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Supplementary Material for

Structure-Guided Transformation of Channelrhodopsin into a Light-Activated Chloride Channel

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Materials and Methods

Structural representations Figures of the C1C2 (pdb: 3UG9 (24)) structure were generated using the PyMOL Molecular Graphics System, Version 1.7.0.1 (Schrödinger, LLC; *PNAS* **98**:10037-41, 2001).

Point mutagenesis of C1C2 C1C2 mutations were introduced using the QuickChange™ Site-Directed mutagenesis kit (Agilent) and purified with QIAprep™ Spin Miniprep Kits (Qiagen) after transformation and amplification in *E. coli*. We used AAV vectors bearing the CaMKIIα promoter for protein expression in neurons, and a pcDNA3.1 vector bearing the CMV promoter for expression in HEK cells. All clones were fused to the eYFP (enhanced yellow fluorescent protein) gene for fluorescence microscopy.

Neuronal culture preparation and imaging Primary hippocampal neurons were cultured from postnatal day 0 (P0) Sprague-Dawley rat pups (Charles River). The CA1 and CA3 regions were isolated, digested with 0.4 mg/mL papain (Worthington), and plated onto 12 mm glass coverslips pre-coated with 1:30 Matrigel (Beckton Dickinson Labware), at a density of 65,000 cells per well in 24-well plates. The cultured cells were maintained in Neurobasal-A medium (Invitrogen) containing 1.25% FBS (HyClone), 4% B-27 supplement (Gibco), 2 mM Glutamax (Gibco) and 2 mg/ml fluorodeoxyuridine (FUDR, Sigma), and kept in a humid culture incubator with 5% CO_2 at 37°C.

Cells were transfected at 6-10 days *in vitro* (DIV). A DNA-$CaCl_2$ mix containing the following was prepared per each well to be transfected: 2 μg of DNA (prepared using an endotoxin-free preparation kit (Qiagen)) 1.875 μl 2M $CaCl_2$, and sterile water to a total volume of 15 μl. An additional 15 μl of 2X filtered HEPES-buffered saline (HBS, in mM: 50 HEPES, 1.5 Na_2HPO_4, 280 NaCl, pH 7.05 with NaOH) was added per DNA-$CaCl_2$ mix, and the resulting DNA-$CaCl_2$-HBS mix was incubated at room temperature for 20 minutes. During this time, the neuronal growth medium was removed from the wells and saved at 37°C, and replaced with 400 μl pre-warmed minimal essential medium (MEM). Once the DNA-$CaCl_2$-HBS mix incubation was complete, the mix was then added dropwise into each well, and the plates were kept in the culture incubator for 45-60 minutes. Afterwards, each well was washed three times with 1 ml of pre-warmed MEM, and then the MEM was replaced with the original neuronal growth medium. The transfected cells were then returned to the culture incubator until recordings.

For confocal images of opsin-eYFP-expressing neurons, coverslips of transfected cells expressing were fixed for 15 minutes in 4% paraformaldehyde and mounted with PVA-DABCO. Images were acquired with a Leica DM600B confocal microscope, and the same settings were used across images.

Electrophysiological recordings in hippocampal neurons The Spectra X Light engine (Lumencor) was coupled to the fluorescence port of an Olympus BX61WI microscope to detect eYFP expression and to deliver light for opsin activation. 475/15 and 632/22 filters

were used for blue light and red light respectively, and light power density through a 40X objective was measured with a power meter (ThorLabs).

Whole-cell recordings were obtained with patch pipettes (4-6 MΩ) pulled from glass capillaries (Sutter Instruments) with a horizontal puller (P-2000, Sutter Instruments). The external recording solution contained (in mM): 135 NaCl, 4 KCl, 10 HEPES, 2 $CaCl_2$, 2 $MgCl_2$, 30 D-glucose, pH 7.3 with synaptic transmission blockers 25 μM D-APV, 10 μM NBQX. The internal solution recording solution contained (in mM): 140 K-gluconate, 10 HEPES, 10 EGTA, 2 $MgCl_2$, pH 7.3. Measurements were corrected for the liquid junction potential of +16 mV. We used 3 M CsCl agar bridges for the reference electrode at all recordings. Series resistance was monitored throughout recordings for stability. Recordings were made using a MultiClamp700B amplifier (Molecular Devices). pClamp10.3 (Molecular Devices), OriginLab8 (OriginLab), and Sigmaplot (SPSS) software was used to record and analyze data.

