Optogenetic manipulation of neuronal activity through excitatory and inhibitory opsins has become an indispensable experimental strategy in neuroscience research. For many applications bidirectional control of neuronal activity allowing both excitation and inhibition of the same neurons in a single experiment is desired. This requires low spectral overlap between the excitatory and inhibitory opsin, matched photocurrent amplitudes and a fixed expression ratio. Moreover, independent activation of two distinct neuronal populations with different optogenetic actuators is still challenging due to blue-light sensitivity of all opsins. Here we report BiPOLES, an optogenetic tool for potent neuronal excitation and inhibition with light of two different wavelengths. BiPOLES enables sensitive, reliable dual-color neuronal spiking and silencing with single- or two-photon excitation, optical tuning of the membrane voltage, and independent optogenetic control of two neuronal populations using a second, blue-light sensitive opsin. The utility of BiPOLES is demonstrated in worms, flies, mice and ferrets.
To prove the necessity and sufficiency of a particular neuronal population for a specific behavior, a cognitive task, or a pathological condition, faithful activation, and inhibition of this population of neurons are required. In principle, optogenetic manipulations allow such interventions. However, excitation and inhibition of the neuronal population of interest are commonly done in separate experiments, where either an excitatory or inhibitory microbial opsin is expressed. Alternatively, if both opsins are co-expressed in the same cells, it is essential to achieve efficient membrane trafficking of both opsins, equal subcellular distributions, and a tightly controlled ratio between excitatory and inhibitory action at the specific wavelengths and membrane potentials, so that neuronal activation and silencing can be controlled precisely and predictably in all transduced cells. Precise co-localization of the two opsins is important when local, subcellular stimulation is required, or when control of individual neurons is intended, for example with two-photon holographic stimulation. Meeting these criteria is particularly challenging in vivo, where the optogenetic actuators are either expressed in transgenic lines or from viral vectors that are exogenously delivered to the target neurons by a single viral vector. Moreover, for expression with fixed stoichiometry, the opsins should be encoded in a single open reading frame (ORF).

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

The second strategy employed a 2A ribosomal skip sequence to express the enhanced opsins ChR2(H134R) and NpHR3.0 as independent proteins at a fixed ratio from the same mRNA. These bicistronic constructs, termed eNPAC, and eNPAC2.0, were used for bidirectional control of neuronal activity in various brain regions in mice. While membrane trafficking of the individual opsins is more efficient compared to the gene fusion strategy, the expression ratio might still vary from cell to cell. Moreover, subcellular targeted co-localization (e.g., at the soma) is not easily achieved. Finally, functionality is limited in some model organisms such as *D. melanogaster*, since rhodopsin pumps are not efficient in these animals.

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

In order to overcome current limitations for bidirectional neuronal manipulations and to facilitate spiking of neuronal populations with orange-red light exclusively, in this work we systematically explore the generation of two-channel fusion proteins that combine red-light activated cation-channels and blue-light activated anion-channels enabling neuronal spiking and inhibition with red and blue light, respectively. With respect to previous bidirectional tools, inversion of the excitatory and inhibitory action spectra restricts depolarization to a narrow, orange-red spectral window since the inhibitory opsin compensates the blue-light-activated currents of the excitatory red-shifted channel. We show that among all tested variants, a combination of GtACR18 and Chrimson14 termed BiPoles (for Bidirectional Pair of Opsins for Light-induced Excitation and Silencing) proves most promising and allows (1) potent and reliable blue-light-mediated silencing and red-light-mediated spiking of pyramidal neurons in hippocampal slices; (2) bidirectional control of single neurons with single-photon illumination and two-photon holographic stimulation; (3) dual-color control of two distinct neuronal populations in combination with a second blue-light-sensitive ChR without cross-talk at light intensities spanning multiple orders of magnitude; (4) precise optical tuning of the membrane voltage between the chloride and cation reversal potentials; (5) bidirectional manipulations of neuronal activity in a wide range of invertebrate and vertebrate model organisms including worms, fruit flies, mice, and ferrets.

