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5 **BIPOLES is an optogenetic tool developed for bidirectional dual-color control of**
6 **neurons**

7
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32 **Abstract**

33 Optogenetic manipulation of neuronal activity through excitatory and inhibitory opsins has
34 become an indispensable experimental strategy in neuroscience research. For many
35 applications bidirectional control of neuronal activity allowing both excitation and inhibition of the
36 same neurons in a single experiment is desired. This requires low spectral overlap between the
37 excitatory and inhibitory opsin, matched photocurrent amplitudes and a fixed expression ratio.
38 Moreover, independent activation of two distinct neuronal populations with different optogenetic
39 actuators is still challenging due to blue-light sensitivity of all opsins. Here we report BiPOLES,
40 an optogenetic tool for potent neuronal excitation and inhibition with light of two different
41 wavelengths. BiPOLES enables sensitive, reliable dual-color neuronal spiking and silencing with
42 single- or two-photon excitation, optical tuning of the membrane voltage, and independent
43 optogenetic control of two neuronal populations using a second, blue-light sensitive opsin. The
44 utility of BiPOLES is demonstrated in worms, flies, mice and ferrets.

45

46

47 **Introduction**

48 To prove necessity and sufficiency of a particular neuronal population for a specific behavior, a
49 cognitive task, or a pathological condition, faithful activation and inhibition of this population of
50 neurons is required. In principle, optogenetic manipulations allow such interventions. However,
51 excitation and inhibition of the neuronal population of interest is commonly done in separate
52 experiments, where either an excitatory or inhibitory microbial opsin is expressed. Alternatively,
53 if both opsins are co-expressed in the same cells, it is essential to achieve efficient membrane
54 trafficking of both opsins, equal subcellular distributions, and a tightly controlled ratio between
55 excitatory and inhibitory action at the specific wavelengths and membrane potentials, so that
56 neuronal activation and silencing can be controlled precisely and predictably in all transduced
57 cells. Precise co-localization of the two opsins is important when local, subcellular stimulation is
58 required, or when control of individual neurons is intended, for example with 2-photon
59 holographic illumination¹. Meeting these criteria is particularly challenging *in vivo*, where the
60 optogenetic actuators are either expressed in transgenic lines or from viral vectors that are
61 exogenously transduced. Ideally, both opsins are expressed from the same gene locus or
62 delivered to the target neurons by a single viral vector. Moreover, for expression with fixed
63 stoichiometry, the opsins should be encoded in a single open reading frame (ORF).

64 Previously, two strategies for stoichiometric expression of an inhibitory and an excitatory opsin
65 from a single ORF were reported using either a gene fusion approach² or a 2A ribosomal skip
66 sequence^{3,4}. In both cases, a blue-light sensitive cation-conducting channel for excitation was
67 combined with a red-shifted rhodopsin pump for inhibition. The gene fusion approach was used
68 to systematically combine the inhibitory ion pumps halorhodopsin (NpHR), bacteriorhodopsin
69 (BR) or archaerhodopsin (Arch) with a number of channelrhodopsin-2 (ChR2) mutants to
70 generate single tandem-proteins². While this strategy ensured co-localized expression of the
71 inhibitory and excitatory opsins at a one-to-one ratio and provided important mechanistic
72 insights in their relative ion-transport rates, membrane trafficking was not as efficient as with
73 individually expressed opsins, thus limiting the potency of these fusion constructs for reliable
74 control of neuronal activity.

75 The second strategy employed a 2A ribosomal skip sequence³ to express the enhanced opsins
76 ChR2(H134R)⁵ and eNpHR3.0 as independent proteins at a fixed ratio from the same RNA⁴.
77 These bicistronic constructs, termed eNPAC, and eNPAC2.0⁶, were used for bidirectional
78 control of neuronal activity in various brain regions in mice⁶⁻⁹. While membrane trafficking of the
79 individual opsins is more efficient compared to the gene fusion strategy, the expression ratio
80 might still vary from cell to cell. Moreover, subcellularly targeted co-localization (e.g. at the
81 soma) is not easily achieved. Finally, functionality is limited in some model organisms such as
82 *D. melanogaster*, since rhodopsin pumps are not efficient in these animals^{10,11}.

83 In addition to activation and inhibition of the same neurons, also independent optogenetic
84 activation of two distinct neuronal populations is still challenging. Although two spectrally distinct
85 opsins have been combined previously to spike two distinct sets of neurons¹²⁻¹⁵, careful
86 calibration and dosing of blue light was required to avoid activation of the red-shifted opsin. This
87 typically leaves only a narrow spectral and energetic window to activate the blue- but not the
88 red-light-sensitive rhodopsin. Thus, dual-color control of neurons is particularly challenging in
89 the mammalian brain where irradiance decreases by orders of magnitude over a few millimeters
90 in a wavelength-dependent manner^{16,17}.

91 In order to overcome current limitations for bidirectional neuronal manipulations and to facilitate
92 spiking of neuronal populations with orange-red light exclusively, in this work we systematically
93 explore the generation of two-channel fusion proteins that combine red-light activated cation-
94 channels and blue-light activated anion-channels enabling neuronal spiking and inhibition with
95 red and blue light, respectively. With respect to previous bidirectional tools, inversion of the
96 excitatory and inhibitory action spectra restricts depolarization to a narrow, orange-red spectral
97 window since the inhibitory opsin compensates the blue-light-activated currents of the excitatory
98 red-shifted channel. We show that among all tested variants, a combination of *GtACR2*¹⁸ and
99 Chrimson¹² termed BiPOLES (for Bidirectional Pair of Opsins for Light-induced Excitation and
100 Silencing) proves most promising and allows 1) potent and reliable blue-light-mediated silencing
101 and red-light-mediated spiking of pyramidal neurons in hippocampal slices; 2) bidirectional
102 control of single neurons with single-photon illumination and 2-photon holographic stimulation;
103 3) dual-color control of two distinct neuronal populations in combination with a second blue-
104 light-sensitive ChR without cross-talk at light intensities spanning multiple orders of magnitude;
105 4) precise optical tuning of the membrane voltage between the chloride and cation reversal
106 potentials; 5) bidirectional manipulations of neuronal activity in a wide range of invertebrate and
107 vertebrate model organisms including worms, fruit flies, mice and ferrets.

108

109 **Results**

110 **Engineering of BiPOLES and biophysical characterization in HEK cells**

111 To identify suitable combinations of opsins for potent membrane voltage shunting or
112 depolarization with blue and red light, respectively, we combined the blue- or green-light
113 sensitive anion-conducting channelrhodopsins (ACRs) Aurora¹¹, iC++¹⁹, *GtACR1* and *GtACR2*¹⁸
114 with the red-light sensitive cation-conducting channelrhodopsin (CCR) Chrimson¹²; or
115 conversely, the blue-light sensitive *GtACR2* with the red-light sensitive CCRs bReaChES²⁰, f-
116 Chrimson, vf-Chrimson²¹ and ChRmine²² (Fig. 1a). We fused these opsin-pairs with different
117 linkers, expanding previous rhodopsin fusion strategies^{2,23} to obtain optimal expression and
118 membrane targeting. The linkers were composed of the Kir2.1 membrane trafficking signal
119 (TS)⁴, different arrangements of a cyan or yellow fluorescent protein, and the transmembrane β
120 helix of the rat gastric H⁺/K⁺ ATPase (β HK) to maintain correct membrane topology of both
121 opsins² (Fig. 1a).

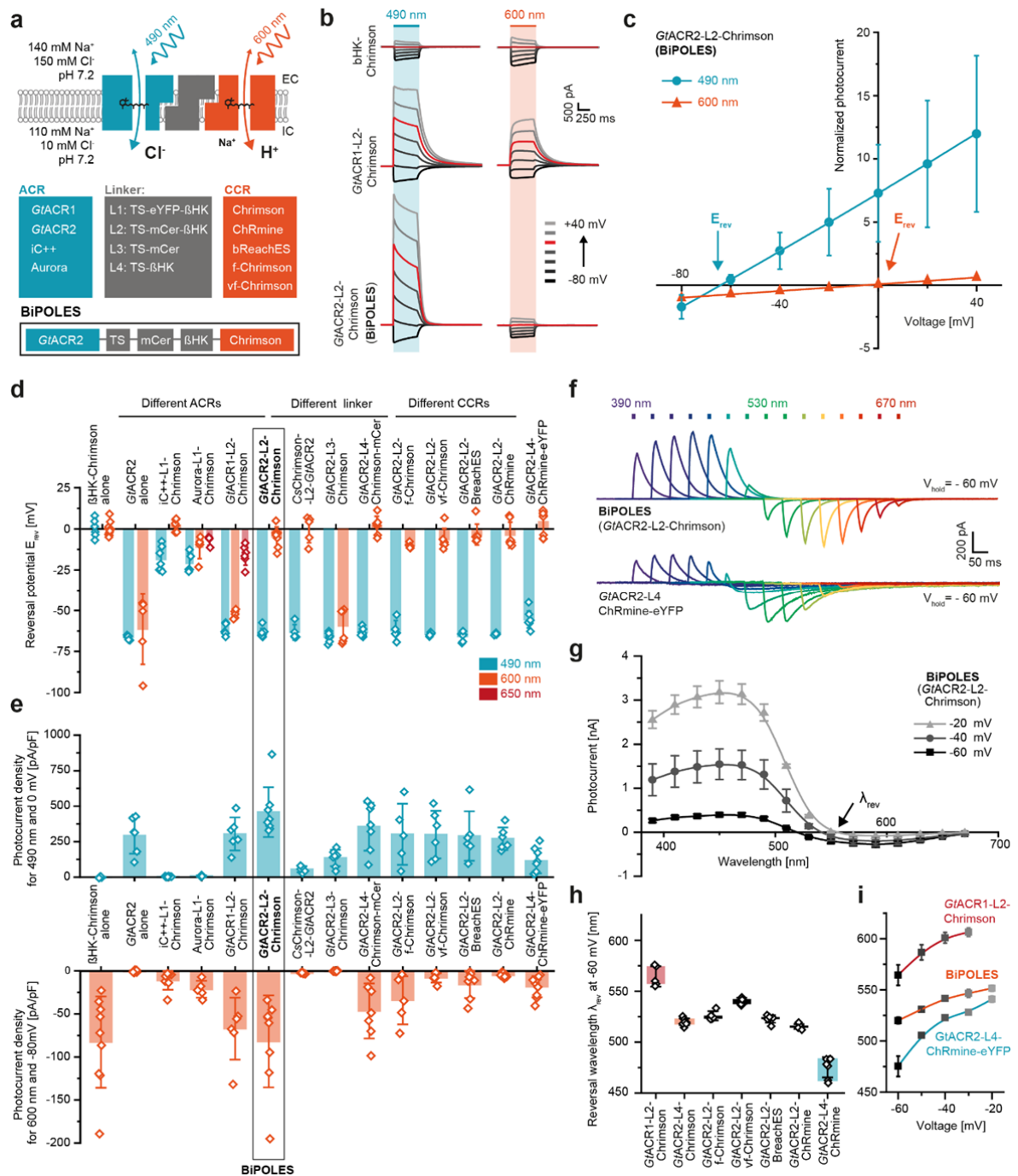
122 For a detailed biophysical evaluation, we expressed all ACR-CCR tandems in human embryonic
123 kidney (HEK) cells and recorded blue- and red-light evoked photocurrents in the presence of a
124 chloride gradient. In all constructs, except the one lacking the β HK-subunit (L3, Fig. 1a), blue-
125 light-activated currents were shifted towards the chloride Nernst potential whereas red-light-
126 activated currents were shifted towards the Nernst potential for protons and sodium (Fig.1b-d,
127 Supplementary Fig. 1), indicating functional membrane insertion of both channels constituting
128 the tandem constructs. Reversal potentials (Fig. 1d) and photocurrent densities (Fig. 1e) varied
129 strongly for the different tandem variants indicating considerable differences in their wavelength-
130 specific anion/cation conductance ratio and their membrane expression. Photocurrent densities
131 were not only dependent on the identity of the fused channels, but also on the sequence of both
132 opsins in the fusion construct as well as the employed fusion linker. In contrast to a previous
133 study², the optimized linker used in this study did not require a fluorescent protein to preserve
134 functionality of both channels (L4, Fig. 1a,d,e). Direct comparison of red- and blue-light evoked
135 photocurrent densities with those of β HK-Chrimson and *GtACR2* expressed alone indicated that
136 most tandem constructs harboring a *GtACR* reached similar membrane expression efficacy as
137 the individually expressed channels (Fig. 1e).

138 At membrane potentials between the Nernst potentials for chloride and protons, blue and red
139 light induced outward and inward currents, respectively in all *GtACR*-fusion constructs. (Fig.1e-
140 g, Supplementary Fig. 1). The specific wavelength of photocurrent inversion (λ_{rev}) was
141 dependent on the absorption spectra and relative conductance of the employed channels as
142 well as on the relative ionic driving forces defined by the membrane voltage and the respective
143 ion gradients (Fig.1g-i). The red-shift of λ_{rev} for the vf-Chrimson-i tandem compared to BiPOLES
144 reflects the reduced conductance of this Chrimson mutant (Fig. 1h, Supplementary Fig. 1c), as

145 already previously shown^{21,24}, whereas the blue-shift of λ_{rev} for the ChRmine tandem with L4
146 (Fig. 1f,h) is explained by the blue-shifted activation spectrum of ChRmine compared to
147 Chrimson²⁵ and its presumably large single channel conductance. Switching the L4 linker to L2
148 shifted λ_{rev} to longer wavelengths for the ChRmine fusion constructs at the expense of ChRmine
149 photocurrents (Fig. 1e,h), pointing to a stronger impact of the protein linker on the ChRmine
150 photocurrent compared to other red-shifted CCRs (Fig. 1e).

151 Among all tested combinations, *GtACR2*-L2-Chrimson – from here on termed BiPOLES – was
152 the most promising variant. First, it showed the largest photocurrent densities of all tested fusion
153 constructs (Fig. 1e,f), second, reversal potentials for blue or red light excitation were close to
154 those of individually expressed channels (-64 ± 3 mV and -5 ± 6 mV for BiPOLES compared to -
155 66 ± 2 mV and 0 ± 5 mV of *GtACR2* and β HK-Chrimson expressed alone, Fig. 1c,d,
156 Supplementary Fig. 1b) and third, peak activity of the inhibitory anion and excitatory cation
157 current had the largest spectral separation among all tested variants (150 ± 5 nm, Fig. 1f,g).
158 Thus, BiPOLES enables selective activation of large anion and cation currents with spectrally
159 well-separated wavelengths (Fig. 1e). BiPOLES was remarkably better expressed in HEK-cells
160 than the previously reported ChR2-L1-NpHR fusion construct² and featured larger photocurrents
161 at -60 mV than the bistronic construct eNPAC2.0⁶ (Supplementary Fig. 2 a-c). Moreover,
162 employing an anion channel with high conductance instead of a chloride pump, which transports
163 one charge per absorbed photon and is weak at negative voltage, yielded chloride currents in
164 BiPOLES expressing cells at irradiances 2 orders of magnitude lower than with eNPAC2.0
165 (Supplementary Fig. 2 d-f). Anion conductance in BiPOLES was sufficiently large to
166 compensate inward currents of Chrimson even at high irradiance, driving the cell back to the
167 chloride Nernst potential, which is close to the resting membrane voltage (Supplementary Fig. 2
168 d-f). We further verified the implementation of an anion-conducting channel by testing whether
169 sufficient blue-light hyperpolarization could be achieved with a rhodopsin pump²⁶ instead of a
170 channel. Replacing *GtACR2* with a putatively potent blue-light sensitive pump led to barely
171 detectable outward currents at the same irradiance due to low ion turnover of the proton pump
172 under the given voltage and ion conditions (Supplementary Fig. 2 d,g).

173



174

175 **Figure 1: Development of BiPOLES and biophysical characterization.** (a) Molecular scheme of
 176 BiPOLES with the extracellular (EC) and intracellular (IC) ionic conditions used for HEK293-cell
 177 recordings. The blue-green-light-activated natural anion channels *GtACR1* and *GtACR2* or the
 178 engineered ChR-chimeras iC++ and Aurora were fused to the red-light-activated cation channels
 179 Chrimson, ChRmine, bReaChES, f-Chrimson or vf-Chrimson by different transmembrane spanning linker
 180 regions consisting of a trafficking signal (ts), a yellow or cyan fluorescent protein (eYFP, mCerulean3)
 181 and the βHK transmembrane fragment. The fusion construct termed BiPOLES is indicated by a black
 182 frame. (b) Representative photocurrents of βHK-Chrimson-mCerulean (top), *GtACR1*-ts-mCerulean-βHK-
 183 Chrimson (middle) *GtACR2*-ts-mCerulean-βHK-Chrimson (BiPOLES, bottom) in whole-cell patch clamp
 184 recordings from HEK293 cells at 490 nm and 600 nm illumination. (c) Normalized peak photocurrents of
 185 BiPOLES at different membrane voltages evoked at either 490 or 600 nm (see panel b, mean ± SD; n = 8
 186 independent cells; normalized to the peak photocurrent at -80 mV and 600 nm illumination). (d) Reversal

187 potential of peak photocurrents during 500-ms illumination with 490, 600, or 650 nm light as shown in (b)
188 (mean \pm SD). **(e)** Peak photocurrent densities for 490 nm and 600 nm excitation at 0 mV (close to the
189 reversal potential of protons and cations) and -80 mV (close to the reversal potential for chloride)
190 measured as shown in (b) (mean \pm SD; for both (d) and (e) $n = 5$ biological independent cells for Aurora-
191 L1-Chrimson, CsChrimson-L2-*GtACR2* and *GtACR2*-L2-f-Chrimson; $n = 6$ for *GtACR2*, *GtACR1*-L2-
192 Chrimson and *GtACR2*-L2-vf-Chrimson; $n = 7$ for iC++-L1-Chrimson, *GtACR2*-L3-Chrimson, *GtACR2*-L4-
193 Chrimson-mCer, *GtACR2*-L2-BreachES and *GtACR2*-L2-ChRmine; $n = 8$ for *GtACR2*-L2-Chrimson and n
194 = 9 for β HK-Chrimson and *GtACR2*-L4-ChRmine-ts-eYFP-er). **(f)** Representative photocurrents of
195 BiPOLES (top) and *GtACR2*-L4-ChRmine-ts-eYFP-er (bottom) with 10 ms light pulses at indicated
196 wavelengths and equal photon flux at -60 mV. **(g)** Action spectra of BiPOLES at different membrane
197 voltages (λ_{rev} = photocurrent reversal wavelength, mean \pm SEM, $n = 9$ independent cells for -60 mV, $n = 4$
198 for -40 mV and $n = 2$ for -20 mV). **(h)** Photocurrent reversal wavelength λ_{rev} at -60 mV (mean \pm SD, $n = 5$
199 independent cells for *GtACR1*-L2-Chrimson and *GtACR2*-L2-f-Chrimson, $n = 6$ for *GtACR2*-L2-vf-
200 Chrimson and *GtACR2*-L2-ChRmine, $n = 7$ for *GtACR2*-L4-ChRmine-ts-eYFP-er, $n = 8$ for *GtACR2*-L2-
201 BreachES and $n = 9$ for *GtACR2*-L2-Chrimson). **(i)** λ_{rev} of *GtACR1*-L2-Chrimson, BiPOLES and *GtACR2*-
202 L4-ChRmine-TS-eYFP-ER at different membrane voltages (mean \pm SD; $n = 5$ biological independent
203 cells for *GtACR1*-L2-Chrimson, $n = 7$ for *GtACR2*-L4-ChRmine-ts-eYFP-er and $n = 9$ for *GtACR2*-L2-
204 Chrimson). The data presented in this figure are provided in the Source Data file.

