

## Supplementary Material for:

### Title:

Artificial anion-conducting channelrhodopsins with tuned spectra, modified kinetics and enhanced light sensitivity

### Authors:

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# equal contribution

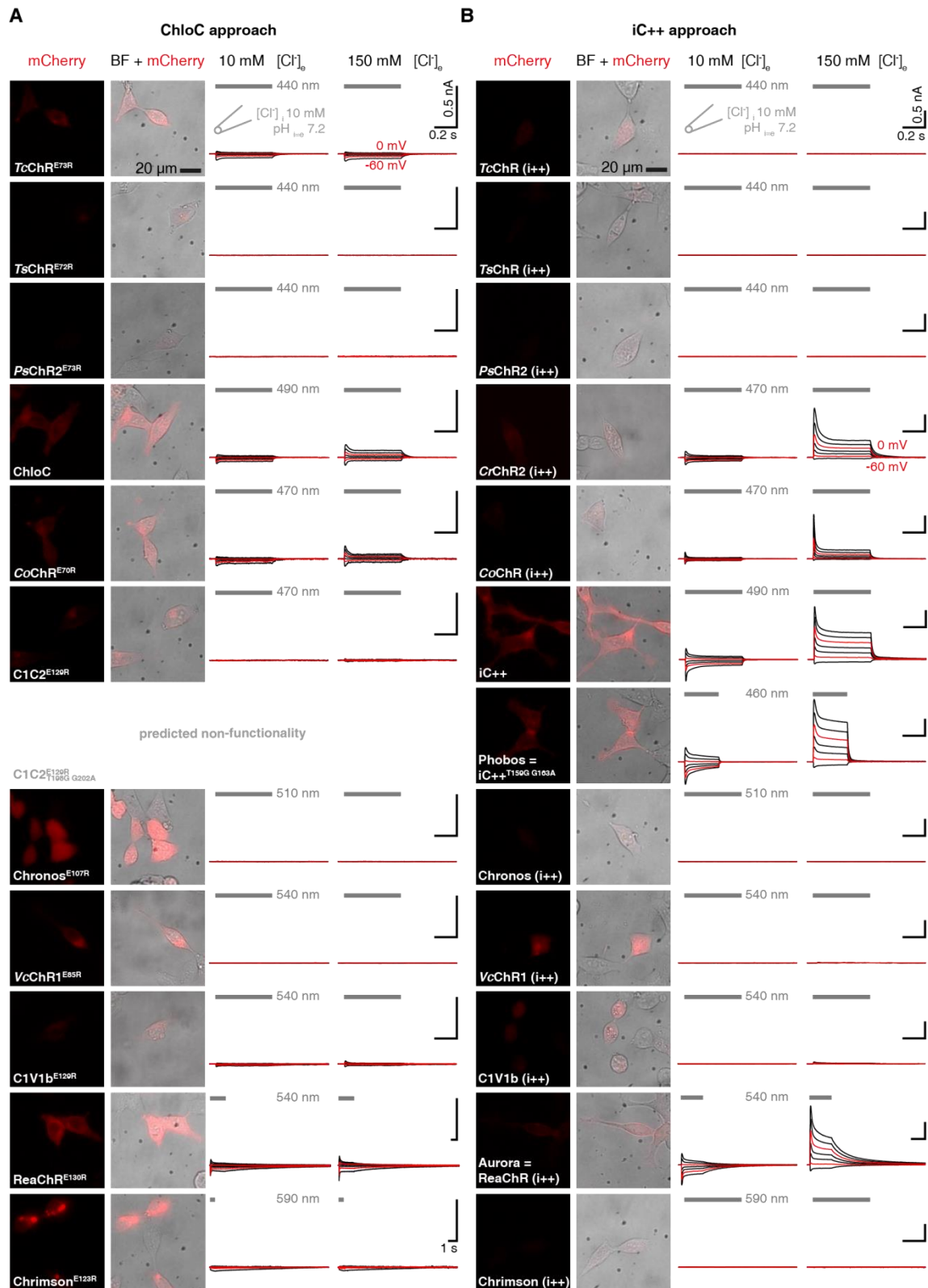
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iChloC	-----MDYG-----G---ALSAVGRELLFVTN--PVVVNGSV--LVP-----EDQCYCAGWIESRGTNGAQTASNVLQWL	59
iC++	-----MDYG-GALSAVGLFQTSYTLNNGSVICIPN-----NGQCFCLAWLKSNGTNAEKLAANILQWIS	59
Phobos	-----MDYG-GALSAVGLFQTSYTLNNGSVICIPN-----NGQCFCLAWLKSNGTNAEKLAANILQWIS	59
Aurora	-----MDYG-GALSAVGLFQTSYTLNNGSVICIPN-----NGQCFCLAWLKSNGTNAEKLAANILQWIS	59
TcChR	-----MGWKNINPLYSDVAILEI-----CKENEMVFGPLWEQKLRALQWFT	42
TsChR	-----MFAINPEYMETVLLDE-----CTPIYLDIGPLWEQVVARVQWFG	41
PsChR2	-----MGFQLNPEYLNELTLLDD-----CTPIYLVNGLWEQKVARVQWFG	42
CrChR2	-----MDYG-----G---ALSAVGRELLFVTN--PVVVNGSV--LVP-----EDQCYCAGWIESRGTNGAQTASNVLQWL	59
CoChR	-----MLNGNSA--IVP-----IDQCFCLAWTDSLGSDEQLVANILQWFA	39
C1C2	---MS-----RRPWLLALALAVALAAGSAGASTGSDATVPVATQDGGPDYVFHRAHERMLFQTSYTLNNGSVICIPN-----NGQCFCLAWLKSNGTNAEKLAANILQWIT	98
ShChR (Chronos)	---MET-----AATMTHAFISAVPSAEAT---IRGLLSAAAVVTPAADAHG-----E---TSNATTAGADHGCFPH-----INHGTTELQHKIAGVGLQWFT	76
VcChR1	-----MDYPVAR-----SLIVRYPTDLNGTVCMP-----RGQCYCEGWLRSRGTSEIKTIAITLQWVV	54
C1V1b	---MS-----RRPWLLALALAVALAAGSAGASTGSDATVPVATQDGGPDYVFHRAHERMLFQTSYTLNNGSVICIPN-----NGQCFCLAWLKSNGTNAEKLAANILQWIT	98
ReaChR	---MVS-----RRPWLLALALAVALAAGSAGASTGSDATVPVATQDGGPDYVFHRAHERMLFQTSYTLNNGSVICIPN-----NGQCFCLAWLKSNGTNAEKLAANILQWVV	99
bReaChES	-----MDYG-GALSAVGLFQTSYTLNNGSVICIPN-----NGQCFCLAWLKSNGTNAEKLAANILQWVV	59
CnChR1	MAELISSATRSLFAAGGNPWPNPYHHE---DMCGG-----GMTPTGECF-----STEWWCDPYSGLSDAGYGYCFVEATGGYLVVGVGKQAWLHRSRGTGPEKIGAQVCQWIA	101

