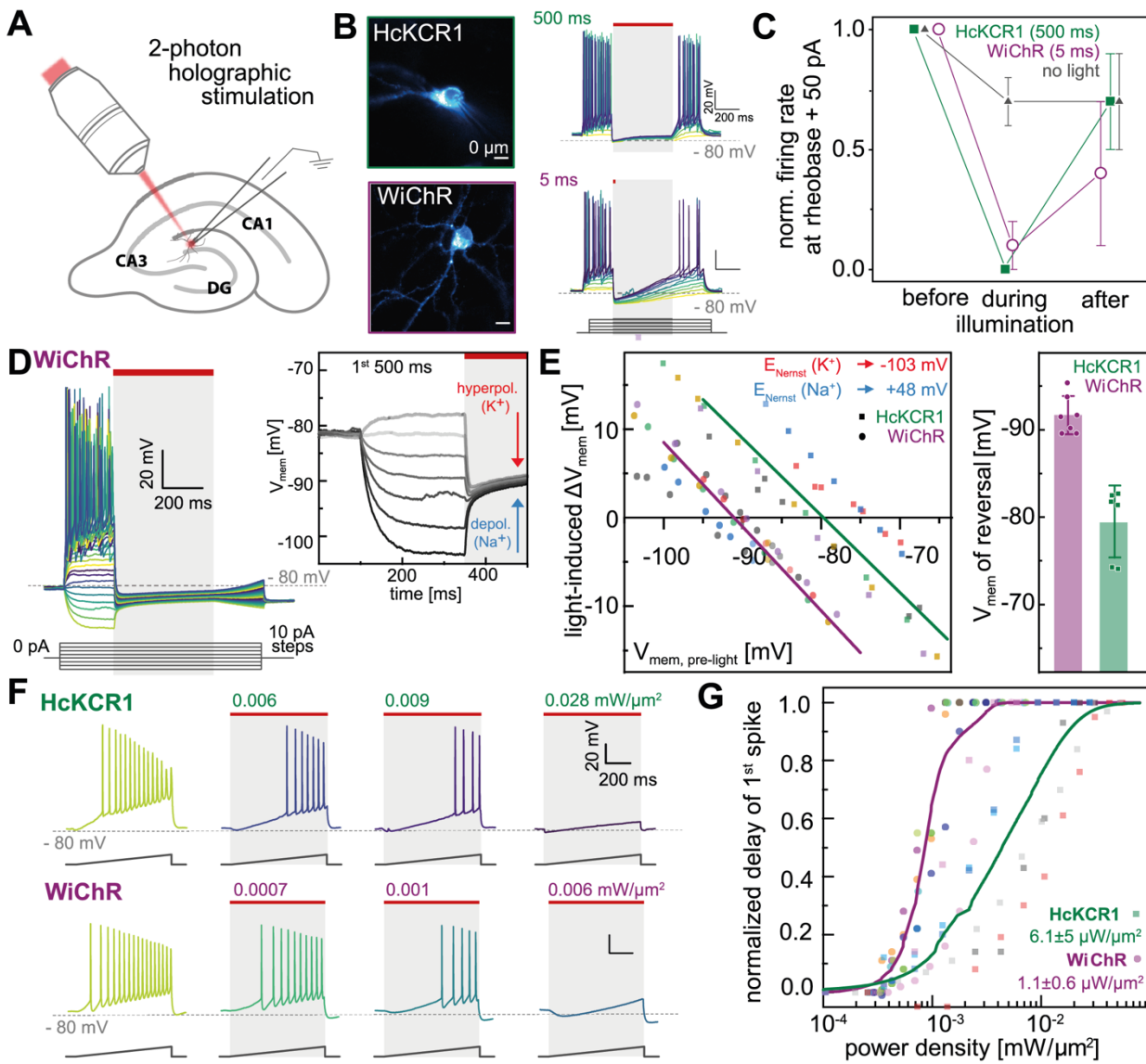
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36 **Fig. 2. Molecular determinants for the  $K^+$ -conductance in KCRs.** (A) Equilibrated  
 37 AlphaFold2 3D model of HcKCR1 with important residues lining the putative pore. (B)  
 38 Representative photocurrents of pore lining HcKCR1 mutants at 110 mM  $K^+_i$  and 110 mM  
 39  $Na^+_e$ . (C) I(E) relations of the normalized peak-photocurrent amplitudes of selected mutants.  