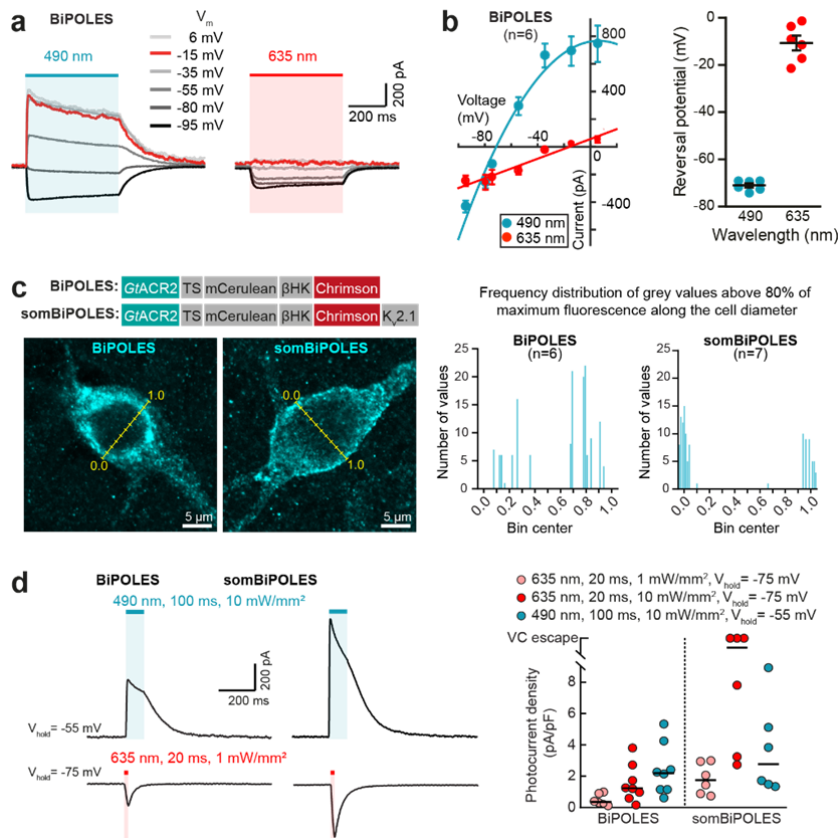
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206 **Evaluation of BiPOLES in CA1 pyramidal neurons**

207 Next, we validated BiPOLES as an optogenetic tool for bidirectional control of neuronal activity.
208 In CA1 pyramidal neurons of rat hippocampal slice cultures, illumination triggered photocurrents
209 with biophysical properties similar to those observed in HEK cells (Fig. 2a,b, Supplementary
210 Fig. 3a-c). We observed membrane-localized BiPOLES expression most strongly in the
211 somatodendritic compartment (Fig. 2c, Supplementary Fig. 3d). However, some fraction of the
212 protein accumulated inside the cell in the periphery of the cell nucleus, indicating sub-optimal
213 membrane trafficking of BiPOLES. To enhance membrane trafficking, we generated a soma-
214 targeted variant (somBiPOLES) by attaching a C-terminal Kv2.1-trafficking sequence²⁷. Soma
215 targeting has the additional benefit of avoiding expression of the construct in axon terminals,
216 where functionality of BiPOLES might be limited due to an excitatory chloride reversal potential
217 and subsequent depolarizing action of *GtACR2*^{28,29}. somBiPOLES showed strongly improved
218 membrane localization restricted to the cell soma and in proximal dendrites with no detectable
219 intracellular accumulations (Fig. 2c, Supplementary Fig. 3d). Compared to BiPOLES, blue- and
220 red-light mediated photocurrents were enhanced and now similar in magnitude to those in
221 neurons expressing either Chrimson or soma-targeted *GtACR2* (som*GtACR2*), alone (Fig. 2d,
222 Supplementary Fig. 4a, 5a,b). Passive and active membrane parameters of BiPOLES- and
223 somBiPOLES-expressing neurons were similar to non-transduced, wild-type neurons
224 (Supplementary Fig. 6), indicative of good tolerability in neurons.

225 To verify the confinement of somBiPOLES to the somatodendritic compartment despite the
226 improved expression, we virally transduced area CA3 in hippocampal slice cultures with
227 somBiPOLES and recorded optically evoked EPSCs in postsynaptic CA1 cells. Local
228 illumination with red light in CA3 triggered large excitatory postsynaptic currents (EPSCs), while

229 local red illumination of axon terminals in CA1 (635 nm, 2 pulses of 5 ms, 40 ms ISI, 50 mW
 230 mm²), did not trigger synaptic release, indicating absence of somBiPOLES from axonal
 231 terminals (Supplementary Fig. 3e,f). Thus, despite enhanced membrane trafficking,
 232 somBiPOLES remained confined to the somatodendritic compartment.



233

234 **Figure 2: Expression and functional characterization of BiPOLES and somBiPOLES in**
 235 **hippocampal neurons.** (a) Representative photocurrent traces of BiPOLES in CA1 pyramidal neurons at
 236 indicated membrane voltages (V_m : from -95 to +6 mV) upon illumination with 490 or 635 nm (500 ms, 10
 237 mW mm⁻²). (b) Left: quantification of photocurrent-voltage relationship (symbols: mean \pm SEM, n = 6
 238 cells, lines: polynomial regression fitting, $R^2 = 0.98$ and 0.94 , for 490 and 635 nm, respectively). Right:
 239 reversal potential under 490 or 635 nm illumination (black lines: mean \pm SEM, n = 6 cells). (c) Left:
 240 Molecular scheme of BiPOLES and somBiPOLES as used in neurons. Representative maximum-intensity
 241 projection images of immunostainings showing expression of BiPOLES or soma-targeted BiPOLES
 242 (somBiPOLES) in CA3 pyramidal neurons of organotypic hippocampal slices. Yellow lines indicate the
 243 bins used to measure fluorescence intensity along the cell equator. Right: Frequency distribution of grey
 244 values above 80% of the maximum fluorescence intensity measured along the cell diameter in BiPOLES-
 245 (n = 6 cells) and somBiPOLES-expressing CA3 cells (n = 7 cells). Note improved trafficking of
 246 somBiPOLES to the cell membrane, shown by the preferential distribution of brighter pixels around bins
 247 0.0 and 1.0. (d) Left: Representative photocurrent traces measured in BiPOLES- or somBiPOLES-
 248 expressing CA1 pyramidal neurons. Inward cationic photocurrents evoked by a 635 nm light pulse (20
 249 ms, 1 mW mm⁻²) were recorded at a membrane voltage of -75 mV, and outward anionic photocurrents
 250 evoked by a 490 nm light pulse (100 ms, 10 mW mm⁻²) were recorded at a membrane voltage of -55 mV.
 251 Right: Quantification of photocurrent densities evoked under the indicated conditions. Note that
 252 photocurrent densities were strongly enhanced for somBiPOLES compared to BiPOLES (black horizontal
 253 lines: medians, $n_{BiPOLES} = 8$ cells, $n_{somBiPOLES} = 6$ cells). The data presented in this figure are provided in the
 254 Source Data file.

255

256

257 Having shown that somBiPOLES is efficiently expressed in CA1 pyramidal cells, we next
258 systematically benchmarked light-evoked spiking and inhibition parameters for somBiPOLES by
259 direct comparison to Chrimson or somGtACR2 expressed in hippocampal CA1 pyramidal
260 neurons, respectively (Fig. 3, Supplementary Figs. 4,5). To compare spiking performance in
261 somBiPOLES or Chrimson expressing CA1 pyramidal cells, we delivered trains of 5-ms blue
262 (470 nm), orange (595 nm) or red (635 nm) light pulses at irradiances ranging from 0.1 to 100
263 mW mm^{-2} . Action potential (AP) probability in somBiPOLES neurons reached 100% at 0.5 mW
264 mm^{-2} with 595 nm and 10 mW mm^{-2} with 635 nm light, similar to neurons expressing Chrimson
265 alone (Fig. 3b,c). In pyramidal cells, APs could be reliably driven up to 10-20 Hz with
266 somBiPOLES (Supplementary Fig. 7c) similar to Chrimson alone, as shown previously¹².
267 Delivering the same number of photons in a time range of 1 - 25 ms did not alter the AP
268 probability, but longer pulses increased sub-threshold depolarization (Supplementary Fig. 7d).

269 In contrast to orange or red light, APs were not evoked at any blue light irradiance in
270 somBiPOLES neurons due to the activity of the blue-light sensitive anion channel. On the contrary,
271 neurons expressing Chrimson alone reached 100% AP firing probability at 10 mW mm^{-2} with
272 470 nm (Fig. 3b,c). Using light ramps with gradually increasing irradiance enabled us to
273 precisely determine the AP threshold and to quantitatively compare spiking efficacy of different
274 excitatory opsins. The irradiance threshold for the first AP was similar for somBiPOLES and
275 Chrimson at 595 nm ($0.74 \pm 0.06 \text{ mW mm}^{-2}$ for somBiPOLES and $0.68 \pm 0.05 \text{ mW mm}^{-2}$ for
276 Chrimson) reflecting that the functional expression levels were similar. In contrast, blue light
277 triggered APs at $0.95 \pm 0.09 \text{ mW mm}^{-2}$ in Chrimson expressing cells, but never in somBiPOLES
278 or BiPOLES neurons (Fig. 3d,e, Supplementary Fig. 7a,b). Thus, somBiPOLES enables
279 neuronal excitation exclusively within a narrow spectral window restricted to orange-red light,
280 avoiding inadvertent blue-light mediated spiking.

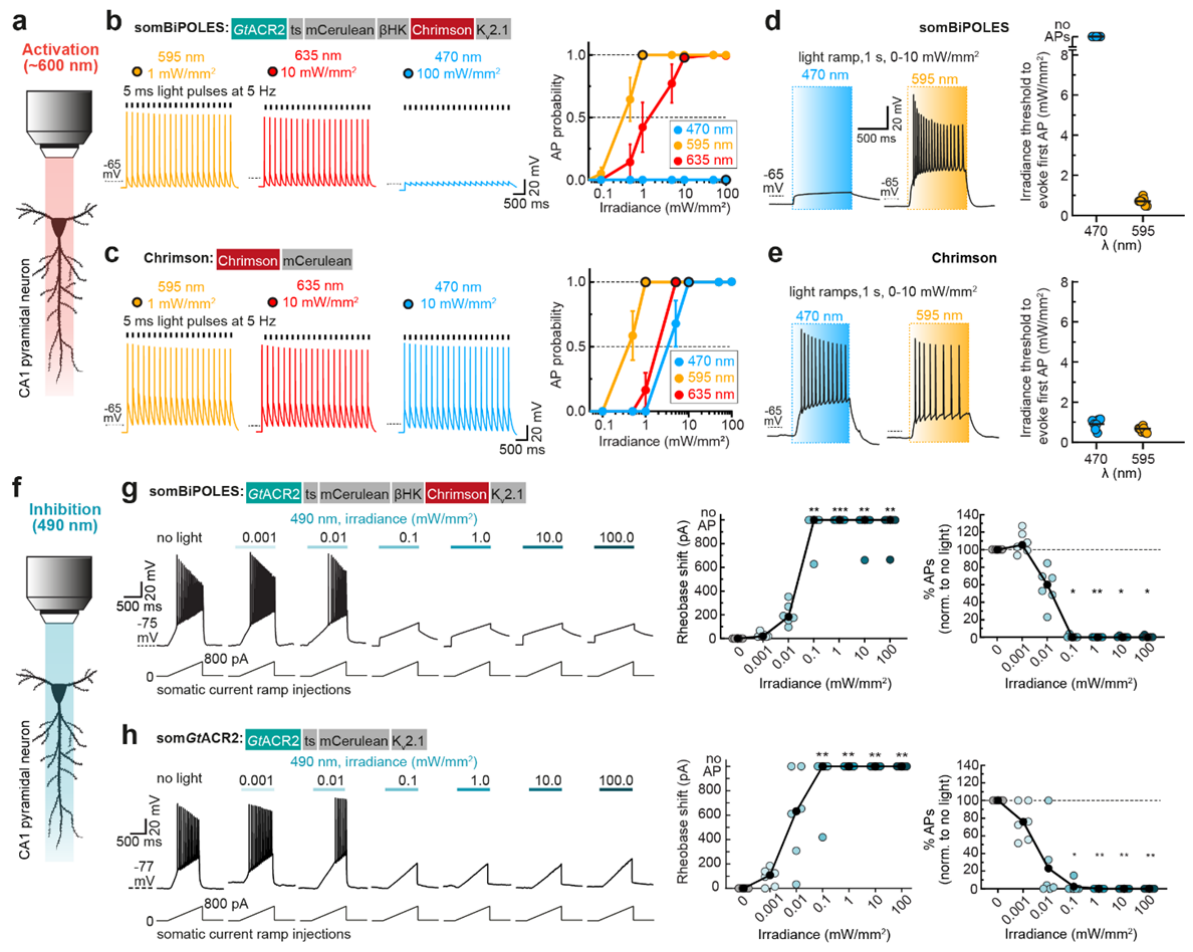
281 Next, we quantified the silencing capacity of somBiPOLES and compared it to somGtACR2
282 alone – the most potent opsin for blue-light mediated somatic silencing^{28,29} – by measuring the
283 capacity to shift the threshold for electrically evoked APs (i.e. rheobase, see Methods). Both
284 variants similarly shifted the rheobase towards larger currents starting at an irradiance of 0.1
285 mW mm^{-2} with 490 nm light, leading to a complete block of APs in most cases (Fig. 3g,h).
286 Neuronal silencing was efficient under 490 nm-illumination, even at high irradiances (up to 100
287 mW mm^{-2} Fig. 3g), showing that blue light cross-activation of Chrimson in somBiPOLES did not
288 compromise neuronal shunting.

289 We compared somBiPOLES with eNPAC2.0, the most advanced optogenetic tool currently
290 available for dual-color excitation and inhibition^{4,6,7}. In eNPAC2.0 expressing CA1 pyramidal

291 neurons, depolarizing and hyperpolarizing photocurrents were present under blue and
292 yellow/orange light, respectively (Supplementary Fig. 8a), consistent with its inverted action
293 spectrum compared to BiPOLES (Supplementary Fig. 2). Compared to BiPOLES
294 (Supplementary Fig. 3c) peak photocurrent ratios were more variable between cells
295 (Supplementary Fig. 8a), indicative of different stoichiometries between ChR2(HR) and
296 eNpHR3.0 in different neurons, probably because membrane trafficking and degradation of both
297 opsins occur independently. Moreover, blue-light-evoked spiking with eNPAC2.0 required
298 approx. 10-fold higher irradiance compared to somBiPOLES and did not reach 100% reliability
299 (Supplementary Fig. 8c), which might be explained by cross-activation of eNpHR3.0 under high
300 blue irradiance (see also Supplementary Fig. 2d). Blue-light-triggered APs could not be reliably
301 blocked with concomitant yellow illumination at 10 mW mm⁻² (Supplementary Fig. 8b). Further
302 on, activation of eNPAC2.0 (i.e., eNpHR3.0) with yellow light (580 nm) caused strong
303 membrane hyperpolarization followed by rebound spikes in some cases (Supplementary Fig.
304 8d). Finally, and consistent with photocurrent measurements in HEK cells (Supplementary Fig.
305 2e,f), silencing of electrically evoked APs required 100-fold higher irradiance with eNPAC2.0,
306 compared to somBiPOLES, until a significant rheobase-shift was observed (Supplementary Fig.
307 8e).

308 In summary, somBiPOLES is suitable for potent, reliable neuronal activation exclusively with
309 orange-red light and silencing with blue light. somBiPOLES displays similar potency for
310 neuronal excitation and inhibition as Chrimson and som*Gt*ACR2 alone.

311



312

313 **Figure 3: somBiPOLES allows potent dual-color spiking and silencing of the same neurons using**
 314 **red and blue light, respectively.** (a) Quantification of neuronal excitation with somBiPOLES or
 315 ChrImson only. (b) Optical excitation is restricted exclusively to the orange/red spectrum in somBiPOLES-
 316 expressing neurons. Left: Example traces of current-clamp (IC) recordings in somBiPOLES-expressing
 317 CA1 pyramidal cells to determine light-evoked action potential (AP)-probability at different wavelengths.
 318 Right: quantification of light-mediated AP probability at indicated wavelengths and irradiances (symbols
 319 correspond to mean \pm SEM, $n = 8$ cells). Black outlined circles correspond to irradiance values shown in
 320 example traces on the left. (c) Same experiment as shown in (b), except that CA1 neurons express
 321 ChrImson only (symbols correspond to mean \pm SEM, $n = 7$ cells) Note blue-light excitation of ChrImson,
 322 but not somBiPOLES cells. (d) Light-ramp stimulation to determine the AP threshold irradiance. Left:
 323 Representative membrane voltage traces measured in somBiPOLES-expressing CA1 pyramidal neurons.
 324 Light was ramped linearly from 0 to 10 mW mm^{-2} over 1 s. Right: Quantification of the irradiance threshold
 325 at which the first AP was evoked (black horizontal lines: medians, $n = 7$ cells). (e) Same experiment as
 326 shown in (b), except that CA1 neurons express ChrImson only (black horizontal lines: medians, $n = 7$
 327 cells). The threshold for action potential firing with 595 nm was similar between somBiPOLES- and
 328 ChrImson-expressing neurons, while somBiPOLES cells were not sensitive to blue light. (f) Quantification
 329 of neuronal silencing with somBiPOLES or somGtACR2 only. (g) somBiPOLES mediates neuronal
 330 silencing upon illumination with blue light. Left: Current ramps (from 0 - 100 to 0 - 900 pA) were injected
 331 into somBiPOLES-expressing CA1 pyramidal cells to induce APs during illumination with blue light at
 332 indicated intensities (from 0.001 to 100 mW mm^{-2}). The injected current at the time of the first action
 333 potential was defined as the rheobase. Right: Quantification of the rheobase shift and the relative change
 334 in the number of ramp-evoked action potentials. Illumination with 490 nm light of increasing intensities
 335 activated somBiPOLES-mediated Cl^- currents shifting the rheobase to higher values and shunting action
 336 potentials. (h) Same experiment as shown in (g), except that CA1 neurons express somGtACR2 only.
 337 Note similar silencing performance of somBiPOLES and GtACR2. In (h, g) black circles correspond to

338 medians, $n_{\text{somBiPOLES}} = 6$ cells, $n_{\text{somGtACR2}} = 6$ cells, one-way Friedman test, * $p < 0.05$, ** $p < 0.01$, *** $p <$
339 0.001. The data presented in this figure and details on the statistical analysis are provided in the Source
340 Data file.