The stationary photocurrent upon light activation was used as the measure of photocurrent amplitude at different membrane potentials. The reversal potential (V_{rev}) was defined as the point where the stationary photocurrent amplitude was 0 pA. Action potential threshold was measured at the voltage deflection point at which the first-order derivative of the membrane potential (dV/dt) exhibited a sharp transition, typically > 10 mV/ms. The resting membrane potential of the cell was measured in current-clamp after attaining whole-cell configuration. Input resistance was calculated from the steady-state current responses evoked by 20 mV hyperpolarizing steps in voltage-clamp. To investigate action potential inhibition, we tested opsin-expressing cells under two different spike induction protocols. Spikes were electrically evoked with intracellular current injections, either with short electrical pulses (30 ms pulse width, 50-280 pA) 10 Hz, or with a continuous 3 s electrical pulse. Light was applied for 1 s (during the 10 Hz train) or 0.5 s (during the continuous pulse) during the middle of the electrical current injection. Spike inhibition probability was calculated as the fraction of electrically-evoked spikes that were blocked during the light pulse epoch of the electrical stimulation.

HEK cell culture preparation Human embryonic kidney cell cultures (HEK-293: ATCC® CRL-1573™) were maintained in 50 ml Dulbecco's Modified Eagle Medium (Life Technologies) containing 100 units/mL of penicillin and 100 μg/mL of streptomycin as well as fetal bovine serum at a dilution of 1:10. HEK cells were grown in incubators at 37 ^{o}C / 5% CO_2 and were transferred to a new 225 cm^2 culture flask (Thermo) every 3 to 4 days at passaging dilutions ranging from 1:5 to 1:8. 24h prior to DNA transfections cells were plated on 2 cm poly-D-lysine coated glass cover slips and maintained in 24 well culture plates (Thermo) with 500 μl growth medium. 24h prior to recordings, HEK cells were transfected with 1.6 μl plasmid DNA per well using 2 μl Lipofectamine 2000 (Life Technologies).

HEK cell electrophysiology ChR-expressing cells were identified by eYFP fluorescence and recorded ~18 to 30 hours after transfection. We used the same equipment and methods as for neurons. Measurements were conducted in voltage clamp at membrane potentials between -75 and +55 mV. An external 3M CsCl agar bridge was used in all

recordings. We first characterized all constructs using the same internal and external solution as in neurons and corrected for the corresponding junction potential, and used stationary photocurrents for data analysis. The activation spectra for C1C2, iC1C2 and NpHR was determined by measuring stationary photocurrents at -75 mV in response to low light intensities at 0.65 mW/mm^2 in order to prevent saturation. We used 20 nm bandbass filters (Thorlabs) to apply light at different wavelengths (in nm): 400, 420, 440, 460, 470, 480, 490, 500, 520, 540, 560, 570, 580, 590, 600, 620, 630, 650. All photocurrent were normalized to reference values at 470 nm (C1C2 and iC1C2) or 570 nm (NpHR). Kinetics of channel closure were quantified by fitting photocurrents after light-off with mono-exponential functions in order to obtain corresponding tau_{off} values. Light sensitivity measurements were carried out at 470 nm (C1C2, iC1C2) or 560 nm (NpHR). We applied light at intensities from 0.0021 to 5 mW/mm^2 and normalized corresponding photocurrents to the value at maximum light intensity.