 40 (D)  $\Delta E_{rev}$  for extracellular buffer exchange from high  $K^+_e$  to  $Na^+_e$  (E) Photocurrent density at -  
 41 40 mV and 110 mM  $Na^+_e$ . All plots show (Mean  $\pm$  SD, n = 3-8)



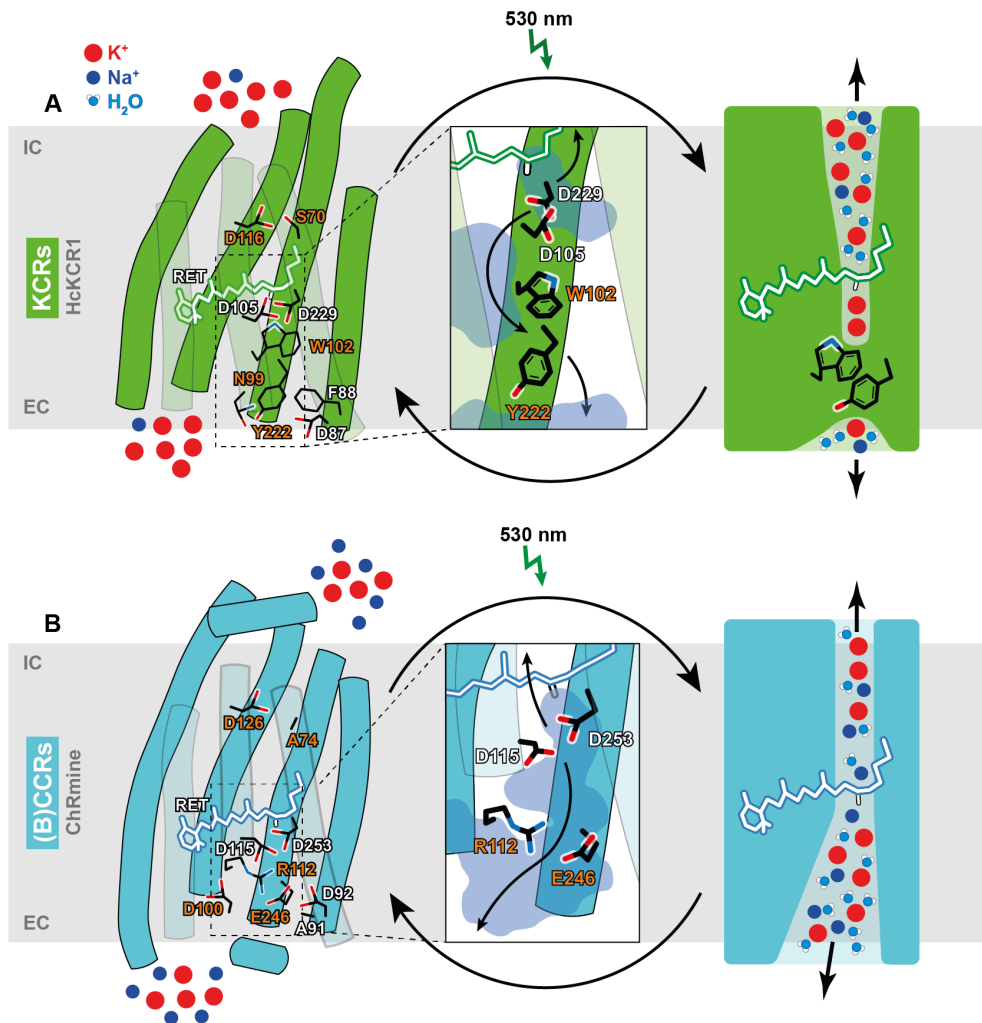
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43 **Fig. 3. Identification and characterization of WiChR.** (A). The 8 amino acid motif of the  