**Results**

**Engineering of BiPoles and biophysical characterization in HEK Cells**. To identify suitable combinations of opsins for potent membrane voltage shunting or depolarization with blue and red light, respectively, we combined the blue-light or green-light sensitive anion-conducting channelrhodopsins (ACRs) Aurora12, iC3+19, GtACR1, and GtACR218 with the red-light sensitive cation-conducting channelrhodopsin (CCR) Chrimson14; or conversely, the blue-light sensitive GtACR2 with the red-light sensitive CCRs bReaChES20, f-Chrimson, vf-Chrimson21, and ChRm22 (Fig. 1a). We fused these opsin-pairs with different linkers, expanding previous rhodopsin fusion strategies to obtain optimal expression and membrane targeting. The linkers were composed of the Kiz2.1 membrane trafficking signal (TS)4, different arrangements of a cyan or yellow fluorescent protein, and the transmembrane β helix of the rat gastric H+;/K+ ATPase (βHK) to maintain the correct membrane topology of both opsins (Fig. 1a).

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

At membrane potentials between the Nernst potentials for chloride and protons, blue and red light induced outward and inward currents, respectively, in all GtACR-fusion constructs. (Fig. 1e–g, Supplementary Fig. 1). The specific wavelength of photocurrent inversion (λ_{rev}) was dependent on the absorption spectra and relative conductance of the employed channels, as well as on the relative ionic driving forces defined by the membrane voltage and the respective ion gradients (Fig. 1g–i).

The red-shift of λ_{rev} for the vf-Chrimson tandem compared to BiPOLES reflects the reduced conductance of this Chrimson mutant (Fig. 1h, Supplementary Fig. 1c), as already previously shown, whereas the blue-shift of λ_{rev} for the ChRmine tandem with L4 (Fig. 1f, h) is explained by the blue-shifted activation spectrum of ChRmine compared to Chrimson and its
presumably large single-channel conductance. Switching the L4 linker to L2 shifted $\lambda_{\text{rev}}$ to longer wavelengths for the ChRmine fusion constructs at the expense of ChRmine photocurrents (Fig. 1e, h), pointing to a stronger impact of the protein linker on the ChRmine photocurrent compared to other red-shifted CCRs (Fig. 1e).

Among all tested combinations, GtACR2-L2-Chrimson—from here on termed BiPOLES—was the most promising variant. First, it showed the largest photocurrent densities of all tested fusion constructs (Fig. 1e,f), second, reversal potentials for blue or red light excitation were close to those of individually expressed channels ($-64 \pm 3$ mV and $-5 \pm 6$ mV for BiPOLES compared to $-66 \pm 2$ mV and $0 \pm 5$ mV of GtACR2 and $\pm 5$ mV of HK-Chrom) expressed alone, Fig. 1c, d, Supplementary Fig. 1b) and third, peak activity of the inhibitory anion and excitatory cation current had the largest spectral separation among all tested variants ($15 \pm 5$ nm, Fig. 1f, g). Thus, BiPOLES enables selective activation of large anion and cation currents with spectrally well-separated wavelengths (Fig. 1e). BiPOLES was remarkably better expressed in HEK-cells than the previously reported ChR2-L1-NpHR fusion construct and featured larger photocurrents at $-60$ mV than the bicistronic construct ENPAC2.6 (Supplementary Fig. 2a–c). Moreover, employing an anion channel with high conductance instead of a chloride pump, which transports one charge per absorbed photon and is weak at a negative voltage, yielded chloride currents in BiPOLES expressing cells at irradiances 2 orders of magnitude lower than with ENPAC2.0 (Supplementary Fig. 2d–f). Anion conductance in BiPOLES was sufficiently large to compensate inward currents of Chrom in even at high irradiance, driving the cell back to the chloride Nernst potential, which is close to the resting membrane voltage (Supplementary Fig. 2d–f). We further verified the implementation of an anion-conducting channel by testing whether sufficient blue-light hyperpolarization could be achieved with a rhodopsin pump instead of a channel. Replacing GtACR2 with a blue-light sensitive proton pump led to barely detectable outward currents at the same irradiance due to low ion turnover of the ion pump under the given voltage and ion conditions (Supplementary Fig. 2d, g).