341

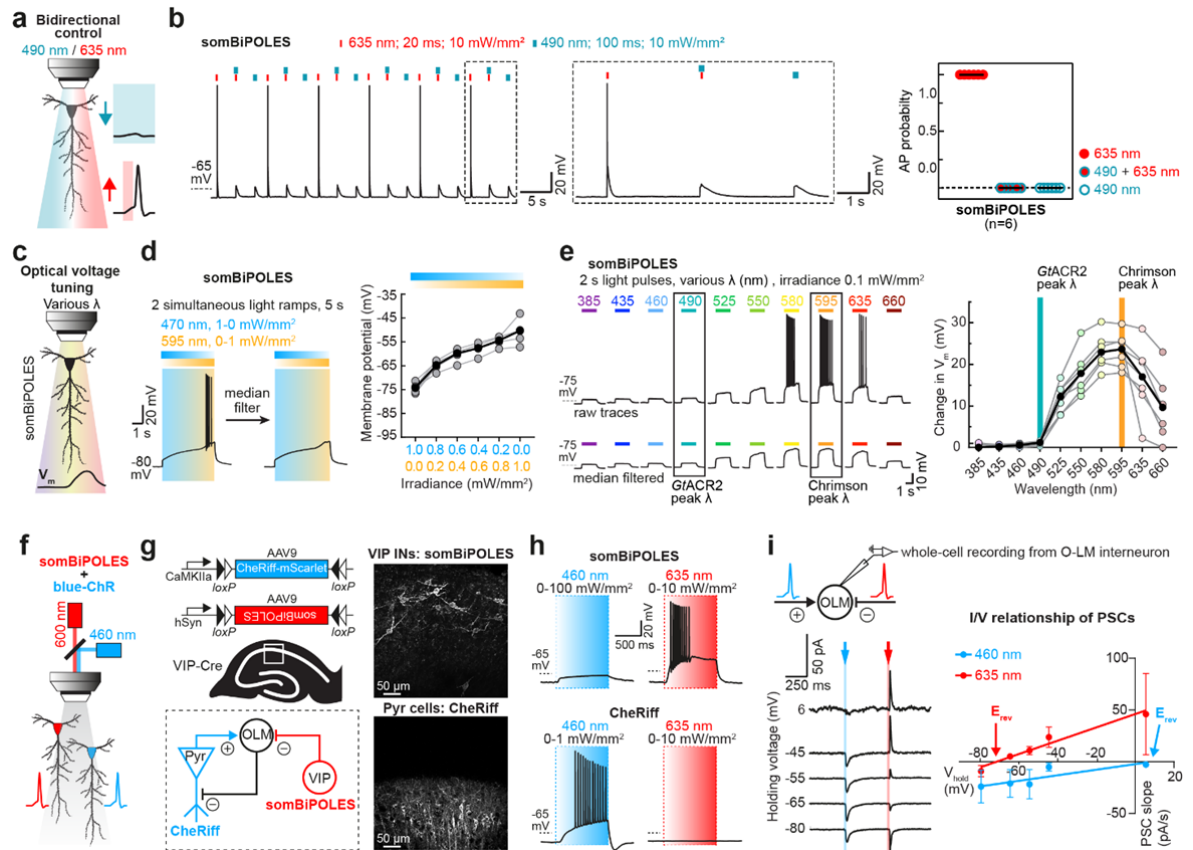
342 **BiPOLES allows various neuronal manipulations with visible light**

343 We evaluated BiPOLES and somBiPOLES in the context of three distinct neuronal applications:
344 bidirectional control of neuronal activity, optical tuning of membrane voltage, and independent
345 spiking of two distinct neuronal populations.

346 We first tested the suitability of BiPOLES and somBiPOLES for all-optical excitation and
347 inhibition of the same neurons (Fig. 4a). Red light pulses (635 nm, 20 ms, 10 mW mm⁻²) reliably
348 triggered action potentials (APs) in somBiPOLES expressing neurons (Fig. 4b), while APs were
349 triggered only in approx. 50% of BiPOLES expressing neurons under these stimulation
350 conditions (Supplementary Fig. 7e), due to a higher irradiance threshold to evoke APs in those
351 cells (Supplementary Fig. 7a,b). Concomitant blue illumination (490 nm, 10 mW mm⁻²) for 100
352 ms reliably blocked red-light evoked APs in all cases. As expected from an anion conducting
353 channel, blue light alone had only a minor impact on the resting membrane voltage, due to the
354 close proximity of the chloride reversal potential to the resting potential of the cell (Fig. 4b,
355 Supplementary Fig. 7e) In contrast, neurons expressing Chrimson alone showed APs under red
356 and blue illumination (Supplementary Fig. 4b).

357 Aside from dual-color spiking and inhibition, a major advantage of the fixed 1:1 stoichiometry
358 between an anion and cation channel with different activation spectra in BiPOLES is the ability
359 to precisely tune the ratio between anion- and cation-conductance with light (Fig. 1f,g,
360 Supplementary Fig. 3c). In neurons this allows to optically tune the membrane voltage between
361 the chloride reversal potential and the action potential threshold (Fig. 4c). Optical membrane
362 voltage tuning was achieved either by a variable ratio of blue and orange light at the absorption
363 peak wavelengths of *GtACR2* and Chrimson (Fig. 4d) or by using a single color with fixed
364 irradiance over a wide spectral range (Fig. 4e). Both approaches yielded reliable and
365 reproducible membrane voltage shifts. Starting from the chloride Nernst potential when only
366 *GtACR2* was activated with blue light at 470 nm, the membrane depolarized steadily with an
367 increasing 595/470 nm ratio, eventually passing the action potential threshold (Fig. 4d).
368 Similarly, tuning a single wavelength between 385 nm and 490 nm clamped the cell near the
369 Nernst potential for chloride, while shifting the wavelength peak further towards red led to
370 gradual depolarization, eventually triggering action potentials at 580 nm (Fig. 4e). Depending on
371 the available light source both methods allow precise control of anion and cation fluxes at a
372 fixed ratio and might be applied for locally defined subthreshold membrane depolarization in
373 single neurons or to control excitability of networks of defined neuronal populations.

374 Since BiPOLES permits neuronal spiking exclusively within the orange-red light window, it
 375 facilitates two-color excitation of genetically distinct but spatially intermingled neuronal
 376 populations using a second, blue-light-activated ChR (Fig. 4f). To demonstrate this, we
 377 expressed somBiPOLES in CA1 VIP interneurons and CheRiff, a blue-light-sensitive ChR (λ_{max}
 378 = 460 nm)³⁰ in CA1 pyramidal neurons (Fig. 4g, see Methods for details). Both CA1 and VIP
 379 neurons innervate Oriens-Lacunosum-Moleculare (OLM) interneurons. Therefore, exclusive
 380 excitation of CA1 cells or VIP interneurons is expected to trigger excitatory (EPSCs) and
 381 inhibitory (IPSCs) postsynaptic currents, respectively. CheRiff-expressing pyramidal cells were
 382 readily spiking upon blue, but not orange-red illumination up to 10 mW mm⁻² (Fig. 4h,
 383 Supplementary Fig. 9). Conversely, as expected, red light evoked APs in somBiPOLES-
 384 expressing VIP neurons, while blue light up to 100 mW mm⁻² did not evoke APs (Fig. 4h). Next,
 385 we recorded synaptic inputs from these two populations onto VIP-negative GABAergic neurons
 386 in stratum-oriens (Fig. 4i). As expected, blue light triggered EPSCs (CheRiff) and red light
 387 triggered IPSCs (somBiPOLES), evident by their respective reversal potentials at 8.8 ± 10.4 mV
 388 and -71.4 ± 13.1 mV (Fig. 4i). Thus, somBiPOLES, in combination with the blue-light sensitive
 389 CheRiff enabled independent activation of two distinct populations of neurons in the same field
 390 of view.



391

392 **Figure 4: Applications of BiPOLES: bidirectional control of neuronal activity, optical voltage**
 393 **tuning and independent dual-color excitation of two distinct neuronal populations. (a) Schematic**

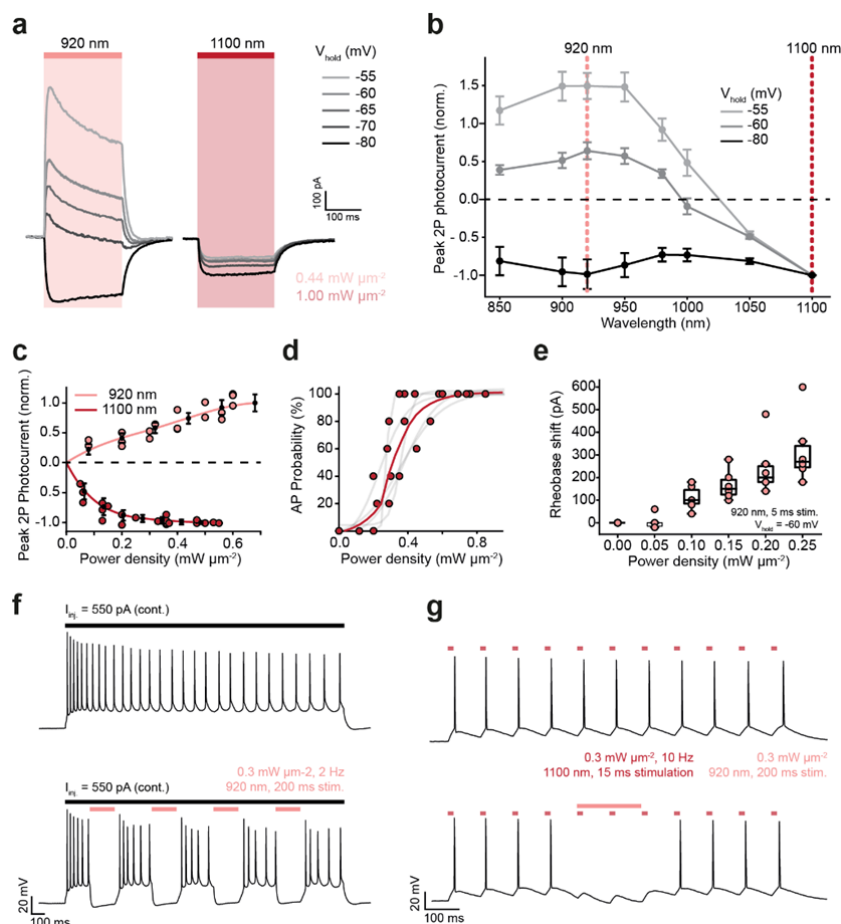
394 drawing illustrating bidirectional control of neurons with blue and red light. **(b)** Current-clamp
395 characterization of bidirectional optical spiking-control with somBiPOLES. Left: Voltage traces showing
396 red-light-evoked APs, which were blocked by a concomitant blue light pulse. Right: quantification of AP
397 probability under indicated conditions (black horizontal lines: medians, $n = 6$ cells). **(c)** Schematic drawing
398 illustrating control of membrane voltage with somBiPOLES. **(d)** Left: Representative membrane voltage
399 traces from a somBiPOLES-expressing CA1 pyramidal cell during simultaneous illumination with 470-
400 and 595-nm light ramps of opposite gradient. Voltage traces were median-filtered to reveal the slow
401 change in membrane voltage during the ramp protocol. Right: Quantification of membrane voltage at
402 different 595/470 nm light ratios (black circles: medians, $n = 5$ cells). **(e)** Left: Representative membrane
403 voltage traces of somBiPOLES in CA1 pyramidal neurons upon illumination with different wavelengths
404 and equal photon flux. As in (d) voltage traces were median-filtered to eliminate action potentials and
405 reveal the slow changes in membrane voltage during the light protocol. Right: Quantification of
406 membrane potential along the spectrum showing optical voltage tuning at the indicated wavelengths.
407 (black circles: medians, an irradiance of 0.1 mW mm^{-2} was kept constant for all wavelengths, $n = 6$ cells).
408 **(f)** Schematic drawing illustrating control of 2 neurons expressing either somBiPOLES (red) or a blue-
409 light-sensitive ChR (blue). **(g)** Left: Cre-On/Cre-Off strategy to achieve mutually exclusive expression of
410 CheRiff-mScarlet in CA1 pyramidal neurons and somBiPOLES in VIP-positive GABAergic neurons. Both
411 cell types innervate OLM interneurons in CA1. Right: Example maximum-intensity projection images of 2-
412 photon stacks showing expression of somBiPOLES in VIP-interneurons (top) and CheRiff-mScarlet in the
413 pyramidal layer of CA1 (bottom). **(h)** IC-recordings demonstrating mutually exclusive spiking of
414 somBiPOLES- and CheRiff-expressing neurons under red or blue illumination. **(i)** Postsynaptic whole-cell
415 voltage-clamp recordings from an OLM cell at indicated membrane voltages showing EPSCs and IPSCs
416 upon blue- and red-light pulses, respectively. Right: quantification of blue- and red-light-evoked PSCs and
417 their reversal potential. Symbols show mean \pm SEM, $n_{460 \text{ nm}} = 8$ cells, $n_{635 \text{ nm}} = 7$ cells, lines: linear
418 regression fit, $R^2 = 0.06$ and 0.20 for blue and red light, respectively. The data presented in this figure are
419 provided in the Source Data file.

420

421 **Bidirectional neuronal control using dual-laser 2-photon holography**

422 Two-photon holographic excitation enables spatially localized photostimulation of multiple
423 neurons with single-cell resolution in scattering tissue¹. We evaluated the feasibility of
424 bidirectional control of single neurons by 2-photon holographic excitation (Supplementary Fig.
425 10a) in hippocampal organotypic slices virally transduced with somBiPOLES expressed from a
426 CaMKII promoter. Single-photon excitation confirmed high potency of somBiPOLES using this
427 expression strategy (Supplementary Fig. 11). The 2-photon action spectrum of somBiPOLES
428 was explored by measuring the peak photocurrents (I_p) at a range of holding potentials (-80 to -
429 55 mV) and excitation wavelengths (850 to 1100 nm). Similar to single-photon excitation, blue-
430 shifted wavelengths ($\lambda_{\text{ex}} < 980 \text{ nm}$) generated large photocurrents, apparently dominated by the
431 flow of chloride ions (outward chloride currents below the chloride Nernst potential and inward
432 chloride currents above the chloride Nernst potential, Fig. 5a-c, Supplementary Fig. 10b). Red-
433 shifted wavelengths ($\lambda_{\text{ex}} > 980 \text{ nm}$) generated photocurrents, which appeared to be dominated
434 by the flow of protons and cations across the membrane (inward currents at physiological
435 neuronal membrane potentials, Fig. 5a-c, Supplementary Fig. 10b). Since 920 nm and 1100 nm
436 illumination generated the largest magnitudes of inhibitory and excitatory photocurrents
437 respectively, these wavelengths were used to evaluate whether neuronal activity could be
438 reliably suppressed or evoked in neurons expressing somBiPOLES. Action potentials could be

439 reliably evoked using short (5 ms) exposure to 1100 nm light (power density: $0.44 \text{ mW}/\mu\text{m}^2$),
 440 with latency ($19.9 \pm 6.3 \text{ ms}$) and jitter ($2.5 \pm 1.5 \text{ ms}$) (Fig. 5d, Supplementary Fig. 10c)
 441 comparable to literature values for Chrimson³¹. 5 ms pulses were also able to induce high-
 442 fidelity trains of APs with frequencies up to 20 Hz (Supplementary Fig. 10d). It is likely that
 443 shorter latency and jitter (and consequently higher rates of trains of APs) could be achieved by
 444 replacing the stimulation laser by one with optimized pulse parameters, in particular, higher
 445 peak energy³². 920 nm excitation effectively inhibited neural activity, increasing the rheobase of
 446 AP firing at power densities above $0.1 \text{ mW } \mu\text{m}^{-2}$ (Fig. 5e). It further enabled temporally precise
 447 elimination of single electrically evoked APs (Supplementary Fig. 10e) and silencing of neuronal
 448 activity over sustained (200 ms) periods (Fig. 5f). Finally, we demonstrate two-photon,
 449 bidirectional control of neurons by co-incident illumination of appropriately titrated 920 nm and
 450 1100 nm light (Fig. 5g). Thus, somBiPOLES is suitable for dual-color 2-photon holographic
 451 manipulation of neuronal activity with cellular resolution with standard lasers typically used for
 452 two-photon imaging.