 44 K<sup>+</sup>-selectivity filter in red (see also Fig S6A and the complete alignment in Fig. S8 and Suppl.  
 45 Data File S4). (B) Phylogenetic relationships among the stramenopile CCRs (see the complete  
 46 phylogenetic tree of the channelrhodopsin family in Fig. S6A and Suppl. Data File S2). Dots  
 47 represent ultra-fast bootstrap support values:  $\geq 80$  (gray) and  $\geq 95$  (black). (C) Cellular  
 48 distribution of the fluorescence tagged KCRs. (D) Representative photocurrents of HcKCR1,  
 49 WiChR, and B1ChR2 at high K<sup>+</sup>, Na<sup>+</sup>, or NMG<sup>+</sup>, and intracellular 110 mM K<sup>+</sup> gluconate.  
 50 (E) & (F) I(V) relations of the peak photocurrent amplitudes of WiChR/WiChR1 (n = 8/8/7)  
 51 and B1ChR2 (n = 7/7/5). (G)  $\Delta E_{rev}$  of stationary photocurrents (after 500 ms light) for  
 52 extracellular buffer exchange from high K<sup>+</sup> to Na<sup>+</sup> (bottom) and high Na<sup>+</sup> to NMG<sup>+</sup> (top)  
 53 (H) stationary photocurrent densities with high Na<sup>+</sup> and -40 mV. (I) photocurrent closure after  
 54 500 ms recordings. (J) light titration of the WiChR (n = 5) and HcKCR1 (n=5) peak  
 55 photocurrents. (K) Action spectra of peak photocurrents (n= 6 for all four channels). All plots  
 56 show (mean  $\pm$  SD).



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**Fig. 4: Neuronal inhibition with 2-photon holographic stimulation of WiChR in organotypic hippocampal slices.** (A) Whole-cell patch clamp recordings of HcKCR1 and WiChR with 2P holographic illumination (1030 nm); liquid junction potential corrected for all recordings (B) Epifluorescence images and recordings of square depolarizing current injections (1 s) with a 500 ms period of photoinhibition within this time; continuous illumination for HcKCR1 and 5 ms for WiChR ( $29 \mu\text{W}/\mu\text{m}^2$ ) (C) Normalized firing rate 50 pA above rheobase for HcKCR1, WiChR (as in B) and without light, mean $\pm$ SD, n=6-8 each. (D) Like (A) with hyperpolarizing current injections (E) Extrapolation of membrane potential where illumination induces depolarization instead of hyperpolarization; points individual recordings and lines average. (F) Depolarizing somatic ramp current injections with continuous illumination of rising power densities during ramp; (G) Normalized light-induced delay of first spike on the ramp relative to the dark; 0 – no delay, 1 – no action potential during ramp; points represent individual recordings and lines averaged logistic fit; n=6, n=7; EC50 values mean $\pm$ SD.



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73 **Fig. 5: K<sup>+</sup>-selectivity filter of KCRs compared to (B)CCRs.** (A) Cartoon model of the  
 74 proposed HcKCR1 dark state and a simplified representation of its conducting state with  
 75 important amino acid side chains highlighted. The central inlet shows the outer pore. Residues  
 76 in light red are involved in K<sup>+</sup>-selectivity. (B) Cartoon model of the ChRmine dark state and a  
 77 simplified representation of its conducting state. The central inlet shows the outer pore.  
 78 Highlighted amino acids are homologous to residues in A. The outer pore in HcKCR1 is  
 79 smaller and more isolated from the bulk than in ChRmine. Here, a hydrophilic and water-filled  
 80 cavity between helices 3, 4, 6 and 7 is delimited from the central gate by W102 and Y106 and  
 81 from the bulk water by W210, Y222 and N99. Geometrical RMSD calculations of aromatic  
 82 residues revealed high flexibility of F88 (2.00 Å), W102 (2.12 Å), Y106 (1.85 Å) and W210  
 83 (2.27 Å) side chains while Y222 was more stable (1.13 Å) due to predicted hydrogen bonds to  
 84 N99 and Q218. (Fig. S10B) This flexibility would allow the formation of a continuous cation  
 85 translocation pathway during the conducting state.