**Evaluation of BiPOLES in CA1 pyramidal neurons.** Next, we validated BiPOLES as an optogenetic tool for bidirectional control of neuronal activity. In CA1 pyramidal neurons of rats hippocampal slice cultures, illumination triggered photocurrents with biophysical properties similar to those observed in HEK cells (Fig. 2a, b, Supplementary Fig. 3a–c). We observed membrane-localized BiPOLES expression most strongly in the somatodendritic compartment (Fig. 2c, Supplementary Fig. 3d). However, some fraction of the protein accumulated inside the cell in the periphery of the cell nucleus, indicating sub-optimal membrane trafficking of BiPOLES. To enhance membrane trafficking, we generated a soma-targeted variant (somBiPOLES) by attaching a C-terminal Kv2.1-trafficking sequence. Soma targeting has the additional benefit of avoiding the expression of the construct in axon terminals, where the functionality of BiPOLES might be limited due to an excitatory chloride reversal potential and subsequent depolarizing action of GtACR2.28,29. somBiPOLES showed strongly improved membrane localization restricted to the cell soma and proximal dendrites with no detectable intracellular accumulations (Fig. 2c, Supplementary Fig. 3d). Compared to BiPOLES, blue-light and red-light mediated photocurrents were enhanced and now similar in magnitude to those in neurons expressing either Chrimson or soma-targeted GtACR2 (somBiACR2), alone (Fig. 2d, Supplementary Fig. 4a, 5a, b). Passive and active membrane parameters of BiPOLES-expressing and somBiPOLES-expressing neurons were similar to non-transduced, wild-type neurons (Supplementary Fig. 6), indicative of good tolerability in neurons.

To verify the confinement of somBiPOLES to the somatodendritic compartment despite the improved expression, we virally transduced area CA3 in hippocampal slice cultures with somBiPOLES and recorded optically evoked EPSCs in postsynaptic CA1 cells. Local illumination with red light in CA3 triggered large excitatory postsynaptic currents (EPSCs), while local red illumination of axon terminals in CA1 ($635$ nm, $2$ pulses of $5$ ms, $40$ ms ISI, $50$ mW mm$^{-2}$), did not trigger synaptic release, indicating the absence of somBiPOLES from axonal terminals (Supplementary Fig. 3e,f). Thus, despite enhanced membrane trafficking, somBiPOLES remained confined to the somatodendritic compartment.

Having shown that somBiPOLES is efficiently expressed in CA1 pyramidal cells, we next systematically benchmarked light-evoked spiking and inhibition parameters for somBiPOLES by direct comparison to Chrimson or somGtACR2 expressed in
Fig. 2 Expression and functional characterization of BiPOLES and somBiPOLES in hippocampal neurons. a Representative photocurrent traces of BiPOLES in CA1 pyramidal neurons at indicated membrane voltages (V_m, from −95 to +6 mV) upon illumination with 490 or 635 nm (500 ms, 10 mW mm^−2). b Left: quantification of photocurrent-voltage relationship (symbols: mean ± SEM, n = 6 cells, lines: polynomial regression fitting, R^2 = 0.98 and 0.94, for 490 and 635 nm, respectively). Right: reversal potential under 490 or 635 nm illumination (black lines: mean ± SEM, n = 6 expressing or somBiPOLES-expressing CA1 pyramidal neurons. Inward cationic photocurrents evoked by a 635 nm light pulse (20 ms, 1 mW mm^−2) strongly enhanced for somBiPOLES compared to BiPOLES (black horizontal lines: medians, V_m = −95 mV, and outward anionic photocurrents evoked by a 490 nm light pulse (100 ms, 10 mW mm^−2) were recorded at a membrane voltage of −75 mV, and outward anionic photocurrents evoked by a 490 nm light pulse (100 ms, 10 mW mm^−2) were recorded at a membrane voltage of −55 mV. Right: Quantification of photocurrent densities evoked under the indicated conditions. Note that photocurrent densities were strongly enhanced for somBiPOLES compared to BiPOLES (black horizontal lines: medians, n_BiPOLES = 8 cells, n_somBiPOLES = 6 cells).