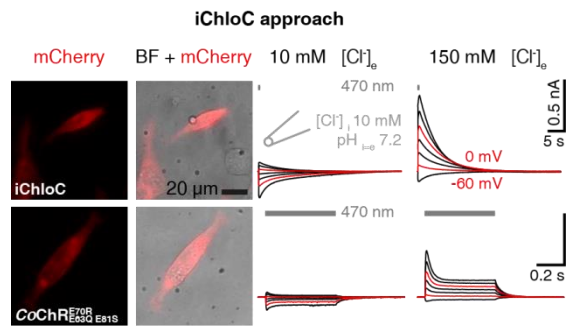
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iChloC	AGFSILLMFYAYQ	TWKSTCGWE	QIYVCAIRMV	KVILEFF	FFSKNPSMLYLATGHRVQWLR	YAEWLLT	CPVLIHLSNLTGLSNDYSRRTMGLLVS	NIC	CI	VWGATSAMATGYVKVIFFC	179		
iC++	FALSALCLMFYAYQ	TWKSTCGWE	NIYVATI	QMIKFIIEYFH	SFDEPAVIYSSNGNKT	RWLRYASWLLT	CPVLIHLSNLTGLANDYNKRTMGLLVS	DIGTIVWGT	TAALS	SKGYVRVIFFL	179		
Phobos	FALSALCLMFYAYQ	TWKSTCGWE	NIYVATI	QMIKFIIEYFH	SFDEPAVIYSSNGNKT	RWLRYASWLLT	CPVLIHLSNLTGLANDYNKRTMGLLVS	DIGTIVWGT	TAALS	SKGYVRVIFFL	179		
Aurora	FALSVA	CLGWYAYQ	AWRATCGWE	NIYVATI	QMIKFIIEYFH	SFDEPAVIYSSNGNKT	RWLRYASWLLT	CPVLIHLSNLTGLKDDYSKRTMGLLVS	DVGCIVWGATSAMCTGWT	KILFFL	179		
TcChR	VILSAIFLAYVY	YSTRATCGWE	EELYVCTVE	FTKVVVEVY	LEYVPPMIYQ	MNGQHTPWLRYE	WLLT	CPVLIHLSNLTGLNDEYSGR	TMSLLT	SDLG	ICAFVLSALAVGWQKILFFG	162	
TsChR	VILSLVFLI	YIWN	TYKATCGWE	EELYVCTVE	FTKIIIELY	FETPPAMIYQ	TNGQHTPWLRYE	WLLT	CPVLIHLSNLTGLNDDYSGR	TMSLLT	SDLGGICMAV	TAALSKGWLKALFFV	161
PsChR2	VILSLAFLI	YIWN	TYKATCGWE	EELYVCTVE	FTKIIIELY	FETPPAMIYQ	TNGQHTPWLRYE	WLLT	CPVLIHLSNLTGLNDDYSGR	TMSLLT	SDLGGICMAV	TAALSKGWLKALFFV	162
CrChR2	AGFSILLMFYAYQ	TWKSTCGWE	EELYVCTVE	FTKIIIELY	FETPPAMIYQ	TNGQHTPWLRYE	WLLT	CPVLIHLSNLTGLNDDYSGR	TMSLLT	SDLGGICMAV	TAALSKGWLKALFFV	179	
CoChR	FGFSILLMFYAYQ	TWRATCGWE	EELYVCCV	ELTKVIEFF	HFDDPSMLYLANGHRVQ	WLR	YAEWLLT	CPVLIHLSNLTGLKDDYSKRTMRL	LVSDVGT	I	VWGATSAMSTGYVKVIFFC	159	
C1C2	FALSALCLMFYAYQ	TWKSTCGWE	NIYVATI	EMIKFIIEYFH	SFDEPAVIYSSNGNKT	VWLRYAEWLLT	CPVLIHLSNLTGLANDYNKRTMGLLVS	DIGTIVWGT	TAALS	SKGYVRVIFFL	218		
ShChR	VIVAIVQLIF	YGWHSFKAT	TGWEEVYV	CVIELVKCFI	ELFHEV	DSPATVYQ	TNGGAVI	WLRYSWLLT	CPVLIHLSNLTGLHEEYSKRTMT	ILVTD	IGNIVWGITAAFTKGLKILFFM	196	
VcChR1	FALSVA	CLGWYAYQ	AWRATCGWE	EELYVCCV	ELTKVIEFF	HFDDPSMLYLANGHRVQ	WLR	YAEWLLT	CPVLIHLSNLTGLKDDYSKRTMRL	LVSDVGT	I	VWGATSAMCTGWT	174
C1V1b	FALSALCLMFYAYQ	TWKSTCGWE	NIYVATI	EMIKFIIEYFH	SFDEPAVIYSSNGNKT	VWLRYAEWLLT	CPVLIHLSNLTGLKDDYSKRTMGLLVS	DIGTIVWGT	TAALS	SKGYVRVIFFL	218		
ReaChR	FALSVA	CLGWYAYQ	AWRATCGWE	EELYVCCV	ELTKVIEFF	HFDDPSMLYLANGHRVQ	WLR	YAEWLLT	CPVLIHLSNLTGLKDDYSKRTMGLLVS	DVGCIVWGATSAMCTGWT	KILFFL	219	
bReaChES	FALSVA	CLGWYAYQ	AWRATCGWE	EELYVCCV	ELTKVIEFF	HFDDPSMLYLANGHRVQ	WLR	YAEWLLT	CPVLIHLSNLTGLKDDYSKRTMGLLVS	DVGCIVWGATSAMCTGWT	KILFFL	179	
CnChR1	FSIAIALTL	FFYGFSAWKAT	CGWE	EELYVCCV	ELFVLTLE	IFKFE	SSPATVY	LSTGNHAY	CLRYFEWLLS	CPVLIHLSNLTGLKDDYSKRTMGLI	VSCVGMIVFGMAAGLATD	WLKLLYI	221