453

454 **Figure 5: Bidirectional control of neuronal activity with somBiPOLES using dual-color 2-photon**
 455 **holography. (a-c)** Voltage clamp (VC) characterization of somBiPOLES in CA1 pyramidal cells. **(a)**
 456 Representative photocurrent traces at different holding potentials, obtained by continuous 200 ms
 457 illumination of 920 and 1100 nm at constant average power density (0.44 and $1.00 \text{ mW } \mu\text{m}^{-2}$). **(b)** Peak
 458 photocurrent as a function of wavelength at different holding potentials (mean \pm SEM, $n = 5$). Data

459 acquired with a constant photon flux of 6.77×10^{26} photons $s^{-1}m^{-2}$. Dashed lines indicate 920 and 1100 nm
460 respectively; the wavelengths subsequently utilized for photo-stimulation and inhibition. **(c)** Peak
461 photocurrent as a function of incident power density at a holding potential of -60 mV (mean \pm SEM, 920
462 nm, n = 4; 1100 nm, n = 5). **(d-g)** Current clamp (IC) characterization of somBiPOLES in CA1 pyramidal
463 cells. **(d)** Probability of photoevoked action potentials under 1100 nm illumination for 5 ms (n = 5, red:
464 average, gray: individual trials). **(e)** Characterization of the efficacy of silencing somBiPOLES expressing
465 neurons under 920-nm illumination by co-injection of current (Box: median, 1st – 3rd quartile, whiskers:
466 1.5x inter quartile range, n = 5). **(f)** Representative voltage traces demonstrating sustained neuronal
467 silencing of neurons by two-photon excitation of somBiPOLES at 920 nm. Upper trace (control): 550 pA
468 current injected (illustrated by black line), no light. Lower trace: continuous injection of 550 pA current, 0.3
469 mW μm^{-2} , 920 nm, 2 Hz, 200 ms illumination. **(g)** Two-photon, bidirectional, control of single neurons
470 demonstrated by co-incident illumination of 920 nm and 1100 nm light. Upper trace: 10 Hz spike train
471 evoked by 15 ms pulses of 1100 nm light. Lower trace: optically induced action potentials shunted using a
472 single, 200 ms pulse of 920 nm light. The data presented in this figure are provided in the Source Data
473 file.
474

475 Considering the reliable performance of BiPOLES in pyramidal neurons we next tested its
476 applicability in the invertebrate model systems *C. elegans* and *D. melanogaster* as well as mice
477 and ferrets, representing vertebrate model systems.

478

479 **Bidirectional control of motor activity in *C. elegans***

480 We expressed BiPOLES in cholinergic motor neurons of *C. elegans* to optically control body
481 contraction and relaxation. Illumination with red light resulted in body-wall muscle contraction
482 and effective body-shrinkage, consistent with motor neuron activation. Conversely, blue light
483 triggered body extension, indicative of muscle relaxation and thus, cholinergic motor neuron
484 inhibition (Fig. 6b). Maximal body length changes of +3% at 480 nm and -10% at 560-600 nm
485 and reversal of the effect between 480-520 nm were consistent with the inhibitory and excitatory
486 action spectrum of BiPOLES (Fig. 6b, Supplementary Fig. 12a). The light effects on body length
487 required functional BiPOLES as light did not affect body length in the absence of all-*trans* retinal
488 (ATR, Fig. 6b). Previous strategies for bidirectional motor control in *C. elegans* using ChR2(HR)
489 and NpHR did not show body contraction and elongation in the same animal³³. Therefore, we
490 tested this directly with light conditions similar to those used for BiPOLES activation. Excitation
491 with blue light resulted in 5% body length decrease, while activation of NpHR at its peak
492 wavelength (575 nm) failed to induce significant changes in body length (Supplementary Fig.
493 12b). Thus, BiPOLES expands the possibilities for bidirectional control of neuronal activity in *C.*
494 *elegans* beyond what is achievable with currently available tools.

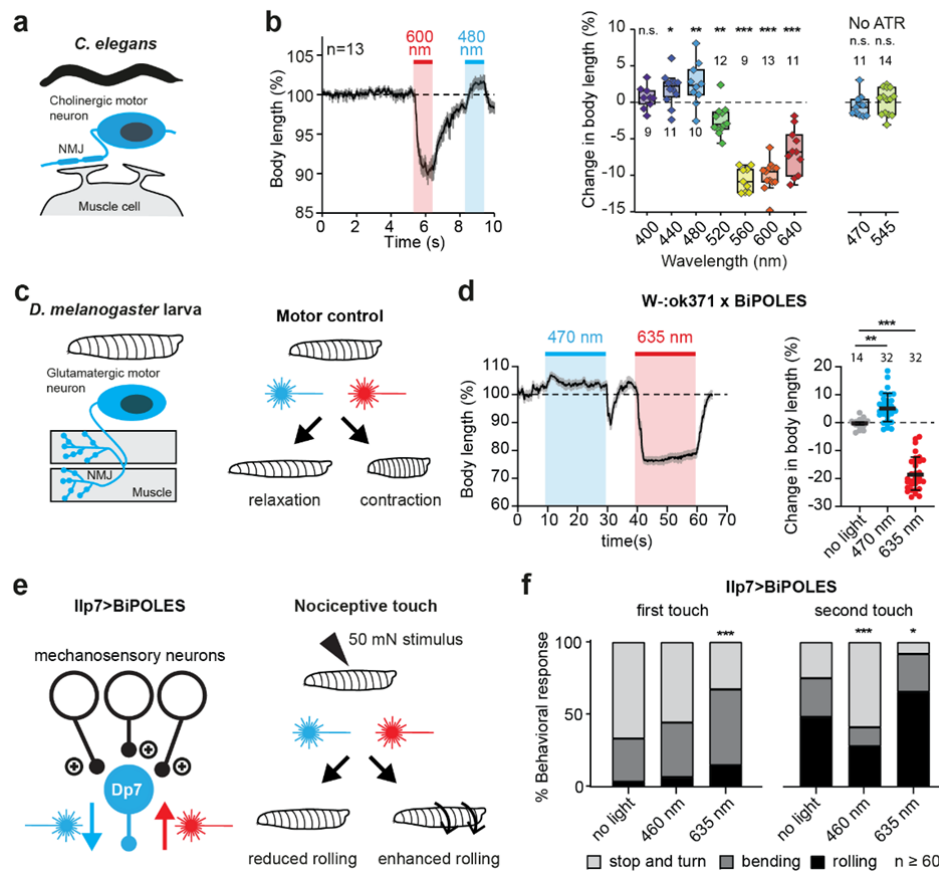
495 **Bidirectional control of motor activity and nociception in *D. melanogaster***

496 Next, we demonstrate bidirectional control of circuit function and behavior with BiPOLES in
497 *Drosophila melanogaster*. *GtACR2* and *CsChrimson* were previously used in separate
498 experiments to silence and activate neuronal activity, respectively¹⁰. In contrast, rhodopsin
499 pump functionality is strongly limited in this organism^{10,11} and bidirectional control of neuronal
500 activity has not been achieved. We therefore expressed BiPOLES in glutamatergic motor

501 neurons of *D. melanogaster* larvae (Fig. 6c). Illumination with blue light led to muscle relaxation
502 and concomitant elongation (Fig. 6d). Change in body length was similar to animals expressing
503 *GtACR2* alone (Supplementary Fig. 12c). Importantly, *GtACR2* activation in BiPOLES overrides
504 blue-light evoked Chrimson activity and thereby eliminates blue-light excitation of neurons, as
505 observed with CsChrimson alone (Supplementary Fig. 12c). Conversely, red illumination of
506 BiPOLES expressing larvae triggered robust muscle contraction and corresponding body length
507 reduction (Fig. 6d). Thus, BiPOLES facilitates bidirectional optogenetic control of neuronal
508 activity in *D. melanogaster* which was not achieved previously.

509 We further tested BiPOLES functionality in a more sophisticated *in vivo* paradigm expressing it
510 in key modulatory neurons (dorsal pair llp7 neurons, Dp7) of the mechanonociceptive circuit.
511 Dp7 neurons naturally exert bidirectional control of the larval escape response to noxious touch
512 depending on their activation level³⁴ (Fig. 6e). Acute BiPOLES-dependent silencing of Dp7
513 neurons with blue light strongly decreased the rolling escape (Fig. 6f), consistent with previously
514 shown chronic silencing of these neurons³⁴. In turn, red light illumination of the same animals
515 enhanced escape responses upon noxious touch showing that BiPOLES activation in Dp7
516 neurons can acutely tune their output and thus the corresponding escape response (Fig. 6f).
517 BiPOLES activation in Dp7 neurons showed a similar ability to block or enhance nociceptive
518 behavior as *GtACR2* or CsChrimson, respectively, while preventing Chrimson-dependent
519 activation with blue light (Supplementary Fig. 12d,e). Taken together, BiPOLES allows robust,
520 acute, and bidirectional manipulation of neuronal output and behavior in *Drosophila*
521 *melanogaster in vivo*.

522



523

524 **Figure 6: BiPOLES allows bidirectional modulation of neuronal activity in *C. elegans* and *D.***
 525 ***melanogaster*.**

526 **(a)** BiPOLES expressed in cholinergic neurons of *C. elegans* enables bidirectional control of body
 527 contraction and relaxation. Scheme of BiPOLES-expressing cholinergic motor neuron innervating a
 528 muscle cell. **(b)** Left: Temporal dynamics of relative changes in body length upon illumination with 600
 529 and 480 nm light (mean \pm SEM, 1.1 mW mm⁻², n = 13 animals). Right: Spectral quantification of maximal
 530 change in body length, compared is the body length before to during light stimulation (seconds 0-4 vs.
 531 seconds 6-9, see Supplementary fig. 12a; Box: median, 1st – 3rd quartile, whiskers: 1.5x inter quartile
 532 range, two-way ANOVA (Sidak's multiple comparisons test), p values: 400 nm (n = 9 animals): 0.99, 440
 533 nm (n = 12): 0.049, 480 nm (n = 10): 0.007, 520 nm (n = 12): 0.002, 560 nm (n = 9): < 0.0001, 600 nm (n
 534 = 13): < 0.0001, 640 nm (n = 11): < 0.0001, no ATR 470 nm (n = 11): 0.24, no ATR 545 nm (n = 14):
 535 0.78). Experiments in absence of all-trans retinal were done with 470/40 nm and 545/30 nm bandpass
 536 filters. **(c)** BiPOLES expressed in glutamatergic neurons of *D. melanogaster* larvae enables bidirectional
 537 control of body contraction and relaxation. Scheme of BiPOLES-expressing glutamatergic motor neuron
 538 innervating muscle fibers. **(d)** Left: Temporal dynamics of relative changes in body length upon
 539 illumination with 470 (17 μ W mm⁻², n = 32 animals) and 635 nm light (25 μ W mm⁻², n = 32). Right:
 540 Quantification of maximal change in body length (mean \pm SEM, no light = 14, 470nm = 32, 635nm = 32,
 541 **p = 0.0152, ***p = 0.0005, one-way ANOVA with Dunnett's multiple comparisons test). **(e)** BiPOLES-
 542 dependent manipulation of Dp7 neurons in the Drosophila larval brain (Ilp7-Gal4>UAS-BiPOLES) and the
 543 resulting change in nociceptive escape behavior following a 50 mN noxious touch. **(f)** Behavioral
 544 response after the first and second mechanical stimulus under blue light (470 nm, 1.7 mW mm⁻²) or red
 545 light (635 nm, 2.5 mW mm⁻²) illumination compared to no light. n = 61 animals *p = 0.034, ***p = 0.0005
 546 (first touch) and 0.0007 (second touch), X²-test. The data presented in this figure and details on the
 547 statistical analysis are provided in the Source Data file.

548

549 **All-optical excitation and inhibition of Locus Coeruleus in mice**

550 To further extend the applications of BiPOLES to vertebrates, we generated various conditional
551 and non-conditional viral vectors, in which the expression of the fusion construct is regulated by
552 different promoters (see Methods, Table 2). Using these viral vectors, we sought to test
553 BiPOLES and somBiPOLES in the mammalian brain. To this end, we conditionally expressed
554 somBiPOLES in TH-Cre mice, targeting Cre-expressing neurons in the Locus Coeruleus (LC)
555 (Fig. 7a). Orange illumination (594 nm) through an optical fiber implanted bilaterally above LC
556 reliably triggered transient pupil dilation, indicative of LC-mediated arousal³⁵ (Fig. 7b-d). Pupil
557 dilation was evident already at 0.7 mW at the fiber tip and gradually increased with increasing
558 light power (Supplementary Fig. 13a). Light-mediated pupil dilation was reverted immediately by
559 additional blue light (473 nm) during the orange-light stimulation, or suppressed altogether when
560 blue-light delivery started before orange-light application (Fig. 7b-d), suggesting that orange-
561 light-induced spiking of somBiPOLES-expressing neurons in LC was efficiently shunted.
562 Illumination of the LC in wt-animals did not influence pupil dynamics (Supplementary Fig. 13b).
563 Thus, LC-neurons were bidirectionally controlled specifically in somBiPOLES expressing
564 animals.

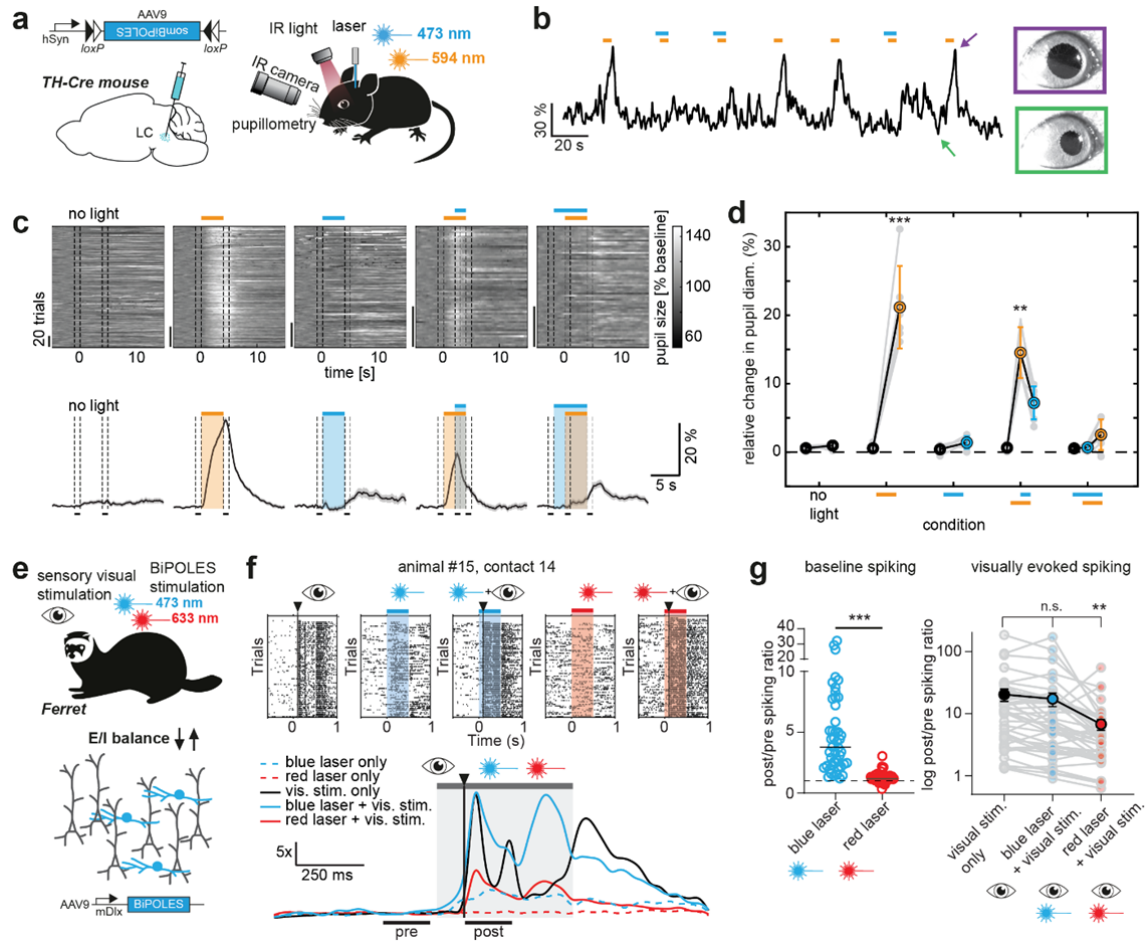
565 We estimated the brain volume accessible to reliable activation and inhibition with somBiPOLES
566 using Monte-Carlo simulations of light propagation¹⁶ under the experimental settings used for
567 the LC-manipulations described above (Supplementary Fig. 14). Based on the light parameters
568 required for neuronal excitation and inhibition determined in Fig. 3, and assuming 1 mW of 473
569 nm and 10 mW of 593 nm at the fiber tip, we estimate that reliable bidirectional control of
570 neuronal activity can be achieved over a distance of >1.5 mm in the axial direction below the
571 fiber tip (Supplementary Fig. 14c).

572 **Manipulation of neocortical excitation/inhibition ratio in ferrets**

573 Finally, we applied BiPOLES to bidirectionally control excitation/inhibition (E/I) ratio in the
574 mammalian neocortex. Therefore, we generated a viral vector using the minimal *Dlx* promoter³⁶
575 (mDlx) to target GABAergic neurons in ferret secondary visual cortex (V2). Functional
576 characterization in GABAergic neurons *in vitro* confirms all-optical spiking and inhibition of
577 GABAergic neurons with mDlx-BiPOLES (Supplementary Fig. 15). Thus, we injected mDlx-
578 BiPOLES in ferret V2 to modulate E/I-ratio during sensory processing (Fig. 7e). Extracellular
579 recordings obtained from linear silicon probes in V2 of isoflurane-anesthetized ferrets provided
580 evidence for modulation of cortical activity by shifts in E/I ratio (Fig. 7f,g). Blue light led to an
581 increase in baseline activity, consistent with deactivation of inhibitory, GABAergic neurons (Fig.
582 7f,g). Activation of GABAergic cells by red light did not further decrease the low cortical baseline
583 activity, but significantly reduced cortical responses triggered by sensory stimuli (Fig. 7f,g).
584 Although effects of blue light on evoked spiking were not significant in the average data, we

585 obtained clear evidence in individual recordings that blue light could enhance late response
 586 components (Fig. 7f), confirming a disinhibitory effect. Overall, these data suggest that
 587 BiPOLES is efficient in bidirectional control of inhibitory mechanisms, demonstrating its
 588 applicability for the control of E/I shifts in the cortical microcircuit *in vivo*.