In contrast to orange or red light, blue light did not evoke APs at any irradiance in somBiPOLES neurons due to the activity of the blue-light sensitive anion channel. On the contrary, neurons expressing Chrimson alone reached 100% AP firing probability at 10 mW mm^−2 with 470 nm (Fig. 3b, c). Using light ramps with gradually increasing irradiance enabled us to precisely determine the AP threshold and to quantitatively compare the spiking efficacy of different excitatory opsins. The irradiance threshold for the first AP was similar for somBiPOLES and Chrimson at 595 nm (0.74 ± 0.06 mW mm^−2 for somBiPOLES and 0.68 ± 0.05 mW mm^−2 for Chrimson) reflecting that the functional expression levels were similar. In contrast, blue light triggered APs at 0.95 ± 0.09 mW mm^−2 in Chrimson expressing cells, but never in somBiPOLES or BiPOLES neurons (Fig. 3d, e, Supplementary Fig. 7a, b). Thus, somBiPOLES enables neuronal excitation...
Fig. 3 somBiPOLES allows potent dual-color spiking and silencing of the same neurons using red and blue light, respectively. a Quantification of neuronal excitation with somBiPOLES or Chrimson only, b Optical excitation is restricted exclusively to the orange/red spectrum in somBiPOLES-expressing neurons. Left: Example traces of current-clamp (IC) recordings in somBiPOLES-expressing CA1 pyramidal cells to determine light-evoked action potential (AP) probability at different wavelengths. Right: quantification of light-mediated AP probability at indicated wavelengths and irradiances (symbols correspond to mean ± SEM, n = 8 cells). Black outlined circles correspond to irradiance values shown in example traces on the left. c Same experiment as shown in b, except that CA1 neurons express Chrimson only (symbols correspond to mean ± SEM, n = 7 cells). Note blue-light excitation of Chrimson, but not somBiPOLES cells. d Light-ramp stimulation to determine the AP threshold irradiance. Left: Representative membrane voltage traces measured in somBiPOLES-expressing CA1 pyramidal neurons. The light was ramped linearly from 0 to 10 mW mm⁻² over 1 s. Right: Quantification of the irradiance threshold at which the first AP was evoked (black horizontal lines: medians, n = 7 cells). e Same experiment as shown in (b), except that CA1 neurons express Chrimson only (black horizontal lines: medians, n = 7 cells). The threshold for action potential firing with 595 nm was similar between somBiPOLES-expressing and Chrimson-expressing neurons, while somBiPOLES cells were not sensitive to blue light. f Quantification of neuronal silencing with somBiPOLES or somGACR2 only. g somBiPOLES mediates neuronal silencing upon illumination with blue light. Left: Current ramps (from 0-100 to 0-900 pA) were injected into somBiPOLES-expressing CA1 pyramidal cells to induce APs during illumination with blue light at indicated intensities (from 0.001 to 100 mW mm⁻²). The injected current at the time of the first action potential was defined as the rheobase. Right: Quantification of the rheobase shift and the relative change in the number of ramp-evoked action potentials. Illumination with 490 nm light of increasing intensities activated somBiPOLES-mediated Cl⁻ currents shifting the rheobase to higher values and shunting action potentials. h Same experiment is shown in g, except that CA1 neurons express somGACR2 only. Note similar silencing performance of somBiPOLES and GACR2. In h, g black circles correspond to medians, n_somBiPOLES = 6 cells, n_somGACR2 = 6 cells, one-way Friedman test, *p < 0.05, **p < 0.01, ***p < 0.001.

exclusively within a narrow spectral window restricted to orange-red light, avoiding inadvertent blue-light mediated spiking.

Next, we quantified the silencing capacity of somBiPOLES and compared it to somGACR2 alone—the most potent opsin for blue-light mediated somatic silencing—by measuring the capacity to shift the threshold for electrically evoked APs (i.e., rheobase, see “Methods” section). Both variants similarly shifted the rheobase towards larger currents starting at an irradiance of 0.1 mW mm⁻² with 490 nm light, leading to a complete block of APs in most cases (Fig. 3g, h). Neuronal silencing was efficient under 490 nm-illumination, even at high irradiances (up to 100 mW mm⁻², Fig. 3g), showing that blue light cross-activation of Chrimson in somBiPOLES did not compromise neuronal shunting.