	<u>H5</u>	<u>H6</u>	<u>H7</u>										
iChloC	LGLCYGANTFFHAAKAYIEGYHTVPKGR	CRQVVTGM	AWLFFVSWG	MFPILFILGPEGPGVLSVYGSTVGH	TIIDLMSKNCWGLLGHYLRVLIHEHILIHGDIRKTTKLNIGGTEIEVETLVEDEAEAGAV	309							
iC++	MGLCYGIYTFNAAKVYIEAYHTVPKGR	CRQVVTGM	AWLFFVSWG	MFPILFILGPEGPGVLSRYGNSVGH	TIIDLMSKQCWGLLGHYLRVLIHSHILIHGDIRKTTKLNIGGTEIEVETLVEDEAEAGAV	309							
Phobos	MGLCYGIYTFNAAKVYIEAYHTVPKGR	CRQVVTGM	AWLFFVSWG	MFPILFILGPEGPGVLSRYGNSVGH	TIIDLMSKQCWGLLGHYLRVLIHSHILIHGDIRKTTKLNIGGTEIEVETLVEDEAEAGAV	309							
Aurora	ISLSYGMYYFHA	AKVYIEAFHTVPKGLCRQ	LVRAMAWLFFVSWG	MFPVLLGPEGPGHISRYGNSIGH	SILLDLIAKQMWGVLGNYLVRVLIHSHILLYGDIRKKQKITIAGQEMEVEVLVAEEED	305							
TcChR	IGCIYGASTFYHAA	CIYIESYHTMPAGKCKRLV	VAMCAVFFTSWF	MFPALFLAGPECFDGLTWSG	STIAHTVADLLSKNIWGLIGHFLRVGIIHEHILVHGDVRRPIEVTIFGKETS	LNCFVNDDEDDV	292						
TsChR	IGCGYGASTFYNA	ACIYIESYHTMPGGICRRL	VLWAGVFFTSWF	MFPGLFLAGPEGTQALS	SWAGTTIGHTVADLLSKNAWGMIGHFLRVEIHKHIIHG	VRRPVTVKALGRQVSNCFVDEKDEEED	293						
PsChR2	IGCCYGASTFYHAA	LIYIESYHTMPHGVC	KMNLAMA	AVFFTSWF	MFPGLFLAGPEGTNALS	SWAGTTIGHTVADLLSKNAWGMIGHFLRVEIHKHIIHG	VRRPVTVNTLGREVTVSCFVDEKDEEED	294					
CrChR2	LGLCYGANTFFHAAKAYIEGYHTVPKGR	CRQVVTGM	AWLFFVSWG	MFPILFILGPEGPGVLSVYGSTVGH	TIIDLMSKNCWGLLGHYLRVLIHEHILIHGDIRKTTKLNIGGTEIEVETLVEDEAEAGAV	311							
CoChR	LGCIYGANTFFHAAKAYIESYHVPKGR	PRVVRIMAWLFFL	LSWGMFPV	LVVVGPEG	DAISVYGSTIGHTI	IDLMSKNCWGLLGHYLRVLIHQHIIYGD	IRKTKINVAGEEMEVE	TMVQDEETV	288				
C1C2	MGLCYGIYTFNAAKVYIEAYHTVPKGR	CRQVVTGM	AWLFFVSWG	MFPILFILGPEGPGVLSVYGSTVGH	TIIDLMSKNCWGLLGHYLRVLIHEHILIHGDIRKTTKLNIGGTEIEVETLVEDEAEAGAV	348							
ShChR	IGLFYGVTCFFQIAK	VYIESYHTLPKGV	CRKICKIMAYV	FFCSWLMFP	VMFIAGHEGLGITP	YTSIGIHLI	LDLISKNTWGF	LGHLLRVKIIHEHILIHGDIRKTTTINVAGEN	MEIETV	FDDEEECGV	325		
VcChR1	ISLSYGMYYFHA	AKVYIEAFHTVPKGL	CRELVRVMAW	TFVAVGMFP	VLLGTEG	PGHISPYGSAIGH	SILLDLIAKNNMWG	VLGNYLVRVLIHEHILLYGDIRKKQKITIAGQEMEVE	TLVAEEED	DTVKQSTAKY	310		
C1V1b	ISLSYGMYYFHA	AKVYIEAFHTVPKGL	CRELVRVMAW	TFVAVGMFP	VLLGTEG	PGHISPYGSAIGH	SILLDLIAKNNMWG	VLGNYLVRVLIHEHILLYGDIRKKQKITIAGQEMEVE	TLVAEEED	DTVKQSTAKY	344		
ReaChR	ISLSYGMYYFHA	AKVYIEAFHTVPKGL	CRELVRVMAW	TFVAVGMFP	VLLGTEG	PGHISPYGSAIGH	SILLDLIAKNNMWG	VLGNYLVRVLIHEHILLYGDIRKKQKITIAGQEMEVE	TLVAEEED	KYES	350		
bReaChES	ISLSYGMYYFHA	AKVYIEAFHTVPKGL	CRELVRVMAW	TFVAVGMFP	VLLGTEG	PGHISPYGSAIGH	SILLDLIAKNNMWG	VLGNYLVRVLIHEHILLYGDIRKKQKITIAGQEMEVE	TLVAEEED	DTVKQSTAKY	305		
CnChR1	VSCIYGGYMYFQA	AKCYVEANHS	VPKGHC	RMVVKL	MAYAYFAS	WSGYPILWAVG	PEGLLKLSPYANS	IGH	SICDIIAKE	FWTFLAHL	RIKIIHEHILIHGDIRKTTKMEIGGEEVEEVEE	DEEED	352