589



590

591 **Figure 7: BiPOLES and somBiPOLES allow bidirectional modulation of neuronal activity in mice**
 592 **and ferrets.**

593 **(a)** Conditional expression of somBiPOLES in Cre-positive neurons of the TH-Cre mouse to modulate
 594 pupil dilation. **(b)** Relative pupil diameter in single trials. Orange and blue bars indicate time of illumination
 595 with 594 (orange) and 473 nm (blue), respectively. Arrows indicate positions of the two example images
 596 of the eye. **(c)** Quantification of normalized pupil size in one animal under various stimulation conditions
 597 for somBiPOLES as indicated. Top: single trials. Bottom: mean \pm SEM. Dashed lines show time windows
 598 used for quantification in the plot on the right. **(d)** Quantification of relative pupil size (n = 6 mice; One-way
 599 analysis of variance; $F = 61.67$, $p = 1.36 \times 10^{-12}$; Tukey's multiple comparison test: ** $p = 0.0028$, *** $p <$
 600 0.0001). **(e)** Modulation of GABAergic neurons (blue) in ferret secondary visual cortex (area 18) with
 601 mDlx-BiPOLES. Red (633 nm) or blue (473 nm) laser light was used to (de-)activate interneurons with or
 602 without a following 10-ms visual flash (white LED; Osram OSOLON Compact) to the ferret's right eye. **(f)**
 603 Example neuronal spiking responses at one contact of the linear probe ($\sim 700 \mu\text{m}$ depth) under indicated
 604 stimulation conditions. Top: Raster-plots of the visual stimulus alone, blue laser (+visual), red laser
 605 (+visual) conditions. Bottom: Normalized to 'pre'-phase averaged spike-density plot (sigma = 20 ms) of
 606 each indicated condition. Gray area: laser-on epoch; black vertical line: visual stimulus onset. Black

607 horizontal lines indicate the 200 ms pre- and post-stim analysis epochs to compute the results in (g). Note
608 the rate-increase after the onset of the blue laser before the onset of the visual stimulus and the reduced
609 answer after red laser illumination. **(g)** Spike-rate ratio of pos-t vs pre-laser-stimulus epoch. Left:
610 quantification of laser-mediated impact on baseline spiking rate (no visual stim.). Right: quantification of
611 the spike-rate change of the same units during only visual and laser+visual stimulation. (n = 46 contacts
612 showing visual responses from 3 animals, **p = 0.0046, ***p < 0.0001). The data presented in this figure
613 and details on the statistical analysis are provided in the Source Data file.

614

615 **Discussion**

616 In summary, BiPOLES is a performance-optimized fusion construct composed of a red-light-
617 activated cation- and a blue-light-activated anion-selective ChR. BiPOLES serves as an
618 optogenetic tool for potent excitation and inhibition of the same neurons with red and blue light,
619 respectively. In addition, it can be applied for exclusive red-light activation of a neuronal
620 subpopulation in multicolor experiments, and for locally defined optical tuning of the membrane
621 voltage between the Nernst potential for chloride and the action potential threshold.

622 BiPOLES performs reliably in invertebrate and vertebrate model systems, showing potent,
623 bidirectional modulation in the *C. elegans* motor system, the *D. melanogaster* motor and
624 nociceptive systems and the ferret visual cortex. Addition of the soma-targeting signal from the
625 mammalian potassium channel Kv2.1 yielded somBiPOLES, leading to further enhancement of
626 trafficking to the plasma membrane at the soma and proximal dendrites while avoiding
627 localization to distal dendrites and axons, as previously shown for individually expressed
628 microbial rhodopsins²⁷⁻²⁹. Thus, eliminating the risk of inadvertent blue-light mediated
629 depolarization of axons^{28,37} while improving bidirectional optogenetic manipulation of the
630 somatodendritic compartment somBiPOLES is optimized for applications in mammalian
631 systems.

632

633 Combining cation and anion channels of overlapping action spectra requires careful
634 consideration of the electrochemical conditions of the neuronal membrane. Since the resting
635 membrane potential is close to the Nernst potential of chloride, anion channels displaying large
636 unitary conductance are needed in order to efficiently shunt depolarizing currents of the red-
637 shifted cation channel, which, in turn, needs to be potent enough to reliably trigger action
638 potentials. Thus, photocurrent amplitudes and spectral sensitivity of the two opsins need to
639 match the aforementioned conditions in order to both reliably silence and drive neuronal activity.
640 If the red-shifted excitatory opsin shows too large, blue-light sensitive photocurrents, it may
641 compromise the silencing capacity of the anion channel. Conversely, if the action spectrum of
642 the blue-light sensitive anion channel extends too far towards longer wavelengths, efficient red-
643 light evoked spiking may get impaired. For the molecular engineering of BiPOLES we focused
644 on a large spectral separation of the anion and the cation conductance. Minimizing the optical

645 cross-talk of both channels favors inhibitory conductance under blue light illumination and
646 increases both the light intensity range and the spectral range that allows exclusive activation of
647 the red shifted cation channel. Due to the large spectral separation, BiPOLES can be controlled
648 with two simple light sources, such as LEDs, without the requirement of sophisticated spectral
649 control, making its use straightforward. The GtACR2-L4-ChRmine-construct might be an
650 interesting alternative if spectrally narrow light sources, such as lasers, are available, because it
651 reaches peak depolarizing currents 60 nm blue-shifted compared to BiPOLES. Thus, inhibition
652 and excitation can be achieved with 430-470 nm and 530-550 nm (Fig. 1f) providing an
653 additional spectral window in the red, that can be used for a third optogenetic actuator or
654 sensor. Finally, a seemingly trivial but equally important advantage of all the tandem systems
655 we present here is their modular architecture allowing easy tailoring of fusion constructs fulfilling
656 specific experimental requirements.

657 Noteworthy, BiPOLES does not represent the first optogenetic tool for bidirectional control of
658 neuronal activity. Different combinations of the excitatory blue-light-sensitive ChR2 and orange-
659 light-sensitive inhibitory ion pumps such as NpHR, bR, or Arch3.0 were generated previously
660 ^{2,4,6}. However, among all these variants, only the combination of ChR2 and NpHR (i.e. eNPAC
661 and eNPAC2.0) was successfully used to address neuroscientific questions in mice⁶⁻⁹.
662 BiPOLES will significantly expand the possibilities of bidirectional neuronal manipulations, since,
663 aside from efficiently expressing in a wide array of different model systems, it also features a
664 number of additional advantages: First, combining two potent channels, rather than a pump and
665 a channel, provides a more balanced ionic flux per absorbed photon for the inhibitory and
666 excitatory rhodopsin. This results in a high operational light sensitivity for both excitation and
667 inhibition by orange and blue light, respectively. In contrast, high irradiance and expression
668 levels are required for the ion pumps that only transport one charge per absorbed photon.
669 Second, due to the use of two channels, BiPOLES-mediated photocurrents do not actively
670 move ions against their gradients, which can cause adverse side-effects³⁷, but rather fixes the
671 neuronal membrane voltage anywhere between the reversal potential of GtACR2 and
672 Chrimson. The membrane voltage can be tuned depending on the ratio of blue/red light or a by
673 a single light source tuned to wavelengths between the absorption peaks of GtACR2 and
674 Chrimson. Third, inverting the color of the excitatory and inhibitory opsin, compared to previous
675 tools, restricts optical excitation in BiPOLES-expressing cells exclusively to the orange/red
676 spectrum. The inverted color-scheme enables scale-free and independent spiking of two
677 neuronal populations in combination with a second, blue-light-sensitive ChR, expressed in a
678 second population of neurons, as the blue-light-activated, inhibitory channel GtACR2 potently
679 shunts Chrimson-mediated, blue-light-activated excitatory photocurrents. Other applications
680 could employ multiplexing with blue-light sensitive cyclases³⁸ or genetically encoded activity-
681 indicators that require blue light for photoconversion^{39,40}. Fourth, compared to the first

682 generation of tandem constructs, BiPOLES was optimized for membrane trafficking and
683 especially the somBiPOLES variant shows strongly improved membrane expression in
684 mammalian neurons, enabling reliable and potent optogenetic spiking and inhibition even in
685 deep brain regions *in vivo*. One additional reason for the superior membrane expression of
686 BiPOLES compared to other rhodopsin-tandems might be the absence of N-terminal,
687 extracellular cysteine residues, which are involved in disulfide bond formation and thus
688 dimerization in all structurally described ChRs⁴¹⁻⁴⁴. The absence of N-terminal cysteines may
689 avoid heteromeric protein networks and undesired clustering of the fused tandem-rhodopsins.
690 Fifth, soma-targeted BiPOLES allows efficient and reliable bidirectional control of neuronal
691 spiking over a wide range of light intensities. This is important for *in vivo* applications in the
692 mammalian brain, where light scattering and absorption lead to an exponential fall-off of the
693 irradiance over distance¹⁷. The color scheme in somBiPOLES in combination with the large
694 conductance of *GtACR2* and its absence from axon terminals enables potent and reliable
695 silencing with blue light over a wide range of intensities. Potential cross-activation of Chrimson
696 by high blue light intensities did not compromise neuronal silencing in pyramidal neurons.
697 Similarly, due to the red-shifted absorption of Chrimson, neuronal spiking can be efficiently
698 achieved with orange light. somBiPOLES reliably mediates silencing and activation at modest
699 intensities of blue and orange light far away from the fiber tip, while maintaining its wavelength-
700 specificity under high-intensity irradiance, as typically present directly under the fiber tip. Thus,
701 somBiPOLES holds the potential to manipulate neuronal activity in large brain areas with single-
702 photon illumination (Supplementary Fig. 14c). Finally, a fusion-protein of two potent channels
703 with opposite charge selectivity targeted to the somatodendritic compartment and displaying a
704 local one-to-one expression ratio in the plasma membrane, enables temporally precise
705 bidirectional control of neuronal activity at single-cell resolution using 2-photon excitation. In
706 contrast to widefield illumination with visible light, 2-photon excitation in combination with soma-
707 targeted opsins allows optogenetic control with single-cell resolution⁴⁵⁻⁴⁷. Bidirectional
708 optogenetic control in the same cells has not been achieved with 2-photon excitation, so far;
709 partially due to the low quantum efficiency of rhodopsin pumps, which limits their 2-photon
710 activation. In contrast, the large conductance of the two channels improves their efficacy with
711 respect to the number of transported ions per absorbed photon, and their presence at equal
712 stoichiometry anywhere on the membrane ensures reliable and reproducible generation of
713 anion- and/or cation currents, which is particularly important under locally confined 2-photon
714 excitation.

715 In principle, also multicistronic vectors encoding both opsins under a single promoter using
716 either an internal ribosomal entry site (IRES)⁴⁸ or a 2A ribosomal skip sequence allow
717 expression of both ion channels at a fixed ratio from a single AAV vector^{3,7}. However, with both
718 of these strategies neither co-localized nor stoichiometric membrane expression of both

719 channels is guaranteed since both channels might get differentially targeted and distributed in
720 the plasma membrane. This may not pose a limitation for experiments that require bidirectional
721 control of large numbers of cells where precise control of single-cell activity or sub-cellular ion
722 gradients is not so crucial. BiPOLES as a covalently linked fusion protein displays a fixed
723 expression of both opsins at a 1:1 stoichiometry anywhere in the membrane and membrane
724 trafficking or degradation of both opsins occur at identical rates, preserving excitatory and
725 inhibitory currents at a fixed ratio in all expressing cells. A fixed stoichiometry anywhere in the
726 cell membrane is important if local, subcellular activation of the opsins is required, such as
727 during 2-photon excitation or when a fixed ratio of cation and anion conductance is desired
728 between different neurons or in particular neuronal compartments, such as single dendrites or
729 dendritic spines.

730 Notably, BiPOLES employs an anion channel for optogenetic silencing and therefore relies on
731 the extra- and intracellular chloride concentration. In the case of a depolarized chloride Nernst
732 potential, opening of the anion channel may produce depolarizing currents, which can trigger
733 action potentials or neurotransmitter release⁴⁹. Unlike for rhodopsin pumps, efficient silencing
734 consequently requires low cytosolic chloride concentrations and is therefore limited in neurons
735 or cellular compartments with a depolarized Nernst potential for chloride, such as immature
736 neurons or axon terminals. Given these caveats, BiPOLES may not be suitable for bidirectional
737 control of developing neurons or presynaptic boutons. In this case, silencing may be more
738 efficient with rhodopsin pumps, despite their own limitations^{37,49} or with G-protein coupled
739 rhodopsins^{50,51}. As with any optogenetic application, neurophysiological parameters need to be
740 considered by the experimenter, guiding the appropriate choice of the tool suitable to address
741 the specific experimental requirements.

742 Since BiPOLES can be used to spike or inhibit the same population of mature neurons *in vivo*, a
743 number of previously inaccessible questions can be addressed. During extracellular recordings,
744 BiPOLES may be useful for optogenetic identification (optotagging) with red light⁵² and
745 optogenetic silencing of the same neurons. This will permit verification of the identity of silenced
746 neurons by their spiking profiles. Moreover, in combination with a second, blue-light sensitive
747 ChR, BiPOLES can be used to map local networks of spatially intermingled neurons. For
748 example, expressed in distinct types of molecularly defined GABAergic neurons, connectivity of
749 these neurons to a postsynaptic target cell can be evaluated. Additional applications for
750 BiPOLES may encompass bidirectional control of engram neurons⁵³ to test both necessity and
751 sufficiency of a particular set of neurons for memory retrieval or switching the valence of a
752 particular experience by inhibiting or activating the same or even two distinct populations of
753 neuromodulatory neurons. In principle, this could even be achieved with cellular resolution using
754 2-photon holography. Due to its utility for a wide range of research questions, its versatile
755 functionality and its applicability in numerous model systems, as demonstrated in this study,

756 BiPOLES fills an important gap in the optogenetic toolbox and might become the tool of choice
757 to address a number of yet inaccessible problems in neuroscience.

758 **Methods**

759 **Molecular Biology**

760 For HEK-cell expression, the coding sequences of Chrimson (KF992060.1), CsChrimson
761 (KJ995863.2) from *Chlamydomonas noctigama*¹², ChRmine from *Rhodomonas lens* although
762 initially attributed to *Tiarina fusus*^{22, 25} (Addgene #130997), bReaChES²⁰, iC++ (Addgene
763 #98165)¹⁹, Aurora (Addgene #98217)¹¹, GtACR1 (KP171708) and GtACR2 (KP171709) from
764 *Guillardia theta*¹⁸ as well as the blue shifted Arch3.0 mutant M128A/S151A/A225T herein
765 described as ArchBlue²⁶ were cloned together with mCerulean3⁵⁴ and a trafficking signal (ts)
766 from the Kir 2.1 channel⁴ into a pCDNA3.1 vector containing the original opsin tandem cassette²
767 with a linker composed of eYFP and the first 105 N-terminal amino acids of the rat gastric
768 H+/K+-ATPase beta subunit (β HK, NM_012510.2), kindly provided by Sonja Kleinlogel
769 (University of Bern, CH). For direct comparison also the bicistronic tool eNPAC2.0⁶ – kindly
770 provided by Karl Deisseroth (Stanford University, CA) - was cloned into the same backbone.
771 Site-directed mutagenesis to introduce the f-Chrimson and vf-Chrimson mutations Y261F,
772 S267M and K176R²¹ was performed using the QuickChange Site-Directed Mutagenesis Kit
773 (Agilent Technologies, Santa Clara, CA) according to the manufacturers' instructions.

774 For neuronal expression, the insert consisting of GtACR2-ts-mCerulean3- β HK-Chrimson was
775 cloned into an AAV2-backbone behind a human synapsin (hSyn) promoter (pAAV-hSyn-
776 BiPOLES-mCerulean; Addgene #154944). A soma-targeted, membrane-trafficking optimized
777 variant was generated by fusing an additional trafficking signal from the potassium channel
778 Kv2.1²⁷ to the C-terminus of Chrimson (pAAV-hSyn-somBiPOLES-mCerulean; Addgene
779 #154945). For expression in GABAergic neurons, BiPOLES and somBiPOLES were cloned into
780 an AAV2-backbone behind the minimal Dlx (mDlx) promoter³⁶ resulting in pAAV-mDlx-
781 BiPOLES-mCerulean (Addgene #154946) and pAAV-mDlx-somBiPOLES-mCerulean (Addgene
782 #154947). For expression in projection neurons, somBiPOLES was cloned into an AAV2-
783 backbone behind the minimal CaMKII promoter⁵⁵ resulting pAAV-CaMKII-somBiPOLES-
784 mCerulean (Addgene #154948). Double-floxed inverted open reading frame variants of
785 BiPOLES and somBiPOLES were generated by cloning these inserts in antisense direction
786 behind the Ef1alpha or hSyn promoter, flanked by two loxP and lox2272 sites (Ef1a-DIO-
787 BiPOLES-mCerulean, Addgene #154949; hSyn-DIO-BiPOLES-mCerulean, Addgene #154950;
788 hSyn-DIO-somBiPOLES-mCerulean, Addgene #154951). Note that in all constructs the
789 mCerulean3-tag is fused between GtACR2-ts and β HK-Chrimson and therefore part of
790 BiPOLES. We nonetheless chose to add "mCerulean" to the plasmid names to remind the
791 reader of the presence of a cyan fluorophore in BiPOLES. BiPOLES stands for "Bidirectional

792 Pair of Opsins for Light-induced Excitation and Silencing". Sequences of all primers used for
793 cloning and sequences of DNA-inserts used in this study are provided in a separate list
794 (Supplementary Data 1).

795

796 **Patch-Clamp experiments in HEK293 cells**

797 ⁵⁶Fusion constructs were expressed under the control of a CMV-promotor in HEK293 cells that
798 were cultured in Dulbecco's Modified Medium (DMEM) with stable glutamine (Biochrom, Berlin,
799 Germany), supplemented with 10% (v/v) fetal bovine serum (FBS Superior; Biochrom, Berlin,
800 Germany), 1 μ M all-*trans* retinal, and 100 μ g ml⁻¹ penicillin/streptomycin (Biochrom, Berlin,
801 Germany). Cells were seeded on poly-lysine coated glass coverslips at a concentration of 1 x
802 10⁵ cells ml⁻¹ and transiently transfected using the FuGENE® HD Transfection Reagent
803 (Promega, Madison, WI). two days before measurement.