We compared somBiPOLES with eNPAC2.0, the most advanced optogenetic tool currently available for dual-color
excitation and inhibition\(^{4,6,7}\). In eNPAC2.0 expressing CA1 pyramidal neurons, depolarizing and hyperpolarizing photocurrents were present under blue and yellow/orange light, respectively (Supplementary Fig. 8a), consistent with its inverted action spectrum compared to BiPOLES (Supplementary Fig. 2). Compared to BiPOLES (Supplementary Fig. 3c) peak photocurrent ratios were more variable between cells (Supplementary Fig. 8a), indicative of different stoichiometries between ChR2(HR) and eNpHR3.0 in different neurons, probably because membrane trafficking and degradation of both opsins occur independently. Moreover, blue-light-evoked spiking with eNPAC2.0-required approx. 10-fold higher irradiance compared to somBiPOLES and did not reach 100% reliability (Supplementary Fig. 8c), which might be explained by cross-activation of eNpHR3.0 under high blue irradiance (see also Supplementary Fig. 2d). Blue-light-triggered APs could not be reliably blocked with concomitant yellow illumination at 10 mW mm\(^{-2}\) (Supplementary Fig. 8b). Further on, activation of eNPAC2.0 (i.e., eNpHR3.0) with yellow light (580 nm) caused strong membrane hyperpolarization followed by rebound spikes in some cases (Supplementary Fig. 8d). Finally, and consistent with photocurrent measurements in HEK cells (Supplementary Fig. 2e, f), silencing of electrically evoked APs required 100-fold higher irradiance with eNPAC2.0, compared to somBiPOLES, until a significant rheobase-shift was observed (Supplementary Fig. 8e).

In summary, somBiPOLES is suitable for potent, reliable neuronal activation exclusively with orange-red light and silencing with blue light, somBiPOLES displays similar potency for neuronal excitation and inhibition as Chrismon and somGtACR2 alone.

**BiPOLES allows various neuronal manipulations with visible light.** We evaluated BiPOLES and somBiPOLES in the context of three distinct neuronal applications: bidirectional control of neuronal activity, optical tuning of the membrane voltage, and independent spiking of two distinct neuronal populations.

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

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

Since BiPOLES permits neuronal spiking exclusively within the orange-red light window, it facilitates two-color excitation of genetically distinct but spatially intermingled neuronal populations using a second, blue-light-activated ChR (Fig. 4f). To demonstrate this, we expressed somBiPOLES in CA1 VIP interneurons and CheRiff, a blue-light-sensitive ChR (\(\lambda_{\text{max}} = 460\) nm\(^{38}\)) in CA1 pyramidal neurons (Fig. 4g, see “Methods” section for details). Both CA1 and VIP neurons innervate Orians-Lacunosum-Molecular (OLM) interneurons. Therefore, exclusive excitation of CA1 pyramidal cells or VIP interneurons is expected to trigger excitatory (EPSCs) and inhibitory (IPSCs) postsynaptic currents, respectively. CheRiff-expressing pyramidal cells were readily spiking upon blue, but not orange-red illumination up to 10 mW mm\(^{-2}\) (Fig. 4h, Supplementary Fig. 9). Conversely, as expected, red light evoked APs in somBiPOLES-expressing VIP neurons, while blue light up to 100 mW mm\(^{-2}\) did not evoke APs (Fig. 4b). Next, we recorded synaptic inputs from these two populations onto VIP-negative GABAergic neurons in stratum-oriens (Fig. 4i). As expected, blue light triggered EPSCs (CheRiff) and red light triggered IPSCs (somBiPOLES), evident by their respective reversal potentials at 8.8 ± 10.4 mV and −71.4 ± 13.1 mV (Fig. 4i). Thus, somBiPOLES, in combination with the blue-light sensitive CheRiff enabled independent activation of two distinct populations of neurons in the same field of view.