We determined ion selectivities by varying ion composition and pH of the internal and external solutions. External solutions contained (in mM) 2 $CaCl_2$, 2 $MgCl_2$, 120 NaCl, 120 CsCl or 120 Na-gluconate, 10 Citric acid/Na-citrate (pH 6) or 10 HEPES (pH 7.3) or 10 Tris (pH 9). Internal solutions contained (in mM) 2 $CaCl_2$, 2 $MgCl_2$, 120 KCl, 120 CsCl or 120 K-gluconate, 10 Citric acid/Na-citrate (pH 6) or 10 HEPES (pH 7.3) or 10 Tris (pH 9). We corrected for junction potential under each condition (in mV): $KCl_{int}/NaCl_{ext}$ = 4, $KCl_{int}/CsCl_{ext}$ = -0.6, $KCl_{int}/NaGluc_{ext}$ = -6.2, $KGluc_{int}/NaCl_{ext}$ = 15.8, $CsCl_{int}/NaCl_{ext}$ = 4.6. We used the Nernst equation to determine the Nernst potential for cations, Cl^- and protons under each external and internal ion composition and pH. We used an adapted Goldman-Hodgkin-Katz equation to calculate the ratio of proton to Cl^- permeability in iC1C2. We assumed that permeability for Na^+ and K^+ is zero, which resulted in:

$$V_{rev} = \frac{RT}{F} ln \frac{P_H[H^+]_{ext} + P_{Na}[Na^+]_{ext} + P_K[K^+]_{ext} + P_{Cl}[Cl^-]_{int}}{P_H[H^+]_{int} + P_{Na}[Na^+]_{int} + P_K[K^+]_{int} + P_{Cl}[Cl^-]_{ext}}$$

$$V_{rev} = \frac{RT}{F} ln \frac{P_H[H^+]_{ext} + P_{Cl}[Cl^-]_{int}}{P_H[H^+]_{int} + P_{Cl}[Cl^-]_{ext}}$$

$$\alpha = \frac{P_{Cl}}{P_H}$$

$$V_{rev} = \frac{RT}{F} ln \frac{[H^+]_{ext} + \alpha[Cl^-]_{int}}{[H^+]_{int} + \alpha[Cl^-]_{ext}}$$

R = Gas constant, F = Faraday constant, T = absolute temperature

Statistical analysis was performed with a t-test or a two-way ANOVA, and a Mann-Whitney test for non-parametric data, using Origin8 (OriginLab) and Sigmaplot (SPSS) software. Data is presented as mean ± s.e.m., and error bars indicate s.e.m. $p<0.05$ is defined to be statistically significant.

Figure S1

Protein alignment of C1C2 and iC1C2 in comparison to channelrhodopsin-2 (ChR2). The mutated residues of iC1C2 are highlighted in red and shown in relation to the transmembrane helices (TM 1-7) of C1C2 (gray bars) (24). Positioning of SwiChR mutations is shown in blue.

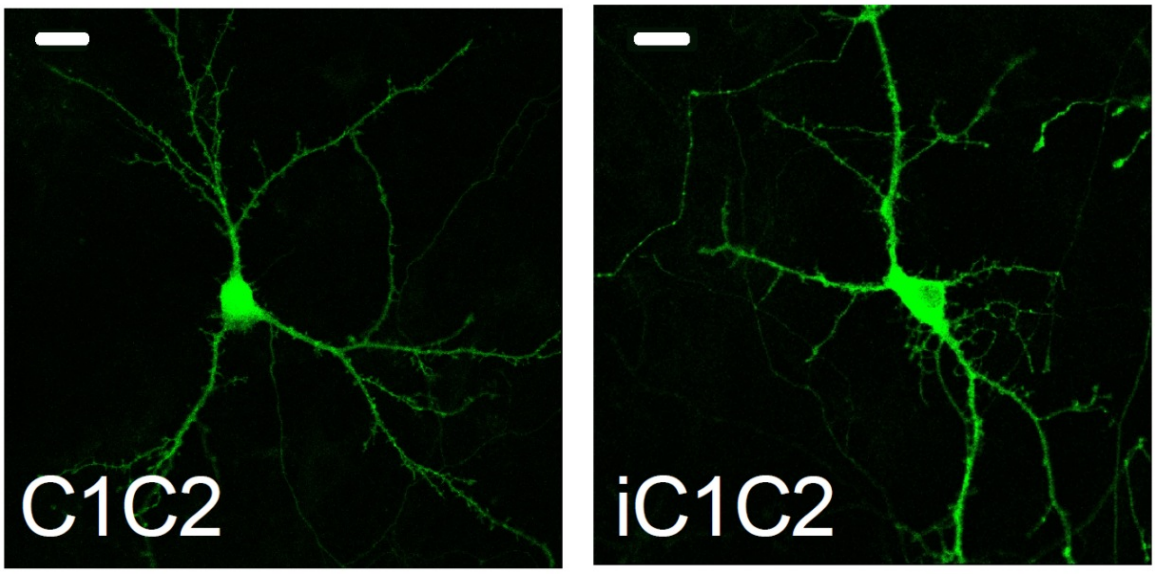

Figure S2

Confocal images of cultured neurons expressing C1C2-eYFP (left) and iC1C2-eYFP (right). Scale bar: 20 μm.