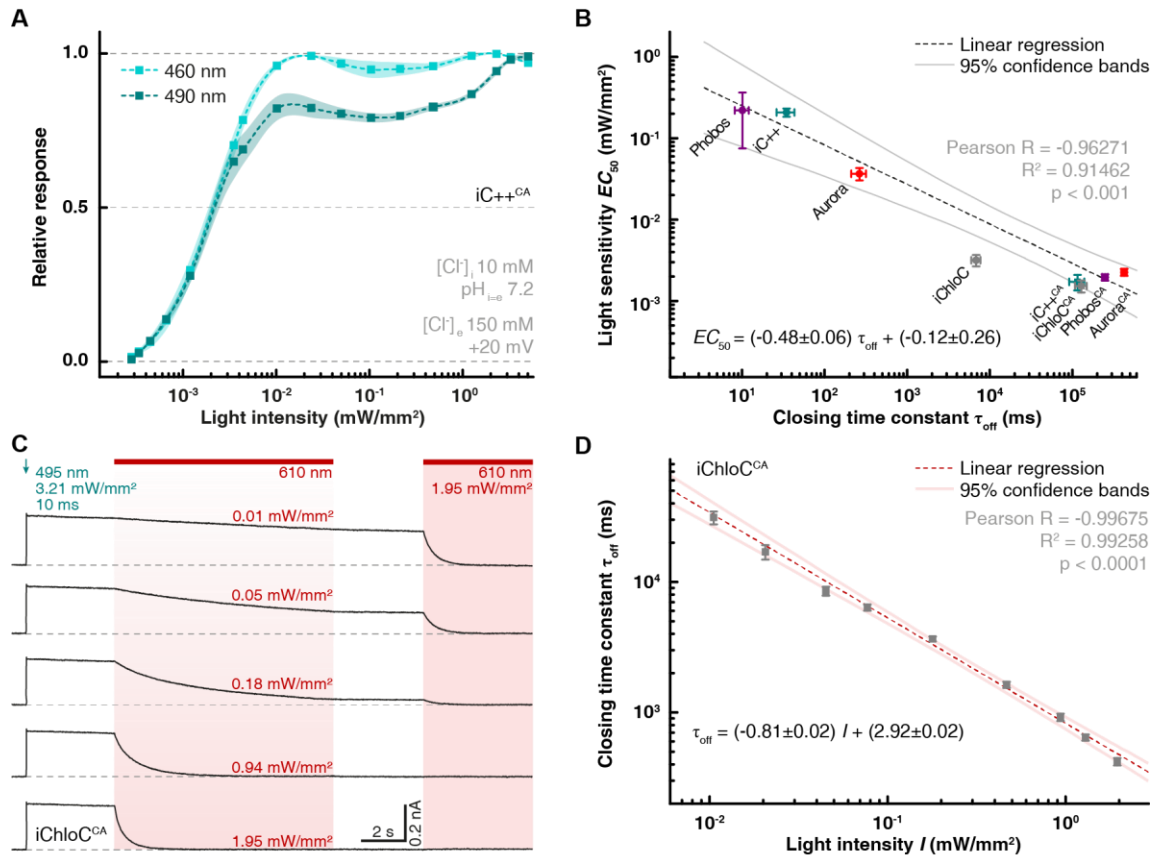
**Figure S1: Sequence comparison of aACRs and CCRs.** Amino acid sequence alignment<sup>1-3</sup> of iChloC (dark gray), iC++ (dark turquoise), Phobos (deep purple), Aurora (dark red) and all cation-conducting ChRs (CCRs) mutated in this study. Mutations yielding the four ACRs are indicated with bold red letters and homologous positions in CCRs are shown in green. In addition, the E90R (bold orange) as well as E83Q and E101S (bold pink) mutations are highlighted in the iChloC sequence. C128, which was mutated to A to generate SFO-ACRs is highlighted in gray. T159G and G163A mutations in blue-shifted opsins are highlighted in purple. The N-termini replaced for the N-terminus of iC++ (dark bold turquoise) are shown in gray. Please note that the Aurora sequence is identical to bReachES<sup>4</sup> except for the introduced mutations. Crossed amino acids were left out for iC++ based approach.



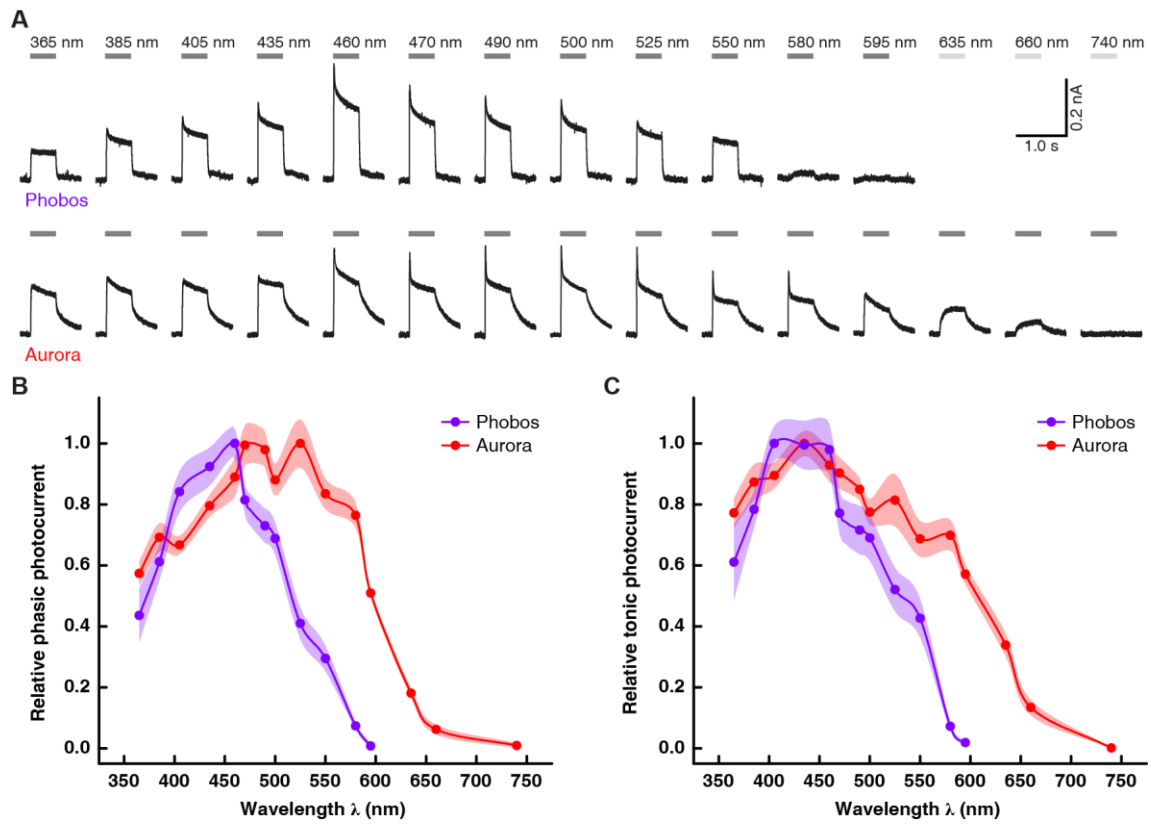
**Figure S2: Screening results of the conversion approaches.** Summary of converted CCRs tested in this study. Epifluorescence images show localization of converted CCRs labeled by mCherry in HEK cells. Corresponding brightfield (BF) images show HEK cell morphology. Photocurrents at holding potentials ranging from -80 to +40 mV (20 mV increments) in low (10 mM, left) or high (150 mM, right) extracellular chloride. The ChloC-strategy is shown in (A), the iC++ approach is shown in (B). Parental CCRs with their mutations are indicated for all constructs. For CCR abbreviations please refer to the main text.