**Bidirectional neuronal control using dual-laser two-photon holography.** Two-photon holographic excitation enables spatially localized photostimulation of multiple neurons with single-cell resolution in scattering tissue\(^1\). We evaluated the feasibility of bidirectional control of single neurons by two-photon holographic excitation (Supplementary Fig. 10a) in hippocampal organotypic slices virally transduced with somBiPOLES expressed from a CaMKII promoter. Single-photon excitation confirmed the high potency of somBiPOLES using this expression strategy (Supplementary Fig. 11). The two-photon action spectrum of somBiPOLES was explored by measuring the peak photocurrents (\(I_p\)) at a range of holding potentials (−80 to −55 mV) and excitation wavelengths (850 to 1100 nm). Similar to single-photon excitation, blue-shifted wavelengths (\(\lambda_{\text{ex}} > 980\) nm) generated large photocurrents, apparently dominated by the flow of chloride ions (outward chloride currents below the chloride Nernst potential and inward chloride currents above the chloride Nernst potential, Fig. 5a–c, Supplementary Fig. 10b). Red-shifted wavelengths (\(\lambda_{\text{ex}} < 980\) nm) generated photocurrents, which appeared to be dominated by the flow of protons and cations across the membrane (inward currents at physiological neuronal membrane potentials, Fig. 5a–c, Supplementary Fig. 10b). Since 920 nm and 1100 nm illumination generated the largest magnitudes of inhibitory and excitatory photocurrents, respectively, these wavelengths were used to evaluate whether the neuronal activity could be reliably suppressed or evoked in neurons expressing...
somBiPOLES. Action potentials could be reliably evoked using short (5 ms) exposure to 1100 nm light (power density: 0.44 mW/μm²), with latency (19.9 ± 6.3 ms) and jitter (2.5 ± 1.5 ms) (Fig. 5d, Supplementary Fig. 10c) comparable to literature values for Chrimson 31. 5 ms pulses were also able to induce high-fidelity trains of APs with frequencies up to 20 Hz (Supplementary Fig. 10d). It is likely that shorter latency and jitter (and consequently higher rates of trains of APs) could be achieved by replacing the stimulation laser with one with optimized pulse parameters, in particular, higher peak energy 32. 920 nm excitation effectively inhibited neural activity, increasing the rheobase of AP firing at power densities above 0.1 mW μm⁻² (Fig. 5e). It further enabled temporally precise elimination of single electrically evoked APs (Supplementary Fig. 10e) and silencing of neuronal activity over sustained (200 ms) periods (Fig. 5f). Finally, we demonstrate two-photon, bidirectional control of neurons by coincident illumination of appropriately titrated 920 nm and 1100 nm light (Fig. 5g). Thus, somBiPOLES is suitable for dual-color
two-photon holographic manipulation of neuronal activity with a cellular resolution with standard lasers typically used for two-photon imaging.

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

**Bidirectional control of motor activity in *C. elegans*.** We expressed BiPOLES in cholinergic motor neurons of *C. elegans* to optically control body contraction and relaxation. Illumination with red light resulted in body-wall muscle contraction and effective body shrinkage, consistent with motor neuron activation. Conversely, blue light triggered body extension, indicative of muscle relaxation and thus, cholinergic motor neuron inhibition (Fig. 6b).

---

**Fig. 5 Bidirectional control of neuronal activity with somBiPOLES using dual-color two-photon holography.** **a-c** Voltage clamp (VC) characterization of somBiPOLES in CA1 pyramidal cells. **a** Representative photocurrent traces at different holding potentials, obtained by continuous 200 ms illumination of 920 and 1100 nm at constant average power density (0.44 and 1.00 mW µm⁻²). **b** Peak photocurrent as a function of wavelength at different holding potentials (mean ± SEM, n = 5). Data acquired with a constant photon flux of $6.77 \times 10^{26}$ photons s⁻¹m⁻². Dashed lines indicate 920 and 1100 nm respectively; the wavelengths subsequently utilized for photo-stimulation and inhibition. **c** Peak photocurrent as a function of incident power density at a holding potential of −60 mV (mean ± SEM, 920 nm, n = 4; 1100 nm, n = 5).

**d-g** Current clamp (IC) characterization of somBiPOLES in CA1 pyramidal cells. **d** Probability of photo evoked action potentials under 1100 nm illumination for 5 ms (n = 5, red: average, gray: individual trials). **e** Characterization of the efficacy of silencing somBiPOLES expressing neurons under 920-nm illumination by co-injection of current (Box: median, 1st-3rd quartile, whiskers: 1.5x inter quartile range, n = 5). **f** Representative voltage traces demonstrating sustained neuronal silencing of neurons by two-photon excitation of somBiPOLES at 920 nm. Upper trace (control): 550 pA current injected (illustrated by the black line), no light. Lower trace: continuous injection of 550 pA current, 0.3 mW µm⁻², 920 nm, 2 Hz, 200 ms illumination. **g** Two-photon, bidirectional, control of single neurons demonstrated by co-incident illumination of 920 nm and 1100 nm light. Upper trace: 10 Hz spike train evoked by 15 ms pulses of 1100 nm light. Lower trace: optically induced action potentials shunted using a single, 200 ms pulse of 920 nm light.
animal. Therefore, we tested this directly with light conditions similar to those used for BiPOLES activation. Excitation with blue light resulted in a 5% body length decrease, while activation of NpHR at its peak wavelength (575 nm) failed to induce significant changes in body length (Supplementary Fig. 12b). Thus, BiPOLES expands the possibilities for bidirectional control of neuronal activity in C. elegans beyond what is achievable with currently available tools.