804 Patch-clamp experiments were performed in transgene expressing HEK293 cells two days after
805 transfection⁵⁶. Patch pipettes were prepared from borosilicate glass capillaries (G150F-3;
806 Warner Instruments, Hamden, CT) using a P-1000 micropipette puller (Sutter Instruments,
807 Novato, CA) and subsequently fire polished. Pipette resistance was between 1.2 and 2.5 M Ω .
808 Single fluorescent cells were identified using an Axiovert 100 inverted microscope (Carl Zeiss,
809 Jena, Germany). Monochromatic light (\pm 7 nm) was provided by a Polychrome V
810 monochromator (TILL Photonics, Planegg, Germany) or by a pE-4000 CoolLED system
811 (CoolLED, Andover, UK) for light titration experiments. Light intensities were attenuated by a
812 motorized neutral density filter wheel (Newport, Irvine, CA) for equal photon flux during action
813 spectra recordings. Light pulses of the Polychrome V were controlled by a VS25 and VCM-D1
814 shutter system (Vincent Associates, Rochester, NY). Recordings were done with an AxoPatch
815 200B amplifier (Molecular Devices, Sunnyvale, CA) or an ELV-03XS amplifier (npi Electronics,
816 Tamm, Germany), filtered at 2 kHz and digitized using a DigiData 1440A digitizer (Molecular
817 Devices, Sunnyvale, CA) at a sampling rate of 10 kHz. The reference bath electrode was
818 connected to the bath solution via a 140 mM NaCl agar bridge. Bath solutions contained 140
819 mM NaCl, 1 mM KCl, 1 mM CsCl, 2 mM CaCl₂, 2 mM MgCl₂ and 10 mM HEPES at pH_e 7.2
820 (with glucose added up to 310 mOsm). Pipette solution contained 110 mM NaGluconate, 1 mM
821 KCl, 1 mM CsCl, 2 mM CaCl₂, 2 mM MgCl₂, 10 mM EGTA and 10 mM HEPES at pH_i 7.2
822 (glucose added up to 290 mOsm). All light intensities were measured in the object plane using a
823 P9710 optometer (Gigahertz-Optik, Türkenfeld, Germany) and normalized to the water Plan-
824 Apochromat 40x/1.0 differential interference contrast (DIC) objective illuminated field (0.066
825 mm²). The irradiance was 2.7 mW mm⁻² at 650 nm, 3.5 mW mm⁻² at 600 nm, 4.2 mW mm⁻² at
826 530 nm, 5.7 mW mm⁻² at 490 nm and 5.2 mW mm⁻² at 450 nm. All electrical recordings were
827 controlled by the pCLAMP™ software (Molecular Devices, Sunnyvale, CA). All whole-cell

828 recordings had a membrane resistance of at least 500 M Ω (usual >1 G Ω) and an access
829 resistance below 10 M Ω .

830

		irradiance (mW mm ⁻²)					
		0.001	0.01	0.1	1	10	100
wavelength (nm)	365	1.84E+18	1.84E+19	1.84E+20	1.84E+21	1.84E+22	1.84E+23
	385	1.94E+18	1.94E+19	1.94E+20	1.94E+21	1.94E+22	1.94E+23
	405	2.04E+18	2.04E+19	2.04E+20	2.04E+21	2.04E+22	2.04E+23
	435	2.19E+18	2.19E+19	2.19E+20	2.19E+21	2.19E+22	2.19E+23
	460	2.32E+18	2.32E+19	2.32E+20	2.32E+21	2.32E+22	2.32E+23
	470	2.37E+18	2.37E+19	2.37E+20	2.37E+21	2.37E+22	2.37E+23
	490	2.47E+18	2.47E+19	2.47E+20	2.47E+21	2.47E+22	2.47E+23
	525	2.65E+18	2.65E+19	2.65E+20	2.65E+21	2.65E+22	2.65E+23
	550	2.77E+18	2.77E+19	2.77E+20	2.77E+21	2.77E+22	2.77E+23
	580	2.92E+18	2.92E+19	2.92E+20	2.92E+21	2.92E+22	2.92E+23
	595	3E+18	3E+19	3E+20	3E+21	3E+22	3E+23
	630	3.18E+18	3.18E+19	3.18E+20	3.18E+21	3.18E+22	3.18E+23
	660	3.33E+18	3.33E+19	3.33E+20	3.33E+21	3.33E+22	3.33E+23

832 **Table 1:** Photon flux given as number of photons sec⁻¹ m⁻²833 **Preparation of organotypic hippocampal slice cultures**

834 All procedures were in agreement with the German national animal care guidelines and
835 approved by the independent Hamburg state authority for animal welfare (Behörde für Justiz
836 und Verbraucherschutz). They were performed in accordance with the guidelines of the German
837 Animal Protection Law and the animal welfare officer of the University Medical Center Hamburg-
838 Eppendorf.

839 Organotypic hippocampal slices were prepared from Wistar rats or VIP-IRES-Cre mice of both
840 sexes (Jackson-No. 031628) at post-natal day 5-7⁵⁷. Dissected hippocampi were cut into 350
841 µm slices with a tissue chopper and placed on a porous membrane (Millicell CM, Millipore).
842 Cultures were maintained at 37°C, 5% CO₂ in a medium containing 80% MEM (Sigma M7278),
843 20% heat-inactivated horse serum (Sigma H1138) supplemented with 1 mM L-glutamine,
844 0.00125% ascorbic acid, 0.01 mg ml⁻¹ insulin, 1.44 mM CaCl₂, 2 mM MgSO₄ and 13 mM D-
845 glucose. No antibiotics were added to the culture medium.

846 **Transgene delivery for single-photon experiments**

847 For transgene delivery in organotypic slices, individual CA1 pyramidal cells were transfected by
848 single-cell electroporation⁵⁸ between DIV 14-16⁵⁸. Except for pAAV-hSyn-eNPAC2.0, which was
849 used at a final concentration of 20 ng µl⁻¹, all other plasmids, namely pAAV-hSyn-BiPOLES-
850 mCerulean, pAAV-hSyn-somBiPOLES-mCerulean, pAAV-hSyn-Chrimson-mCerulean, and
851 pAAV-hSyn-somGtACR2-mCerulean were used at a final concentration of 5 ng µl⁻¹ in K-
852 gluconate-based solution consisting of (in mM): 135 K-gluconate, 10 HEPES, 4 Na₂-ATP, 0.4
853 Na-GTP, 4 MgCl₂, 3 ascorbate, 10 Na₂- phosphocreatine (pH 7.2). A plasmid encoding hSyn-

854 mKate2 or hSyn-mCerulean (both at 50 ng μl^{-1}) was co-electroporated with the opsin-
855 mCerulean or eNPAC2.0 plasmids, respectively, and served as a morphology marker. An
856 Axoprotector 800A (Molecular Devices) was used to deliver 50 hyperpolarizing pulses (-12 V, 0.5
857 ms) at 50 Hz. During electroporation slices were maintained in pre-warmed (37°C) HEPES-
858 buffered solution (in mM): 145 NaCl, 10 HEPES, 25 D-glucose, 2.5 KCl, 1 MgCl₂ and 2 CaCl₂
859 (pH 7.4, sterile filtered). In some cases, slice cultures were transduced with adeno-associated
860 virus (see Table 2 for details) at DIV 3-5⁵⁹. The different rAAVs were locally injected into the
861 CA1 region using a Picospritzer (Parker, Hannafin) by a pressurized air pulse (2 bar, 100 ms)
862 expelling the viral suspension into the slice. During virus transduction, membranes carrying the
863 slices were kept on pre-warmed HEPES-buffered solution.

864

Adeno-associated virus (AAV9)	Titer used for transduction of hippocampal organotypic slice cultures (vg/ml)	Addgene plasmid reference
mDlx-BiPOLES-mCerulean	2.8×10^{13}	154946
hSyn-DIO-BiPOLES-mCerulean	7.0×10^{13}	154950
hSyn-DIO-somBiPOLES-mCerulean	3.4×10^{13}	154951
CaMKIIa(0.4)-somBiPOLES-mCerulean	2.5×10^{13}	154948
CaMKIIa(0.4)-DO-CheRiff-ts-mScarlet-ER	8.15×10^{11}	n.a.
mDlx-H2B-EGFP	2.8×10^{10}	n.a.
CaMKIIa-Cre	3.0×10^{12}	n.a.

865 **Table 2:** List of adeno-associated viral vectors used for experiments in organotypic hippocampal slices.
866 Viruses were transduced at the indicated titers. n.a. : not applicable.

867

868 **Preparation of organotypic hippocampal slice cultures for two-photon holographic**
869 **stimulation of somBiPOLES**

870 All experimental procedures were conducted in accordance with guidelines from the European
871 Union and institutional guidelines on the care and use of laboratory animals (council directive
872 2010/63/EU of the European Union). Organotypic hippocampal slices were prepared from mice
873 (Janvier Labs, C57Bl6J) at post-natal day 8 (P8). Hippocampi were sliced into 300 μm thick
874 sections in a cold dissecting medium consisting of GBSS supplemented with 25 mM D-glucose,

875 10 mM HEPES, 1 mM Na-Pyruvate, 0.5 mM α -tocopherol, 20 nM ascorbic acid and 0.4%
876 penicillin/streptomycin (5000 U ml⁻¹).

877 Slices were placed onto a porous membrane (Millicell CM, Millipore) and cultured at 37°C, 5%
878 CO₂ in a medium consisting of 50% Opti-MEM (Fisher 15392402), 25% heat-inactivated horse
879 serum (Fisher 10368902), 24% HBSS and 1% penicillin/streptomycin (5000 U ml⁻¹). This
880 medium was supplemented with 25 mM D-glucose, 1 mM Na-Pyruvate, 20 nM ascorbic acid
881 and 0.5 mM α -tocopherol. After three days in-vitro, the medium was replaced with one
882 containing 82% neurobasal-A, 15% heat-inactivated horse serum (Fisher 11570426), 2% B27
883 supplement (Fisher, 11530536), 1% penicillin/streptomycin (5000 U ml⁻¹), which was
884 supplemented with 0.8 mM L-glutamine, 0.8 mM Na-Pyruvate, 10 nM ascorbic acid and 0.5 mM
885 α -tocopherol. This medium was removed and replaced once every 2-3 days.

886 Slices were transduced with AAV9-CaMKII-somBiPOLES-mCerulean at DIV 3 by bulk
887 application of 1 μ l of virus (final titer: 2.5×10^{13} vg ml⁻¹) per slice. Experiments were performed
888 between DIV 13-17.

889

890 **Slice culture electrophysiology with single-photon stimulation**

891 At DIV 19-21, whole-cell patch-clamp recordings of transfected or virus-transduced CA1
892 pyramidal or GABAergic neurons were performed. Experiments were done at room temperature
893 (21-23°C) under visual guidance using a BX 51WI microscope (Olympus) equipped with Dodt-
894 gradient contrast and a Double IPA integrated patch amplifier controlled with SutterPatch
895 software (Sutter Instrument, Novato, CA). Patch pipettes with a tip resistance of 3-4 M Ω were
896 filled with intracellular solution consisting of (in mM): 135 K-gluconate, 4 MgCl₂, 4 Na₂-ATP, 0.4
897 Na-GTP, 10 Na₂-phosphocreatine, 3 ascorbate, 0.2 EGTA, and 10 HEPES (pH 7.2). Artificial
898 cerebrospinal fluid (ACSF) consisted of (in mM): 135 NaCl, 2.5 KCl, 2 CaCl₂, 1 MgCl₂, 10 Na-
899 HEPES, 12.5 D-glucose, 1.25 NaH₂PO₄ (pH 7.4). In experiments where synaptic transmission
900 was blocked, 10 μ M CPPene, 10 μ M NBQX, and 100 μ M picrotoxin (Tocris, Bristol, UK) were
901 added to the recording solution. In experiments analyzing synaptic inputs onto O-LM
902 interneurons, ACSF containing 4 mM CaCl₂ and 4 mM MgCl₂ was used to reduce the overall
903 excitability. Measurements were corrected for a liquid junction potential of -14,5 mV. Access
904 resistance of the recorded neurons was continuously monitored and recordings above 30 M Ω
905 were discarded. A 16 channel LED light engine (CoolLED pE-4000, Andover, UK) was used for
906 epifluorescence excitation and delivery of light pulses for optogenetic stimulation (ranging from
907 385 to 635 nm). Irradiance was measured in the object plane with a 1918 R power meter
908 equipped with a calibrated 818 ST2 UV/D detector (Newport, Irvine CA) and divided by the
909 illuminated field of the Olympus LUMPLFLN 60XW objective (0.134 mm²).

910 For photocurrent density measurements in voltage-clamp mode CA1 cells expressing BiPOLES,
911 somBiPOLES, Chrimson or somGtACR2 were held at -75 or -55 mV to detect inward (cationic)
912 or outward (anionic) currents elicited by red (635 nm, 20 ms, 1 and 10 mW mm⁻²) and blue light
913 (490 nm, 100 ms, 10 mW mm⁻²), respectively. For each cell, the peak photocurrent amplitude (in
914 pA) was divided by the cell membrane capacitance (in pF) which was automatically recorded by
915 the SutterPatch software in voltage-clamp mode ($V_{\text{hold}} = -75$ mV).

916 In current-clamp experiments holding current was injected to maintain CA1 cells near their
917 resting membrane potential (-75 to -80 mV). To assess the suitability of BiPOLES and
918 somBiPOLES as dual-color neuronal excitation and silencing tools, alternating pulses of red
919 (635 nm, 20 ms, 10 mW mm⁻²), blue (490 nm, 100 ms, 10 mW mm⁻²) and a combination of
920 these two (onset of blue light 40 ms before red light) were delivered to elicit and block action
921 potentials. For eNPAC2.0 alternating pulses of blue (470 nm, 20 ms, 10 mW mm⁻²), yellow (580
922 nm, 100 ms, 10 mW mm⁻²) and a combination of these two (onset of yellow light 40 ms before
923 blue light) were used.

924 In experiments determining the spiking probability of somBiPOLES and Chrimson under
925 illumination with light of different wavelengths (470, 595 and 635 nm), a train of 20 light pulses
926 (5 ms pulse duration) was delivered at 5 Hz. For each wavelength, irradiance values from 0.1 to
927 100 mW mm⁻² were used. For comparisons with eNPAC2.0, only light of 470 nm was used,
928 which is the peak activation wavelength of Chr2(HR). AP probability was calculated by dividing
929 the number of light-triggered APs by the total number of light pulses.

930 To compare the irradiance threshold needed to spike CA1 cells with BiPOLES, somBiPOLES,
931 eNPAC2.0, Chrimson, and CheRiff across different wavelengths, 470, 525, 595 and 635 nm
932 light ramps going from 0 to 10 mW mm⁻² over 1 s were delivered in current-clamp mode. In the
933 case of BiPOLES and somBiPOLES the blue light ramp went up to 100 mW mm⁻² to rule out
934 that very high blue-light irradiance might still spike neurons. The irradiance value at the time of
935 the first spike was defined as the irradiance threshold (in mW mm⁻²) needed to evoke action
936 potential firing.

937 To measure the ability of BiPOLES, somBiPOLES, and somGtACR2 to shift the rheobase upon
938 blue-light illumination, depolarizing current ramps (from 0–100 to 0–900 pA) were injected into
939 CA1 neurons in the dark and during illumination with 490 nm light at irradiance values ranging
940 from 0.001 to 100 mW mm⁻². The injected current at the time of the first spike was defined as
941 the rheobase. The relative change in the number of ramp-evoked action potentials (APs) was
942 calculated counting the total number of APs elicited during the 9 current ramp injections (from
943 0–100 to 0–900 pA) for each irradiance and normalized to the number of APs elicited in the
944 absence of light. The same experiment was conducted for eNPAC2.0, but using 580 nm light
945 ranging from 0.01 to 100 mW mm⁻². Statistical significance was calculated using Friedman test.

946 To optically clamp the neuronal membrane potential using somBiPOLES, simultaneous
947 illumination with blue and orange light at varying ratios was used. In current-clamp experiments,
948 470 and 595 nm light ramps (5 s) of opposite gradient (1 to 0 mW mm⁻² and 0 to 1 mW mm⁻²,
949 respectively) were applied. Alternatively, optical clamping of the membrane potential was
950 achieved by tuning a single wavelength between 385 and 660 nm (2 s light pulses, 0.1 mW mm⁻²,
951 ²). Voltage traces were median-filtered to remove orange/red-light-mediated spikes and reveal
952 the slow change in membrane voltage during illumination.

953 For independent optogenetic activation of two distinct populations of neurons, organotypic slice
954 cultures from VIP-Cre mice were transduced with 2 adeno-associated viral vectors: 1, a double-
955 floxed inverted open reading frame (DIO) construct encoding somBiPOLES (hSyn-DIO-
956 somBiPOLES-mCerulean, see Table 2 for details) to target VIP-positive interneurons, and 2, a
957 double-floxed open reading frame (DO) construct encoding CheRiff (hSyn-DO-CheRiff-ts-
958 mScarlet-ER, see Table 2 for details) to target CA1 pyramidal neurons and exclude expression
959 in VIP-positive cells. Synaptic input from these two populations was recorded in VIP-negative
960 stratum-oriens GABAergic neurons (putative O-LM cells). In CA1, O-LM neurons receive
961 innervation both from local CA1 pyramidal cells and VIP-positive GABAergic neurons⁶⁰. To
962 facilitate identification of putative GABAergic post-synaptic neurons in stratum oriens, slices
963 were transduced with an additional rAAV encoding mDlx-H2B-EGFP. In the absence of synaptic
964 blockers light-evoked EPSCs and IPSCs were recorded while holding the postsynaptic cell at
965 different membrane potentials (-80, -65, -55, -45 and 6 mV) in whole-cell voltage clamp mode. A
966 blue (460 nm, 0.03 - 84.0 mW mm⁻²) and a red (635 nm, 6.0 - 97.0 mW mm⁻²) light pulse were
967 delivered 500 ms apart from each other through a Leica HC FLUOTAR L 25x/0.95 W VISIR
968 objective.

969 To functionally assess the putative expression of somBiPOLES in the axon terminals of CA3
970 pyramidal cells, slice cultures were transduced with an AAV9 encoding for CaMKIIa(0.4)-
971 somBiPOLES-mCerulean (see Table 2 for details). Red-light evoked EPSCs were recorded in
972 postsynaptic CA1 cells during local illumination either in CA3 at the somata (two light pulses of
973 5 ms delivered 40 ms apart using a fiber-coupled LED (400 μm fiber, 0.39 NA, 625 nm,
974 Thorlabs) controlled by a Mightex Universal 4-Channel LED Driver (1.6 mW at fiber tip), or in
975 CA1 at axon terminals of somBiPOLES-expressing CA3 cells (two light pulses of 5 ms delivered
976 40 ms apart through the 60x microscope objective, 635 nm, 50 mW mm⁻²). Axonal light
977 stimulation was done in the presence of tetrodotoxin (TTX, 1 μM) and 4-aminopyridine (4-AP,
978 100 μM) to avoid antidromic spiking of CA3 cells.

979 To determine the high-frequency spiking limit with somBiPOLES, action potentials were
980 triggered in CA1 cells at frequencies ranging from 10 to 100 Hz using 40 light pulses (595 nm, 3
981 ms pulse width, 10 mW mm⁻²). AP probability was calculated by dividing the number of light-
982 triggered APs by the total number of light pulses.

983 To characterize the spectral activation of BiPOLES, eNPAC2.0 and somGtACR2,
984 photocurrents were recorded from CA1 cells in voltage-clamp mode in response to 500 ms
985 illumination with various wavelengths (from 385 to 660 nm, 10 mW mm⁻²). BiPOLES- and
986 somGtACR2-expressing cells were held at a membrane voltage of -55 mV, more positive than
987 the chloride Nernst potential, to measure light-mediated outward chloride currents. Photocurrent
988 recordings from eNPAC2.0-expressing cells were done at a holding voltage of -75 mV. For
989 BiPOLES and eNPAC2.0 the photocurrent ratio between excitatory and inhibitory photocurrents
990 was calculated in each cell by dividing the amplitude of the photocurrents evoked by 490/595 nm
991 (for BiPOLES) and 460/580nm (for eNPAC2.0).