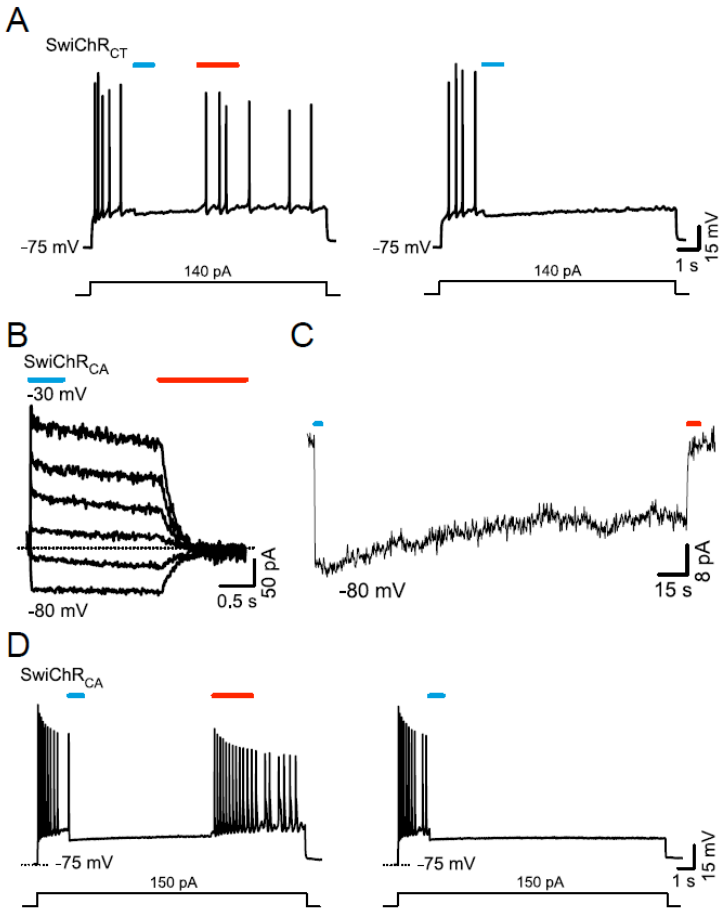

Figure S3

Bistable inhibition with SwiChRs. **(A)** Bistable spiking modulation with SwiChR$_{CT}$. Spiking was induced by a continuous electrical pulse (3 s), stably inhibited by 475 nm light (blue bar) delivered at 5 mW/mm^2, and resumed after 632 nm light application (red bar) (left). Prolonged spiking modulation in the same cell after only 475 nm light delivery (blue bar) at 5 mW/mm^2 (right) with the same current injection as in left panel. **(B)** Current-voltage relationship of SwiChR$_{CA}$ recorded at membrane potentials from -80 mV to -30 mV upon 470 nm activation pulses (blue bar) followed by 632 nm (red bar) light pulses. **(C)** Representative photocurrent of SwiChR$_{CA}$ upon 470 nm activation (blue bar) followed by a second light pulse at 632 nm (red bar). Light power density was 5 mW/mm^2. **(D)** (left) Bistable spiking modulation by SwiChR$_{CA}$. Spiking was induced by a continuous electrical pulse (3 s) and stably inhibited by 475 nm light (blue bar) delivered at 5 mW/mm^2. Spiking resumed after 632 nm light application (red bar). **(E)** Prolonged spiking modulation of SwiChR$_{CA}$ of the same cell as in (D) with 475 nm light (blue bar) only, delivered at 5 mW/mm^2; the same current injection was used to induce spiking as in (D).