Bidirectional control of motor activity and nociception in D. melanogaster. Next, we demonstrate bidirectional control of circuit function and behavior with BiPOLES in Drosophila melanogaster. GtACR2 and CsChrimson were previously used in separate experiments to silence and activate neuronal activity, respectively. In contrast, rhodopsin pump functionality is strongly limited in this organism and bidirectional control of neuronal activity has not been achieved. We, therefore, expressed BiPOLES in glutamatergic motor neurons of D. melanogaster larvae (Fig. 6c). Illumination with blue light led to muscle relaxation and concomitant elongation (Fig. 6d). The change in body length was similar to animals expressing BiPOLES alone (Supplementary Fig. 12c). Importantly, GtACR2 activation in BiPOLES overrides blue-light evoked Chrimson activity and...
mediated arousal reliably triggered transient pupil dilation, indicative of LC through an optical in the Locus Coeruleus (LC) (Fig. 7a). Orange illumination (594 nm) in TH-Cre mice, targeting Cre-expressing neurons in mammalian brain. To this end, we conditionally expressed viral vectors, we sought to test BiPOLES and somBiPOLES in the Drosophila model systems, showing potent, bidirectional modulation in the somatic motor system, the D. melanogaster motor, and the action potential threshold.

Discussion

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

BiPOLES performs reliably in invertebrate and vertebrate model systems, showing potent, bidirectional modulation in the C. elegans motor system, the D. melanogaster motor and nociceptive systems, and the ferret visual cortex. The addition of the soma-targeting signal from the mammalian potassium channel Kv2.1 yielded somBiPOLES, leading to further enhancement of trafficking to the plasma membrane at the soma and proximal dendrites while avoiding localization to distal dendrites and axons, as previously shown for individually expressed microbial rhodopsins. Thus, eliminating the risk of inadvertent blue-light mediated depolarization of axons while improving bidirectional optogenetic manipulation of the somatodendritic compartment somBiPOLES is optimized for applications in mammalian systems.

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

All-optical, bidirectional control of pupil size in mice. To further extend the applications of BiPOLES to vertebrates, we generated various conditional and non-conditional viral vectors, in which the expression of the fusion construct is regulated by different promoters (see “Methods” section, Table 1). Using these viral vectors, we sought to test BiPOLES and somBiPOLES in the mammalian brain. To this end, we conditionally expressed somBiPOLES in TH-Cre mice, targeting Cre-expressing neurons in the Locus Coeruleus (LC) (Fig. 7a). Orange illumination (594 nm) through an optical fiber implanted bilaterally above LC reliably triggered transient pupil dilation, indicative of LC-mediated arousal (Fig. 7b–d). Pupil dilation was evident already at 0.7 mW at the fiber tip and gradually increased with increasing light power (Supplementary Fig. 13a). Light-mediated pupil dilation was reverted immediately by additional blue light (473 nm) during the orange-light stimulation or suppressed altogether when blue-light delivery started before orange-light application (Fig. 7b–d), suggesting that orange-light-induced spiking of somBiPOLES-expressing neurons in LC was efficiently shunted. Illumination of the LC in wt-animals did not influence pupil dynamics (Supplementary Fig. 13b). Thus, LC neurons were bidirectionally controlled specifically in somBiPOLES expressing animals.