992 Passive and active membrane parameters were measured in somBiPOLES-expressing and
993 non-transduced, wild-type CA1 pyramidal cells. Resting membrane potential, membrane
994 resistance and capacitance were automatically recorded by the SutterPatch software in voltage-
995 clamp mode ($V_{\text{hold}} = -75$ mV) in response to a voltage test pulse of 100 ms and -5 mV. The
996 number of elicited action potentials were counted in response to a somatic current injection of
997 300 pA in current-clamp mode (0 pA holding current). For the 1st elicited AP, the voltage
998 threshold, peak and amplitude were measured.

999

1000 **Slice culture immunohistochemistry and confocal imaging**

1001 The subcellular localization of BiPOLES and somBiPOLES in hippocampal neurons was
1002 assessed 20 days after virus transduction (AAV9-hSyn-DIO-BiPOLES-mCerulean + CaMKIIa-
1003 Cre, and CaMKIIa(0.4)-somBiPOLES-mCerulean, respectively. See Table 2 for details).
1004 Hippocampal organotypic slice cultures were fixed in a solution of 4% (w/v) paraformaldehyde
1005 (PFA) in PBS for 30 min at room temperature (RT). Next, slices were washed in PBS (3 x 10
1006 min), blocked for 2 h at RT (10% [v/v] normal goat serum [NGS] in 0.3% [v/v] Triton X-100
1007 containing PBS) and subsequently incubated for 48 h at 4°C with a primary antibody against
1008 GFP to amplify the mCerulean signal (chicken, anti-GFP, Invitrogen, A10262, Lot 1972783) at
1009 1:1000 in carrier solution (2% [v/v] NGS, in 0.3% [v/v] Triton X-100 containing PBS). Following 3
1010 rinses of 10 min with PBS, slices were incubated for 3 h at RT in carrier solution (same as
1011 above) with an Alexa Fluor® dye-conjugated secondary antibody (goat, anti-chicken Alexa-488,
1012 Invitrogen; A11039, Lot 2079383, 1:1000). Slices were washed again, transferred onto glass
1013 slides and mounted for visualization with Shandon Immu-Mount (Thermo Scientific; 9990402).

1014 Confocal images were acquired using a laser-scanning microscope (Zeiss, LSM 900) equipped
1015 with a 40x oil-immersion objective lens (Zeiss EC Plan-Neofluar 40x/1.3 oil). Excitation/emission
1016 filters were appropriately selected for Alexa 488 using the dye selection function of the ZEN
1017 software. The image acquisition settings were optimized once and kept constant for all images
1018 within an experimental data set. Z-stack images were obtained using a 1 µm z-step at a

1019 1024×1024-pixel resolution scanning at 8 μs per pixel. Fiji⁶¹ was used to quantify fluorescence
1020 intensity values along a line perpendicular to the cell equator and spanning the cell diameter.
1021 For each cell, grey values above 80% of the maximum intensity were distributed in 10 bins
1022 according to their location along the line.

1023

1024 **Slice culture two-photon imaging**

1025 Neurons in organotypic slice cultures (DIV 19-21) were imaged with two-photon microscopy to
1026 check for the live expression of hSyn-DIO-somBiPOLES-mCerulean, CaMKIIa(0.4)-DO-CheRiff-
1027 ts-mScarlet-ER, mDlx-BiPOLES-mCerulean and CaMKIIa(0.4)-somBiPOLES-mCerulean. The
1028 custom-built two-photon imaging setup was based on an Olympus BX-51WI upright microscope
1029 upgraded with a multiphoton imaging package (DF-Scope, Sutter Instrument), and controlled by
1030 ScanImage 2017b software (Vidrio Technologies). Fluorescence was detected through the
1031 objective (Leica HC FLUOTAR L 25x/0.95 W VISIR) and through the oil immersion condenser
1032 (numerical aperture 1.4, Olympus) by two pairs of GaAsP photomultiplier tubes (Hamamatsu,
1033 H11706-40). Dichroic mirrors (560 DXCR, Chroma Technology) and emission filters
1034 (ET525/70m-2P, ET605/70m-2P, Chroma Technology) were used to separate cyan and red
1035 fluorescence. Excitation light was blocked by short-pass filters (ET700SP-2P, Chroma
1036 Technology). A tunable Ti:Sapphire laser (Chameleon Vision-S, Coherent) was set to 810 nm to
1037 excite mCerulean on BiPOLES and somBiPOLES. An Ytterbium-doped 1070-nm pulsed fiber
1038 laser (Fidelity-2, Coherent) was used at 1070 nm to excite mScarlet on CheRiff. Maximal
1039 intensity projections of z-stacks were generated with Fiji⁶¹.

1040

1041 **Electrophysiology for two-photon photostimulation of somBiPOLES**

1042 At DIV 13-17, whole-cell patch-clamp recordings of somBiPOLES-infected excitatory neurons
1043 were performed at room temperature (21 - 23°C). An upright microscope (Scientifica,
1044 SliceScope) was equipped with an infrared (IR) source (Thorlabs, M1050L4), oblique
1045 condenser, microscope objective (Nikon, CFI APO NIR, 40X, 0.8 NA), tube lens (Thorlabs,
1046 AC508-300-B) and an CMOS camera (Point Grey, CM3-U3-31S4M-CS) to collect IR light
1047 transmitted through the sample. Recordings were performed using an amplifier (Molecular
1048 Devices, Multiclamp 700B), a digitizer (Molecular Devices, Digidata 1550B) at a sampling rate
1049 of 10 kHz and controlled using pCLAMP11 (Molecular Devices). During experimental sessions,
1050 slice cultures were perfused with artificial cerebrospinal fluid (ACSF) comprised of 125 mM
1051 NaCl, 2.5 mM KCl, 1.5 mM CaCl₂, 1 mM MgCl₂, 26 mM NaHCO₃, 0.3 mM ascorbic acid, 25 mM
1052 D-glucose, 1.25 mM NaH₂PO₄. Synaptic transmission was blocked during all experiments by
1053 addition of 1 μM AP5 (Abcam, ab120003), 1 μM NBQX (Abcam, ab120046), and 10 μM
1054 picrotoxin (Abcam, ab120315) to the extracellular (recording) solution. Continuous aeration of

1055 the recording solution with 95% O₂ and 5% CO₂, resulted in a final pH of 7.4. Patch pipettes
1056 with a tip resistance of 4-6 MΩ were filled with intracellular solution consisting of 135 mM K-
1057 gluconate, 4 mM KCl, 4 mM Mg-ATP, 0.3 mM Na-GTP, 10 mM Na₂-phosphocreatine and 10
1058 mM HEPES (pH 7.35). Only recordings with an access resistance below 30 MΩ were included
1059 in subsequent analysis.

1060 During experiments performed using whole-cell voltage clamp, neurons were held at -60 mV
1061 (the average resting potential of neurons in hippocampal organotypic slices). The soma of each
1062 patched neuron was precisely positioned in the center of the field of view. When recording the
1063 photocurrent as a function of membrane potential (holding potentials: -80, -70, -65, -60, -55
1064 mV), neurons were temporarily held at each holding potential 5 s before and after
1065 photostimulation. For data presented in Fig. 5a-d, two-photon photoactivation was performed by
1066 continuous, 200 ms, illumination of each patched neuron using a 12-μm-diameter holographic
1067 spot (wavelengths: 850, 900, 920, 950, 980, 1000, 1050, 1100 nm), which was precisely
1068 positioned in the center of the field of view.

1069 Data presented in Fig 7d-g was acquired in current clamp experiments. Where necessary,
1070 current was injected to maintain neurons at the resting membrane potential (-60 mV).

1071 The ability of two-photon holographic excitation to evoke action potentials was first assessed
1072 using a protocol consisting of 5, 5 ms pulses of 1100 nm light for power densities ranging
1073 between 0.16 – 1.00 mW μm². The latency and jitter of light-evoked action potentials,
1074 respectively defined as the mean and standard deviation of the time between the onset of
1075 stimulation to the peak of the action potential, were measured using an identical protocol. Trains
1076 of light pulses with frequencies between (2 – 30 Hz) were used to verify that trains of action
1077 potentials could be reliably induced using 5 ms 1100 nm illumination.

1078 The potency of two-photon inhibition was evaluated by measuring the rheobase shift induced by
1079 920 nm illumination. Depolarizing current was injected for 5 ms into recorded neurons (from 0 -
1080 1.2 nA in steps of 20 pA). The protocol was stopped when action potentials were observed for 3
1081 consecutive current steps. The rheobase was defined as the amount of current injected to
1082 evoke the first of these 3 action potentials. The rheobase shift was measured by repeating the
1083 protocol with co-incident, 5 ms, illumination of the neuron with a 920 nm holographic spot
1084 (power densities between 0.05 - 0.25 mW μm²). Co-incident trains of light pulses (15 ms) and
1085 injected current (10 ms) with frequencies between (2 - 30 Hz) were used to verify that two-
1086 photon inhibition could precisely and reliably eliminate single spikes.

1087 Sustained neuronal silencing by two-photon excitation of somBiPOLES under 920 nm
1088 illumination was characterized by continuously injecting current above the rheobase for 1 s. The
1089 protocol was repeated with 200 ms co-incident illumination using a 920 nm holographic spot
1090 (power densities between 0.05 - 0.3 mW μm²).

1091 Two-photon, bidirectional, control of single neurons was demonstrated by co-incident
1092 illumination of titrated 920 nm and 1100 nm light. A 10-Hz train of 15 ms pulses of 1100 nm light
1093 was used to evoke a train of action potentials which were shunted using a continuous 200 ms
1094 pulse of 920 nm light.

1095

1096 **Two-photon photostimulation of somBiPOLES in hippocampal organotypic slices**

1097 Two-photon photostimulation was performed using a tunable femtosecond laser (Coherent
1098 Discovery, 80 MHz, 100 fs, tuned between 850 – 1100 nm). A schematic diagram of the
1099 experimental setup is presented in Supplementary Fig. 10. A telescope formed of two lenses
1100 (L1 (Thorlabs, AC508-100-B) and L2 (Thorlabs, AC508-400-B)) expanded the beam onto a
1101 Spatial Light Modulator (SLM, Hamamatsu, LCOS 10468-07, 600 x 800 pixels, 20 μm pitch). In
1102 the schematic diagram, the reflective SLM is shown as transmissive for illustrative purposes.
1103 The SLM, controlled using custom-built software⁶², was used to modulate the phase of the
1104 beam. Holograms designed to generate 12 μm holographic spots at the focal plane of the
1105 microscope were computed using an iterative Gerchberg-Saxton algorithm⁶³. The zeroth
1106 diffraction order from the SLM was removed using a physical beam block. The modulated field
1107 was relayed and de-magnified using a pair of telescopes (formed of lenses L3 (Thorlabs,
1108 AC508-750-B), L4 (Thorlabs, AC508-750-B), L5 (Thorlabs, AC508-500-B) and L6 (Thorlabs,
1109 AC508-300-B)) to fill the back-aperture of the microscope objective (Nikon, CFI APO NIR, 40X,
1110 0.8 NA) which projected the holograms onto the focal plane. Phase masks were calculated such
1111 that holographic spots for light of different wavelengths overlapped laterally and axially. The
1112 anti-reflective coating of the lenses used is optimized for wavelengths 650 - 1050 nm, and
1113 losses incurred at 1100 nm result in the system being power limited at this wavelength. Hence,
1114 spectral characterisation was performed by normalizing the power density at all wavelengths to
1115 the maximum transmitted at 1100 nm. The power incident on the sample plane was adjusted
1116 using a high-speed modulator (Thorlabs, OM6NH/M), which was calibrated for each
1117 experimental session for each wavelength used, to ensure a photon flux of 6.77×10^{26} photons
1118 $\text{s}^{-1} \text{m}^{-2}$ for all data presented in Fig. 5a. All powers were measured in the object plane using a
1119 power meter (Thorlabs, S121C). This experimental configuration was used for all data
1120 presented in Fig. 5a, along with all data acquired using 1100 nm illumination. Two-photon
1121 inhibition was performed using a femtosecond laser with fixed wavelength (Spark Alcor, 80
1122 MHz, 100 fs, 920 nm) which was combined with the beam from the tunable laser using a
1123 dichroic mirror (Thorlabs, DMLP950R). A liquid crystal variable retarder (Thorlabs, LCC1111-B)
1124 and polarizing beam splitter (Thorlabs, PBS253) were combined to modulate the maximum
1125 power of the fixed 920 nm beam independently of that of the tunable laser. The power densities
1126 used in each experiment are specified alongside the relevant data in Fig. 5 and Supplementary
1127 Fig. 10.

1128

1129 **Transgenic *C. elegans* lines and transgenes**

1130 The strain ZX417 (*zxEx34[punc17::NpHR-ECFP;punc17::CHOP-2(H134R)::eYFP;rol-6]*) was
1131 generated by injection of plasmid DNA (plasmids pRF4 (*rol-6d*), *punc-17::NpHR-eCFP*, and
1132 *punc-17::Chr2(H134R)-eYFP*; each at 80 ng/μl) into the germline of *C. elegans* wildtype
1133 hermaphrodites. Transgenic animals were picked from the F1 generation and one line (ZX417)
1134 was selected out of several transgenic F2 lines for further experiments³³. For expression in
1135 cholinergic neurons of *C. elegans*, BiPOLES (*GtACR2::ts::mCerulean3::βHK::Chrimson*) was
1136 subcloned into the *punc-17* vector RM#348p (a gift from Jim Rand) via Gibson Assembly based
1137 on the plasmid *CMV_GtACR2_mCerulean_βHK_Chrimson*, using the restriction enzyme *NheI*
1138 and the primers *ACR2_Chrimson_fwd* (5'-atcttcaggaggacccttgATGGCATCACAGGTCGTC-3')
1139 and *ACR2_Chrimson_rev* (5'-ataccatggtaccgtcgacgTCACACTGTGTCCTCGTC-3'), resulting in
1140 the construct pAB26. The respective transgenic strain ZX2586 (wild type; *zxEx1228[punc-*
1141 *17::GtACR2::ts::mCerulean3::βHK::Chrimson; pelt-2::GFP]*), was generated via microinjection⁶⁴
1142 of both 30 ng μl⁻¹ plasmid and co-marker plasmid DNA *pelt-2::GFP*. Animals were cultivated on
1143 nematode growth medium (NGM), seeded with *E. coli* OP-50 strain, in 6 cm petri dishes. To
1144 obtain functional rhodopsins in optogenetic experiments, the OP-50 bacteria were
1145 supplemented with all-*trans* retinal ATR (0.25 μl of a 100 mM stock (in ethanol) mixed with 250
1146 μl OP-50 bacterial suspension).

1147

1148 ***C. elegans* stimulation and behavioral experiments**

1149 For body-length measurements, L4 stage transgenic animals were cultivated on ATR plates
1150 overnight. Video analysis of light-stimulation protocols provided information on depolarized and
1151 hyperpolarized states, based on contracted or relaxed body-wall muscles (BWMs)⁶⁵. Prior to
1152 experiments, animals were singled on plain NGM plates to avoid imaging artefacts. They were
1153 manually tracked with an Axio Scope.A1 microscope (Zeiss, Germany), using a 10x objective
1154 (Zeiss A-Plan 10x/0,25 Ph1 M27) and a Powershot G9 digital camera (Canon, USA). For light-
1155 stimulation of optogenetic tools, transgenic worms were illuminated with 5 s light pulses at 1.1
1156 mW mm⁻² of different wavelengths as indicated in Fig. 6d (monochromatic light source,
1157 Polychrome V, Till Photonics or 100W HBO mercury lamp with 470/40 ET Bandpass or 575/40
1158 ET Bandpass filters, AHF Analysentechnik), controlled via an Arduino-driven shutter (Sutter
1159 Instrument, USA). Videos were processed and analyzed using a custom written MATLAB
1160 script⁶⁶ (MathWorks, USA). For the analysis of data, the animals' body length was normalized to
1161 the recording period prior to illumination.

1162

1163 **Transgenic *D. melanogaster* lines and transgenes**

1164 BiPOLES-mCerulean cDNA was cloned via blunt-end ligation into pJFRC7⁶⁷. BILOES was cut
1165 with BamHI/HindIII and the vector was cut with NotI/XbaI. A transgenic line inserted into the
1166 attP2 site on the 3rd chromosome⁶⁸ was generated by phiC31-mediated site-specific
1167 transgenesis (FlyORF Injection Service, Zurich, Switzerland). A Gal4 line expressing in
1168 glutamatergic neurons including motor neurons (*OK371-Gal4¹¹*) was used for locomotion
1169 experiments, a Dp7-expressing line (*Ilp7-Gal4³⁴*) was used for mechanonociception
1170 experiments.

1171

1172 **Locomotion and mechanonociception assays in *D. melanogaster* larvae**

1173 *D. melanogaster* larvae were staged in darkness on grape agar plates and fed with yeast paste
1174 containing 5 mM all-trans-retinal. Third instar larvae (96 h ± 2 h after egg laying) were used for
1175 all experiments.

1176 For locomotion and body length analyses, animals were carefully transferred under minimum
1177 red light conditions to a 2% agar film on a FTIR (frustrated total internal reflection) based
1178 tracking system (FIM, University of Münster)⁶⁹. Five freely moving larvae/trial were video-
1179 captured and stimulated with 470nm (17 μW mm⁻²) or 635nm (25 μW mm⁻²) light (CoolLED
1180 PE4000) for activation of BiPOLES. Animal locomotion was tracked with 10 frames per s for up
1181 to 70 s and then body length was analyzed using the FIMtracking software (FIM, University of
1182 Münster). For analysis, only animals displaying continuous locomotion before the light stimulus
1183 were kept. Larval body length was analyzed over time and was displayed with a 1 s moving
1184 average. The body length was normalized to the average of the first 5 s of recording. Relative
1185 body length changes during the experiment were then analyzed and plotted.

1186 For mechanonociception, staged larvae were placed on 2% agar plates with a 1 ml water film
1187 added. Experiments were performed under minimum light conditions (no activation) with
1188 calibrated von-Frey-filaments (50 mN). For activation of BiPOLES, larvae were illuminated
1189 during the assay with either 470 nm (17 μW mm⁻²) or 635 nm (25 μW mm⁻²). Larvae were
1190 stimulated twice on mid-abdominal segments (a3–a6) within 2 s. Behavioral responses (stop
1191 and turning, bending, rolling) were noted, analyzed and plotted. Staging and experiments were
1192 done in a blinded and randomized fashion.