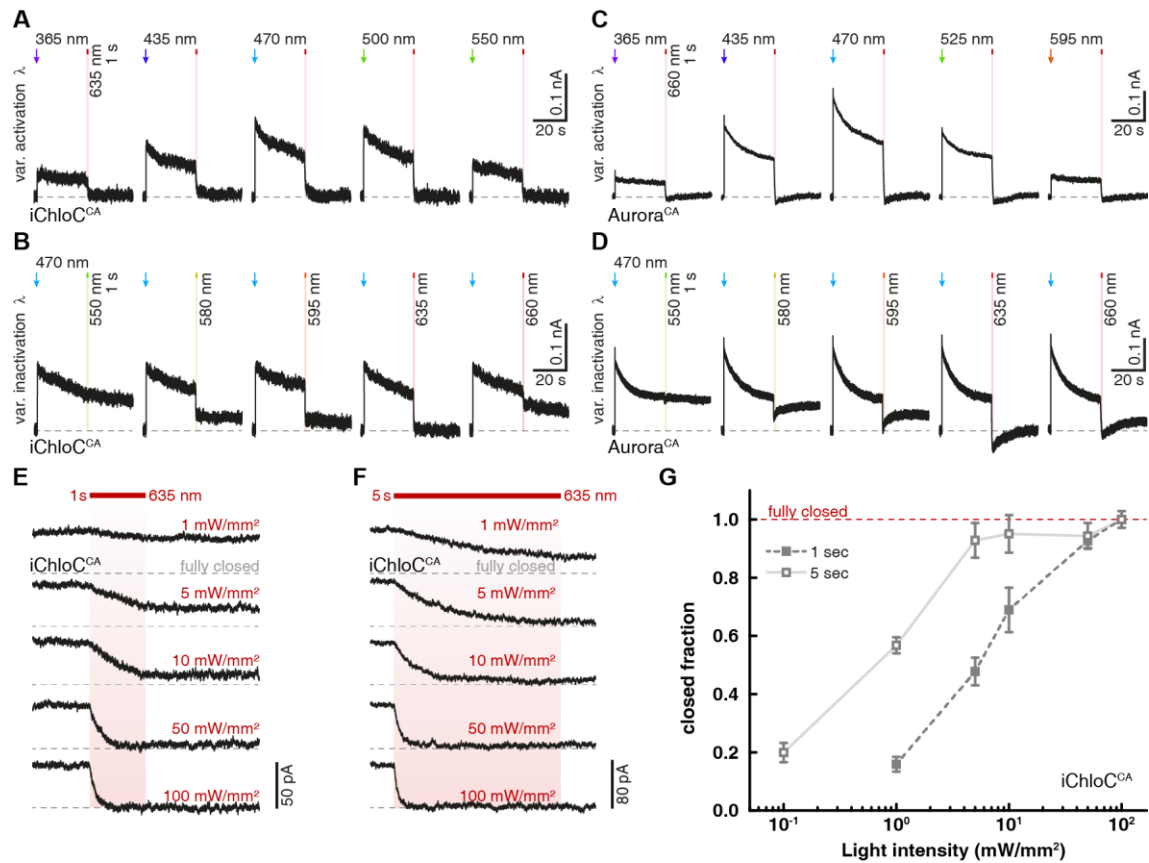
**Figure S3: Characterization of CrChR2 and CoChR converted with the iChloC approach.** Epifluorescence images show localization of converted CCRs labeled by mCherry in HEK cells. Corresponding brightfield (BF) images show HEK cell morphology. Photocurrents at holding potentials ranging from -80 to +40 mV (20 mV increments) in low (10 mM, left) or high (150 mM, right) extracellular chloride.



**Figure S4: Biophysical properties of step-function-aACRs.** (A) Light titration of  $iC_{++}^{CA}$  termed SwitchR++ in,<sup>5</sup> shows reduced partial inactivation for 460 nm ( $n = 3$ ) compared to activation with 490 nm ( $n = 6$ ). (B) Light sensitivity vs. closing time constant of aACR variants linearly correlates except for  $iChloC$  that shows a higher sensitivity compared to other aACRs with respect to its closing time constant. Fitting statistics are listed in the figure panel. (C and D) Dependence of accelerated  $iChloC^{CA}$  channel closing on light dose. (C) Example traces from a single experiment. (D) Data from multiple experiments ( $n = 6$  HEK cells) show a linear relationship between closing speed and light intensity. Fitting statistics are listed in the figure panel.