1193

1194 **Modulation of noradrenergic neurons in the mouse locus coeruleus**

1195 *Animals:* All procedures were in agreement with the German national animal care guidelines
1196 and approved by the Hamburg state authority for animal welfare (Behörde für Justiz und
1197 Verbraucherschutz) and the animal welfare officer of the University Medical Center Hamburg-
1198 Eppendorf. Experiments were performed on mice of either sex between 2.5 and 4 months of

1199 age at the start of the experiment. Mice were obtained from The Jackson Laboratory, bred and
1200 maintained at our own colony (12/12h light-dark cycle, 22°C room temperature, ~40% relative
1201 humidity, food and water ad libitum). Transgenic mice expressing Cre recombinase in tyrosine
1202 hydroxylase positive neurons (TH-Cre, Stock No: 008601)⁷⁰ were injected with a suspension of
1203 rAAV9 viral particles encoding hSyn-DIO-somBiPOLES to target Cre-expressing neurons in the
1204 locus coeruleus. Control experiments were performed in non-injected wildtype littermates.

1205 *Virus injection and implantation of optic fibers:* General anesthesia and analgesia was achieved
1206 by intraperitoneal injections of midazolam/medetomidine/fentanyl (5.0/0.5/0.05 mg kg⁻¹, diluted
1207 in NaCl). After confirming anesthesia and analgesia by absence of the hind limb withdrawal
1208 reflex, the scalp of the animal was trimmed and disinfected with Iodide solution (Bettaisodona;
1209 Mundipharma, Germany). The animal was placed on a heating pad to maintain body
1210 temperature, fixed in a stereotactic frame, and eye ointment (Vidisic; Bausch + Lomb, Germany)
1211 was applied to prevent drying of the eyes. To bilaterally access the LC, an incision (~1 cm) was
1212 made along the midline of the scalp, the skull was cleaned, and small craniotomies were drilled
1213 -5.4 mm posterior and ± 1 mm lateral to Bregma. 0.4 µl of virus suspension were injected into
1214 each LC (-3.6 mm relative to Bregma) at a speed of ~100-200 nl min⁻¹ using a custom-made air
1215 pressure system connected to a glass micropipette. After each injection, the micropipette was
1216 left in place for a minimum of 5 minutes before removal. After virus injection, cannulas housing
1217 two ferrule-coupled optical fibers (200 µm core diameter, 0.37 NA, 4 mm length) spaced 2 mm
1218 apart (TFC_200/245-0.37_4mm_TS2.0_FLT; Doric Lenses, Canada) were inserted just above
1219 the injection site to a depth of -3.5 mm relative to Bregma using a stereotactic micromanipulator.
1220 The implant, as well as a headpost for animal fixation during the experiment, were fixed to the
1221 roughened skull using cyanoacrylate glue (Pattex; Henkel, Germany) and dental cement (Super
1222 Bond C&B; Sun Medical, Japan). The incised skin was glued to the cement to close the wound.
1223 Anesthesia was antagonized by intraperitoneally injecting a cocktail of
1224 atipamezole/flumazenil/buprenorphine (2.5/0.5/0.1 mg kg⁻¹, diluted in NaCl). Carprofen (4 mg
1225 kg⁻¹) was given subcutaneously for additional analgesia and to avoid inflammation. In addition,
1226 animals received meloxicam mixed into softened food for 3 days after surgery.

1227 *Optogenetic stimulation:* 4 - 6 weeks after surgery, mice were habituated to head fixation and
1228 placement in a movement-restraining plastic tube for at least one session. Bilateral optogenetic
1229 stimulation of LC neurons was achieved by connecting the fiber implant to a 1×2 step-index
1230 multimode fiber optic coupler (200 µm core diameter, 0.39 NA; TT200SL1A, Thorlabs,
1231 Germany) in turn connected to a laser combiner system (LightHUB; Omicron, Germany)
1232 housing a 473 nm (LuxX 473-100; Omicron, Germany) and a 594 nm diode laser (Obis 594 nm
1233 LS 100 mW; Coherent, Germany) for activation the *GtACR2* and Chrimson components of
1234 somBiPOLES, respectively. Coupling to the implant was achieved with zirconia mating sleeves
1235 (SLEEVE_ZR_1.25; Doric lenses, Canada) wrapped with black tape to avoid light emission from

1236 the coupling interface. Following a habituation period of ~3 min after placing in the setup, stimuli
1237 were generated and presented using custom-written MATLAB scripts (MathWorks, US)
1238 controlling a NI-DAQ-card (PCIe-6323; National Instruments, US) to trigger the lasers via digital
1239 input channels. For activation of Chrimson, pulse trains (594 nm, ~10 mW at each fiber end,
1240 20 ms pulse duration, 20 Hz repetition rate) of 4 s duration were presented, while *GtACR2* was
1241 activated by continuous illumination (473 nm, ~10 mW at each fiber end) of 2 - 6 seconds
1242 duration. 30 - 40 trials of 473 nm pulses, 594 nm pulse trains, and combinations thereof, were
1243 presented at an inter-train-interval of 20 - 30 seconds in each session.

1244 *Data acquisition:* A monochrome camera (DMK 33UX249; The Imaging Source, Germany)
1245 equipped with a macro objective (TMN 1.0/50; The Imaging Source, Germany) and a 780 nm
1246 long-pass filter (FGL780; Thorlabs, Germany) was pointed towards one eye of the mouse.
1247 Background illumination was provided with an infrared spotlight (850 nm), while a UV LED (395
1248 nm; Nichia, Japan) was adjusted to maintain pupil dilation of the mouse at a moderate baseline
1249 level. Single frames were triggered at 30 Hz by an additional channel of the NI-DAQ-card that
1250 controlled optogenetic stimulation, and synchronization was achieved by simultaneous
1251 recording of all control voltages and their corresponding timestamps.

1252 *Data analysis:* Pupil diameter was estimated using a custom-modified, MATLAB-based
1253 algorithm developed by McGinley et al⁷¹. In short, an intensity threshold was chosen for each
1254 recording to roughly separate between pupil (dark) and non-pupil (bright) pixels. For each
1255 frame, a circle around the center of mass of putative pupil pixels and with an area equivalent to
1256 the amount of pupil pixels was then calculated, and putative edge pixels were identified by
1257 canny edge detection. Putative edge pixels that were more than 3 pixels away from pixels below
1258 the threshold (putative pupil) or outside an area of $\pm 0.25 - 1.5$ times the diameter of the fitted
1259 circle were neglected. Using least-squares regression, an ellipse was then fit on the remaining
1260 edge pixels, and the diameter of a circle of equivalent area to this ellipse was taken as the pupil
1261 diameter. Noisy frames (e.g. no visible pupil due to blinking or blurry pupil images due to
1262 saccades of the animal) were linearly interpolated, and the data was low-passed filtered (< 3
1263 Hz; 3rd order Butterworth filter). Pupil data was segmented from 5 s before to 15 s after onset of
1264 each stimulus and normalized to the median pupil diameter of the 5 s preceding the stimulus
1265 onset, before individual trials were averaged. Randomly chosen segments of pupil data of the
1266 same duration served as a control. The difference in median pupil diameter one second before
1267 and after stimulation (as indicated in Fig. 7c) was used to calculate potential changes in pupil
1268 diameter for each condition. Statistical significance was calculated using one-way analysis of
1269 variance and Tukey's post-hoc multiple comparison tests.

1270

1271 ***In-vivo recordings from ferret visual cortex***

1272 Data were collected from 3 adult female ferrets (*Mustela putorius*). All experiments were
1273 approved by the independent Hamburg state authority for animal welfare (Behörde für Justiz
1274 und Verbraucherschutz) and were performed in accordance with the guidelines of the German
1275 Animal Protection Law and the animal welfare officer of the University Medical Center Hamburg-
1276 Eppendorf.

1277 For injection of rAAV9 viral particles encoding mDlx-BiPOLES-mCerulean (see table 1) animals
1278 were anesthetized with an injection of ketamine (15 mg kg⁻¹), medetomidine (0.02 mg kg⁻¹),
1279 midazolam (0.5 mg kg⁻¹) and atropine (0.15 mg kg⁻¹). Subsequently, they were intubated and
1280 respired with a mixture of 70:30 N₂/O₂ and 1-1.5% isoflurane. A cannula was inserted into the
1281 femoral vein to deliver a bolus injection of enrofloxacin (15 mg kg⁻¹) and rimadyl (4 mg kg⁻¹) and,
1282 subsequently, continuous infusion of 0.9% NaCl and fentanyl (0.01 mg kg⁻¹ h⁻¹). Body
1283 temperature, heart rate and end-tidal CO₂ were constantly monitored throughout the surgery.
1284 Before fixing the animal's head in the stereotaxic frame, a local anesthetic (Lidocaine, 10%) was
1285 applied to the external auditory canal. The temporalis muscle was folded back, such that a small
1286 craniotomy (ø: 2.5mm) could be performed over the left posterior cortex and the viral construct
1287 was slowly (0.1µl min⁻¹) injected into secondary visual cortex (area 18). The excised piece of
1288 bone was put back in place and fixed with tissue-safe silicone (Kwikcast; WPI). Also, the
1289 temporalis muscle was returned to its physiological position and the skin was closed. After the
1290 surgery the animals received preventive analgesics (Metacam, 0.1 mg) and antibiotics
1291 (Enrofloxacin, 15 mg kg⁻¹) for ten days.

1292 After an expression period of at least 4 weeks, recordings of cortical signals were carried out
1293 under isoflurane anesthesia. Anesthesia induction and maintenance were similar to the
1294 procedures described above, except for a tracheotomy performed to allow for artificial
1295 ventilation of the animal over an extended period. The i.v. infusion was supplemented with
1296 pancuronium bromide (6 µg kg⁻¹ h⁻¹) to prevent slow ocular drifts. To keep the animal's head in
1297 a stable position throughout the placement of recording electrodes and the measurements, a
1298 headpost was fixed with screws and dental acrylic to the frontal bone of the head. Again, the
1299 temporalis muscle was folded back and a portion of the cranial bone was resected. The dura
1300 was removed before introducing an optrode with 32 linearly distributed electrodes (A1x32-
1301 15mm-50(100)-177, NeuroNexus Technologies) into the former virus-injection site (area 18).
1302 The optrode was manually advanced via a micromanipulator (David Kopf Instruments) under
1303 visual inspection until the optic fiber was positioned above the pial surface and the uppermost
1304 electrode caught a physiological signal, indicating that it had just entered the cortex.

1305 During electrophysiological recordings the isoflurane level was maintained at 0.7%. To ensure
1306 controlled conditions for sensory stimulation, all experiments were carried out in a dark, sound-
1307 attenuated anechoic chamber (Acoustair, Moerkapelle, Netherlands). Visual stimuli were
1308 created via an LED placed in front of the animal's eye. In separate blocks, 150 laser stimuli of

1309 different colors ('red', 633nm LuxXplus and 'blue', 473nm LuxXplus, LightHub-4, Omicron) were
1310 applied through the optrode for 500 ms, each, at a variable interval of 2.5-3 seconds. Randomly,
1311 75 laser stimuli were accompanied by a 10ms LED-flash, starting 100ms after the respective
1312 laser onset. For control, one block of 75 LED-flashes alone were presented at comparable
1313 interstimulus intervals.

1314 Electrophysiological signals were sampled with an AlphaLab SnR recording system (Alpha
1315 Omega Engineering, Nazareth, Israel) or with a self-developed neural recording system based
1316 on INTAN digital head-stages (RHD2132, Intantech). Signals recorded from the intracortical
1317 laminar probe were band-pass filtered between 0.5 Hz and 7.5 kHz and digitized at 22-44 kHz
1318 or 25 kHz, respectively. All analyses of neural data presented in this study were performed
1319 offline after the completion of experiments using MATLAB scripts (MathWorks). To extract
1320 multiunit spiking activity (MUA) from broadband extracellular recordings, we high-pass filtered
1321 signals at 500Hz and detected spikes at negative threshold (>3.5 SD)⁷⁴.

1322

1323 **Data availability**

1324 Source data are provided with this paper. All data generated in this study are provided in the
1325 Source Data file.

1326

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1343

1344 **Author contributions**

1345 Conceptualization: JV, SRR, PH, JSW; Investigation: JV, SRR, AD, FP, RS, FT, ACFB, IB, FZ,
1346 NZ, JA, SA, KS, JSW; Data Curation: JV, SRR, AD, FP, RS, FT, ACFB, JA; Analysis: JV, SRR,
1347 AD, FP, RS, FT, ACFB, IB, FZ, JA, JSW; Software: AD, FP; Supervision: EP, AG, PS, VE, AKE,
1348 PH, JSW; Funding acquisition: EP, AG, PS, VE, AKE, PH, JSW; Project administration: PH,
1349 JSW; Writing: JV, SRR, FP, RS, JSW with contributions from all authors.

1350

1351 **Competing interests**

1352 The authors declare no competing interests

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1355

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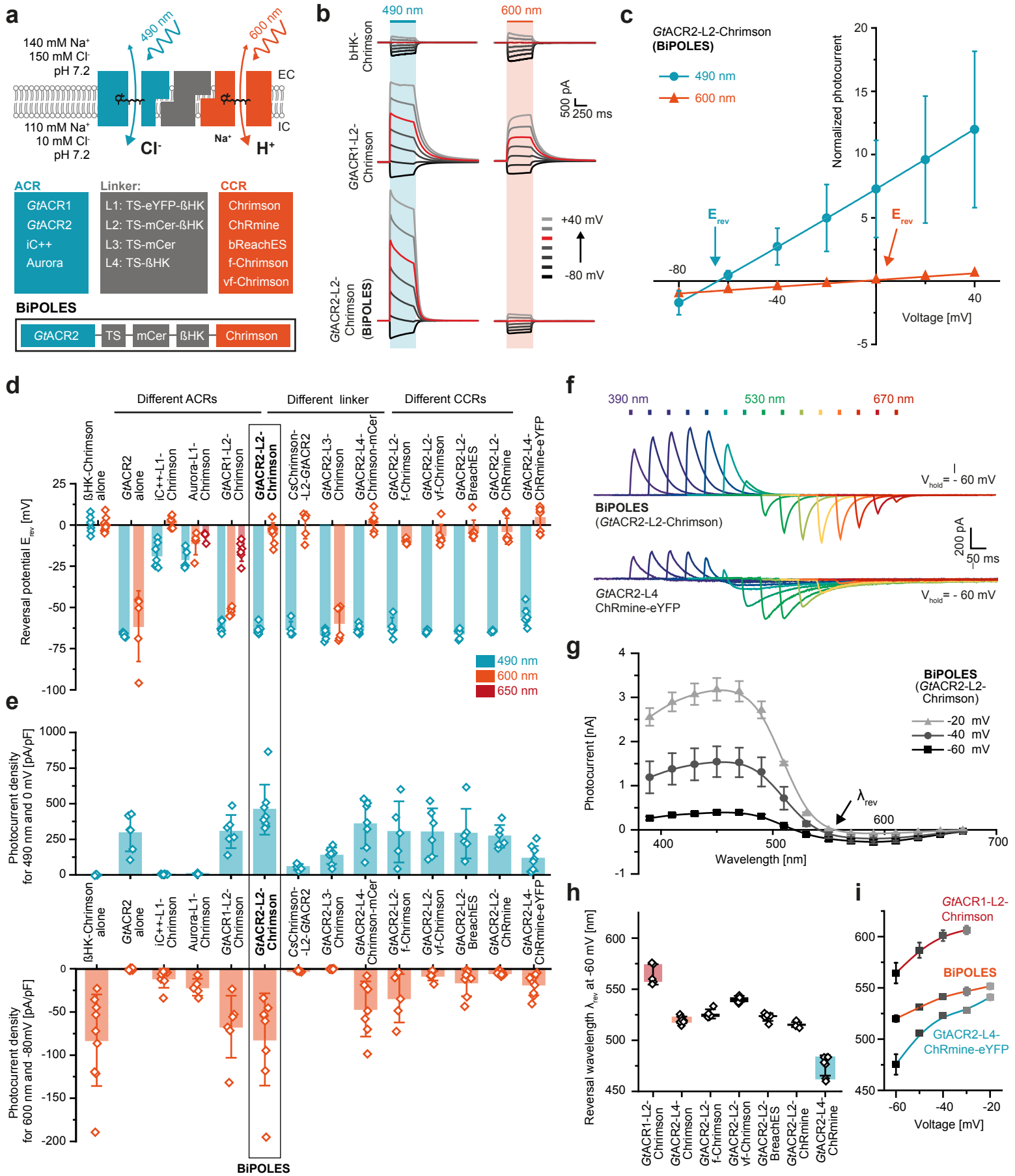
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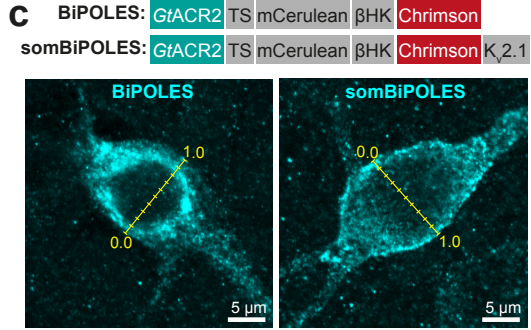
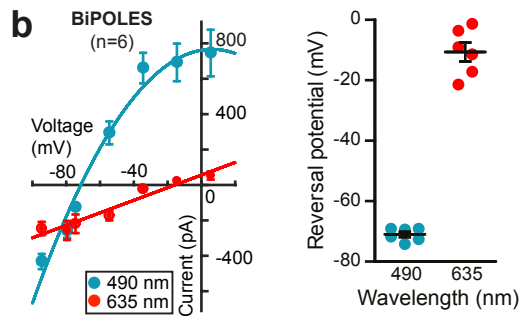
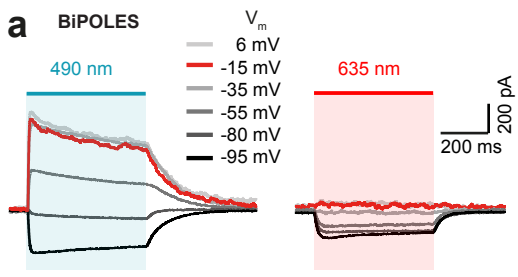
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Frequency distribution of grey values above 80% of maximum fluorescence along the cell diameter