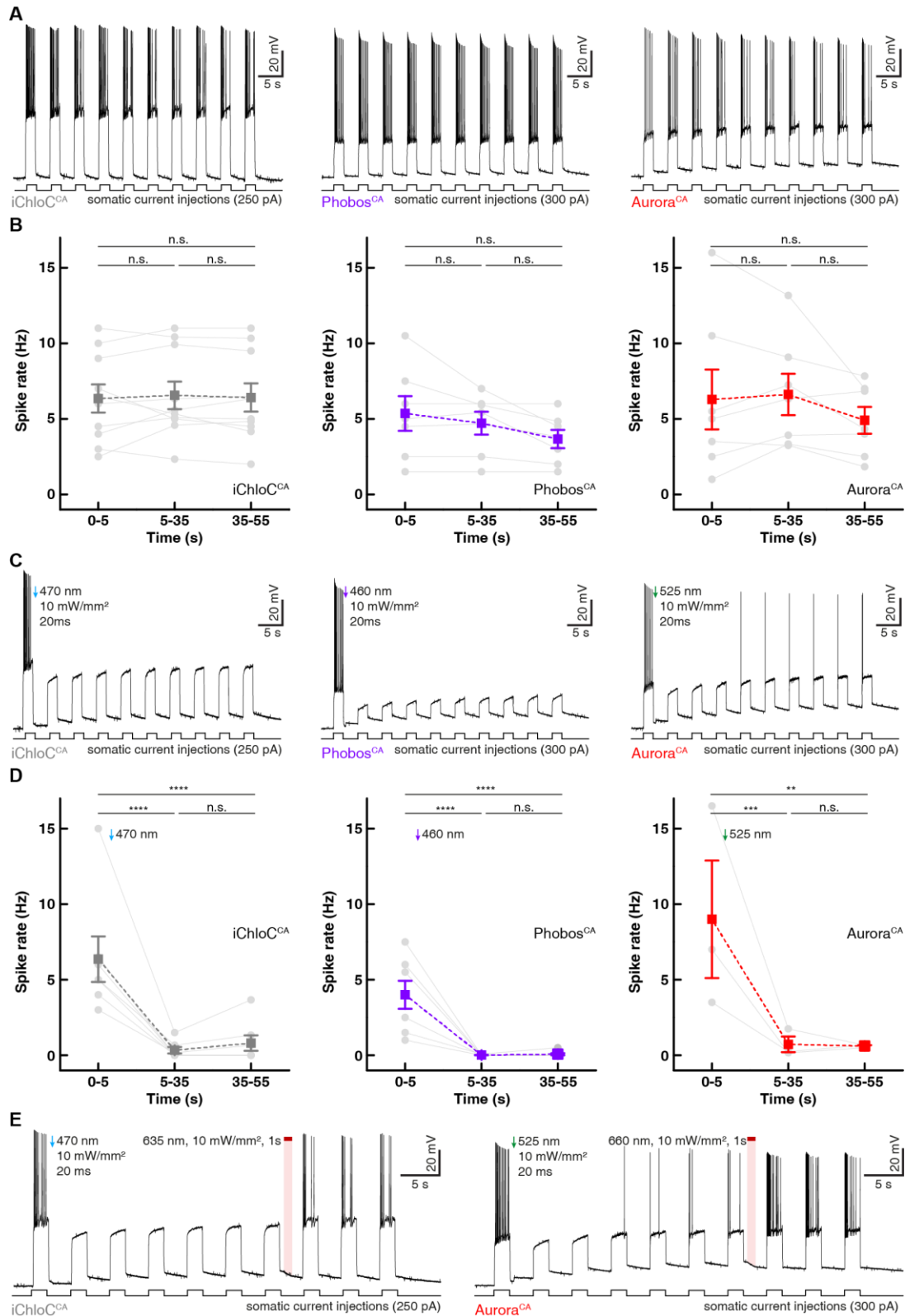


**Figure S5: Action spectra of Phobos and Aurora photocurrents in neurons.** (A) Example traces of Phobos (top row) or Aurora photocurrents (bottom row) evoked at indicated wavelengths (500 ms, 10 mW/mm<sup>2</sup>) in CA1 pyramidal neurons clamped at -50 mV. (B) and (C) Action spectra of normalized peak (B) and tonic (C) Phobos and Aurora photocurrents.



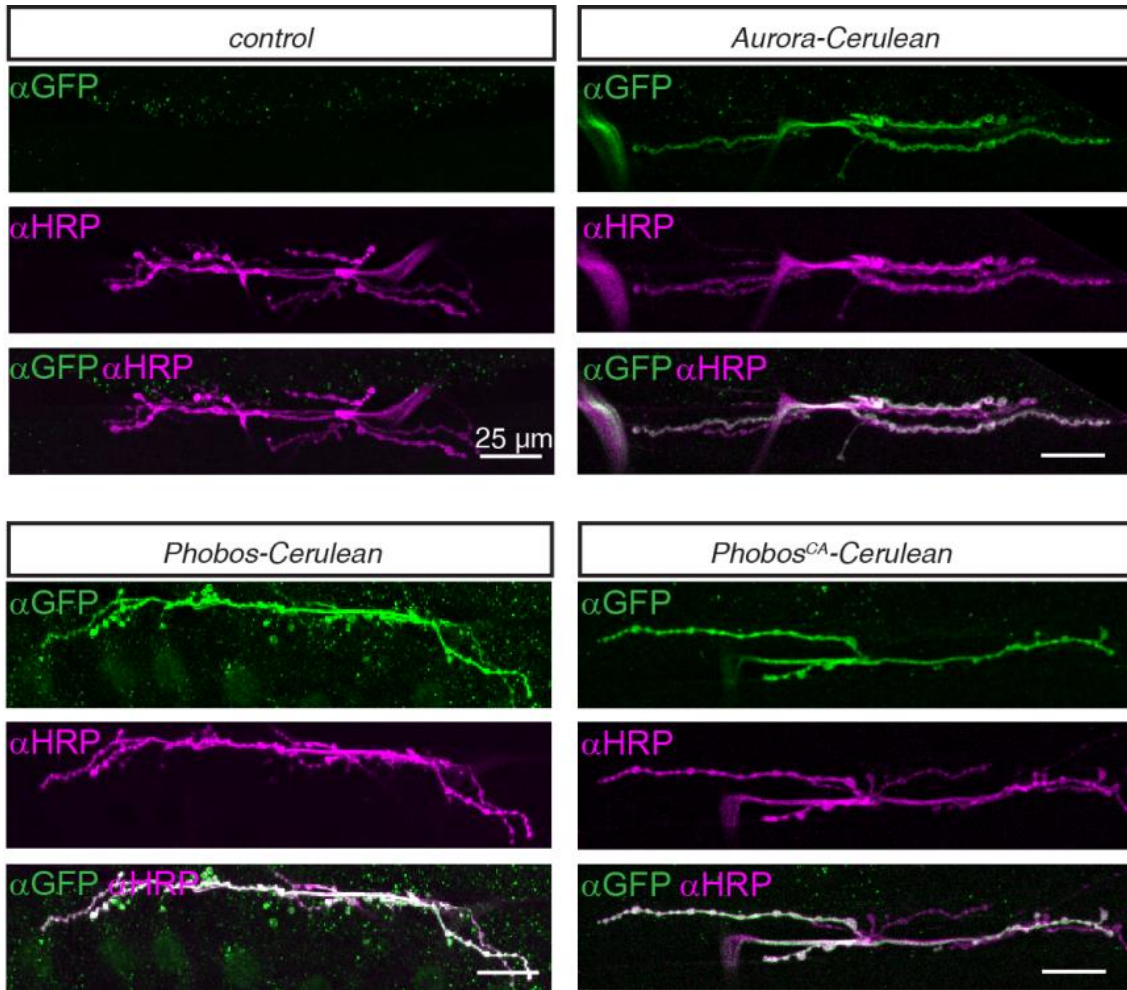
**Figure S6: Light-dependent activation and inactivation of SFO-aACRs in CA1 pyramidal neurons.** (A) Representative recordings of photocurrents in an iChloC<sup>CA</sup>-Citrine expressing CA1 cell. Photocurrents were evoked with different activation wavelengths and shutoff with 635 nm light. (B) Photocurrent traces in the same cell evoked with 470 nm light and shutoff with at indicated wavelengths (10 mW/mm<sup>2</sup>). (C) Representative recordings of photocurrents in an Aurora<sup>CA</sup>-Citrine expressing CA1 cell. Photocurrents were evoked with different activation wavelengths and shutoff with 660 nm light. (D) Photocurrent traces in the same cell evoked with 470 nm light and shutoff with at indicated wavelengths (10 mW/mm<sup>2</sup>). (E) and (F) Example recordings showing light-accelerated channel closing of iChloC<sup>CA</sup>-Citrine in CA1 pyramidal neurons. Channel closing was accelerated by illumination with 635 nm light at indicated powers for 1s (E) or 5s (F). (G) Quantification of experiments shown in (E) and (F). Full channel closing could be achieved with 5 mW/mm<sup>2</sup> over 5 sec or 50 mW/mm<sup>2</sup> over 1 sec (n = 5 neurons in 5 slice cultures). Averages are shown as rectangular symbols with SEM.



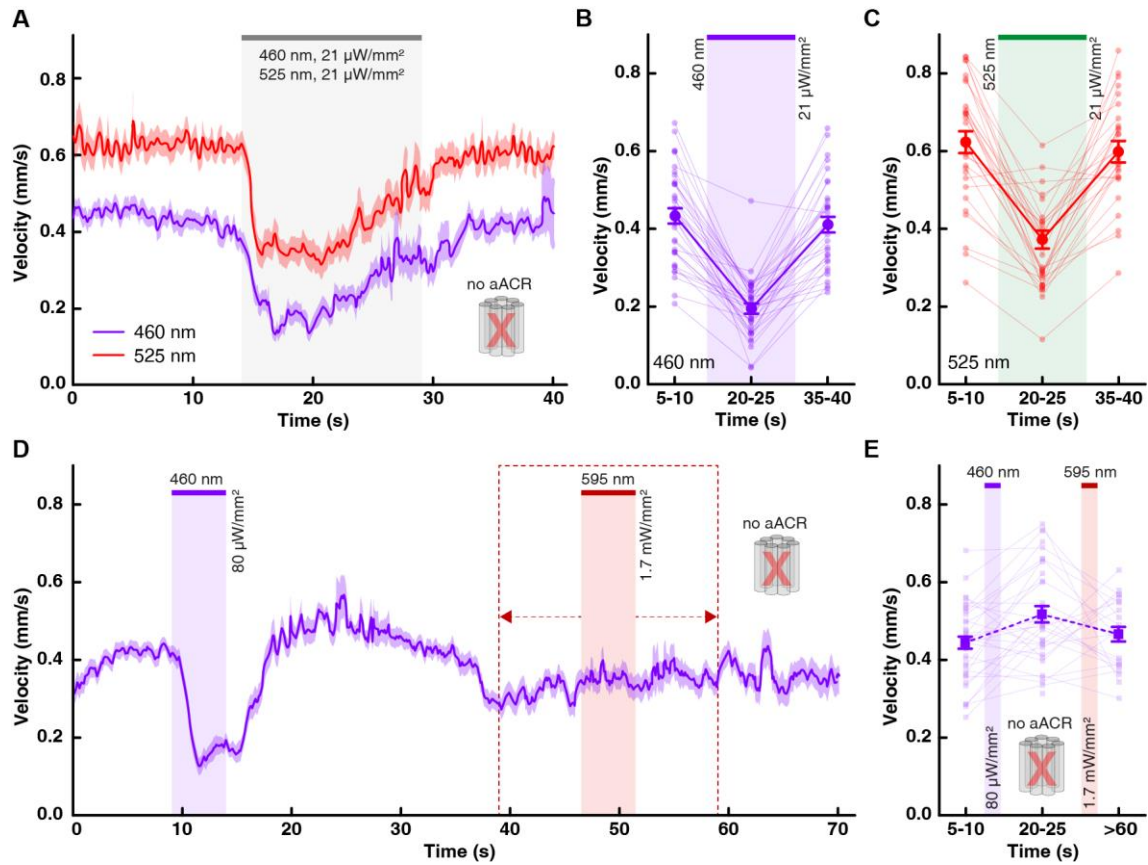


**Figure S7: Performance of SFO-aACRs in CA1 pyramidal neurons.** (A) Membrane voltage traces show reliable depolarization-induced action potential firing of Phobos<sup>CA</sup>-Citrine, iChloC<sup>CA</sup>-Citrine and Aurora<sup>CA</sup>-Citrine expressing CA1 pyramidal neurons in the absence of light. (B) Quantification of the spike rate during current injection at indicated time intervals (n = 10, 7, 5 for iChloC<sup>CA</sup>, Phobos<sup>CA</sup> and Aurora<sup>CA</sup>, respectively, repeated measures one-way ANOVA followed by Tukey's multiple comparisons test). (C) Membrane voltage traces show inhibition of depolarization-induced action potential firing of Phobos<sup>CA</sup>-Citrine, iChloC<sup>CA</sup>-Citrine and Aurora<sup>CA</sup>-Citrine expressing CA1 pyramidal neurons in response to a 20 ms light pulse. (D) Quantification of the spike rate during current injection at indicated time intervals before (0-5 s) and after the light pulse (5-35 and 35-55 s). n = 7, 7, 3 for iChloC<sup>CA</sup>, Phobos<sup>CA</sup> and Aurora<sup>CA</sup>, respectively, \*\*: p < 0.01, \*\*\*: p < 0.001, \*\*\*\*: p < 0.0001, repeated measures one-way ANOVA followed by Tukey's multiple comparisons test). (E) Membrane voltage traces showing reversible suppression of depolarization-induced spiking by photoswitching iChloC<sup>CA</sup> (left) and Aurora<sup>CA</sup> (right) between open and closed state. Light gray symbols indicate individual experiments. Averages are shown as rectangular symbols with SEM.

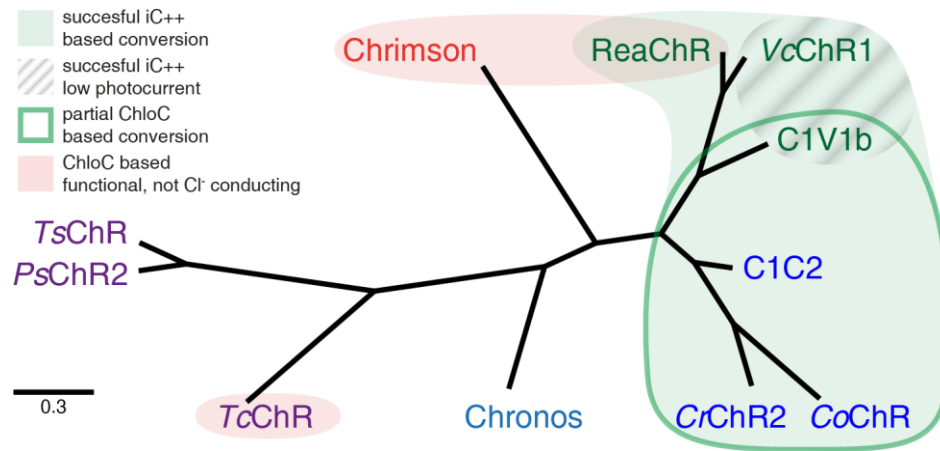




**Figure S8: Expression and localization of Aurora, Phobos and Phobos<sup>CA</sup>.** Representative images of neuromuscular junctions (NMJs) at muscle 6/7 for control, Aurora-Cerulean, Phobos-Cerulean and Phobos<sup>CA</sup>-Cerulean expressing motor neurons (*ok371-Gal4*). aACR expression and localization was visualized by anti-GFP immunohistochemistry together with a neuronal surface marker anti-HRP (horseradish peroxidase). aACRs did not obviously affect NMJ morphology and showed extensive colocalization with  $\alpha$ HRP at the neuronal cell surface. Scale bar: 25 $\mu$ m.



**Figure S9: Innate responses to light during *Drosophila* locomotion behavior.** (A) Larval velocity over time is plotted for control animals ( $w$ ), with 15 s of light stimulation with 460 nm or 525 nm. ( $n = 37$  animals for 460 nm and  $n = 34$  for 525 nm, mean  $\pm$  SEM). (B and C) Averaged velocity from experiments in (A) analyzed in 5 s time bins before (5-10 s), during (20-25 s) and after (35-40 s) exposure to the indicated light intensity and wavelength ( $n = 37$  for 460 nm in B and  $n = 34$  for 525 nm in C, mean  $\pm$  SEM). (D) Velocity of control animals ( $w$ ) over time under the same conditions of light exposure as *Phobos*<sup>CA</sup> using 5 s of 460 nm light exposure and 5 s of 595 nm light ( $n = 38$ , mean  $\pm$  SEM). All animals on an agar plate were sequentially illuminated (5 s each) for channel closing during the time period indicated by the dashed box. (E) Averaged velocity from experiments in (D) analyzed in 5 s time bins before (5 – 10 s), during (20 – 25 s) and after (>60 s) exposure to the indicated light intensity and wavelength ( $n = 37$ , mean  $\pm$  SEM).



**Figure S10: Phylogenetic tree of converted CCRs.** A phylogenetic tree was generated for all CCRs listed in Figure S1 using phylogeny.fr<sup>6</sup>. N-terminal amino acids were excluded up to the position where the N-termini were replaced for the chimeric C2C1 N-terminus of iC++. Successfully converted CCRs cluster in the two right clades. CCR names are color coded according to their spectral absorption.

**Table S1. Neuronal membrane parameters in the dark.**

	wt (n = 10)	SD	Aurora (n = 9)	SD	Phobos (n = 7)	SD	Aurora <sup>CA</sup> (n = 11)	SD	Phobos <sup>CA</sup> (n = 8)	SD	iChloC <sup>CA</sup> (n = 9)	SD	ANOVA
AP threshold (mV)	-50.70	2.47	-46.45	4.17	-44.99	3.73	-42.79	8.06	-47.48	4.29	-46.15	4.89	**
AP peak (mV)	35.82	2.94	32.31	2.47	34.49	3.08	35.82	4.15	32.71	2.23	35.98	4.68	n.s.
AP amplitude (mV)	114.05	5.37	107.57	3.88	108.31	4.71	107.02	7.59	112.14	4.35	115.32	3.65	*
n AP's	13.70	2.93	17.00	2.26	11.29	2.19	8.45	5.25	13.50	3.81	15.33	3.77	*
E <sub>Rest</sub> (mV)	-78.23	4.61	-75.26	2.86	-73.82	3.35	-72.95	6.22	-79.43	3.74	-79.34	3.97	*
R <sub>m</sub> (Mohm)	152.17	67.28	143.40	66.03	147.76	66.62	71.93	18.24	124.25	20.64	172.09	42.10	*

Electrical parameters of untransfected (wt) pyramidal CA1 neurons and neurons expressing the indicated aACR-Citrine fusion construct together with mCerulean. Action potentials (APs) were evoked by a square current pulse (500 ms, 500 pA) in current clamp mode. Threshold, peak voltage and amplitude were calculated for the first AP. Membrane resistance (R<sub>m</sub>) was measured in voltage clamp mode in response to a square voltage pulse (-5 mV, 100 ms). E<sub>rest</sub> = resting membrane potential, S.D. = standard deviation. Right column indicates p-values from one-way ANOVA for each parameter. n.s. no significant differences, \* P < 0.05. Gray field marks value significantly different to non-transfected (wt) neurons. All measurements were liquid junction potential corrected.

## References

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- 3 Sievers, F. *et al.* Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Mol Syst Biol* **7**, 539, doi:10.1038/msb.2011.75 (2011).
- 4 Rajasethupathy, P. *et al.* Projections from neocortex mediate top-down control of memory retrieval. *Nature* **526**, 653-659, doi:10.1038/nature15389 (2015).
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