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

Manipulation of neocortical excitation/inhibition ratio in ferrets. Finally, we applied BiPOLES to bidirectionally control the excitation/inhibition (E/I) ratio in the mammalian neocortex. Therefore, we generated a viral vector using the minimal Dlx promoter to target GABAergic neurons in the ferret secondary visual cortex (V2). Functional characterization in GABAergic neurons in vitro confirms all-optical spiking and inhibition of GABAergic neurons with mDlx-BiPOLES (Supplementary Fig. 15). Thus, we injected mDlx-BiPOLES in ferret V2 to modulate E/I ratio during sensory processing (Fig. 7e). Extracellular recordings obtained from linear silicon probes in V2 of isoflurane-anesthetized ferrets provided evidence for modulation of cortical activity by shifts in the E/I ratio (Fig. 7f, g). Blue light led to an increase in baseline activity, consistent with the deactivation of inhibitory, GABAergic neurons (Fig. 7f, g). Activation of GABAergic cells by red light did not further decrease the low cortical baseline activity, but significantly reduced cortical responses triggered by sensory stimuli (Fig. 7f, g). Although effects of blue light on evoked spiking were not significant in the average data, we obtained clear evidence in individual recordings that blue light could enhance late response components (Fig. 7f), confirming a disinhibitory effect. Overall, these data suggest that BiPOLES is efficient in bidirectional control of inhibitory mechanisms, demonstrating its applicability for the control of E/I shifts in the cortical microcircuit in vivo.
illumination and increases both the light intensity range and the spectral range that allows exclusive activation of the red-shifted cation channel. Due to the large spectral separation, BiPOLES can be controlled with two simple light sources, such as LEDs, without the requirement of sophisticated spectral control, making its use straightforward. The GaCR2-L4-ChRmine-construct might be an interesting alternative if spectrally narrow light sources, such as lasers, are available, because it reaches peak depolarizing currents 60 nm blue-shifted compared to BiPOLES. Thus, inhibition and excitation can be achieved with 430–470 nm and 530–550 nm (Fig. 1f) providing an additional spectral window in the red, that can be used for a third optogenetic actuator or sensor. Finally, a seemingly trivial but equally important advantage of all the tandem systems we present here is their modular architecture allowing easy tailoring of fusion constructs fulfilling specific future experimental requirements.

Noteworthy, BiPOLES does not represent the first optogenetic tool for bidirectional control of neuronal activity. Different combinations of the excitatory blue-light-sensitive ChR2 and orange-light-sensitive inhibitory ion pumps such as NpHR, bR, or
Arch3.0 were generated previously. However, among all these variants, only the combination of ChR2 and NpHR (i.e., eN PAC and eNPAC2.0) was successfully used to address neuroscientific questions in mice. BiPOLES will significantly expand the possibilities of bidirectional neuronal manipulations, since, aside from efficient expression in a wide array of different model systems, it also features a number of additional advantages: First, combining two potent channels, rather than a pump and a channel, provides a more balanced ionic flux per absorbed photon for the inhibitory and excitatory rhodopsins. This results in a high operational light sensitivity for both excitation and inhibition by orange and blue light, respectively. In contrast, high irradiance and expression levels are required for the ion pumps that only transport one charge per absorbed photon. Second, due to the use of two channels, BiPOLES-mediated photocurrents do not actively move ions against their gradients, which can cause adverse side-effects, but rather fixes the neuronal membrane voltage anywhere between the reversal potential of GaCr2 and Chrimson. The membrane voltage can be tuned depending on the ratio of blue/red light or a single light source tuned to wavelengths between the absorption peaks of GaCr2 and Chrimson. Third, inverting the color of the excitatory and inhibitory opsins, compared to previous tools, restricts optical excitation in BiPOLES-expressing cells exclusively to the orange/red spectrum. The inverted color scheme enables scale-free and mutually exclusive spiking of two neuronal populations in combination with a second, blue-light-sensitive ChR, expressed in the second population of neurons, as the blue-light-activated, inhibitory channel GaCr2 potently shunts Chrimson-mediated, blue-light-activated excitatory photocurrents. Other applications could employ multiplexing with blue-light sensitive cyclases or genetically encoded activity-indicators that require blue light for activation.

Bidirectional optogenetic control in the same cells has not been achieved with two-photon excitation, so far; partially due to the low quantum efficiency of rhodopsin pumps, which limits their two-photon activation. In contrast, the large conductance of the two channels improves their efficacy with respect to the number of transported ions per absorbed photon, and their presence at equal stoichiometry anywhere on the membrane ensures the reliable and reproducible generation of anion currents and/or cation currents, which is particularly important under locally confined two-photon excitation.

In principle, also multicistronic vectors encoding both opsins under a single promoter using either an internal ribosomal entry site (IRES) or a 2A ribosomal skip sequence allow expression of both ion channels at a fixed ratio from a single AAV vector. However, with both of these strategies, neither co-localized nor stoichiometric membrane expression of both channels is guaranteed since both channels might get differentially targeted and distributed in the plasma membrane. This may not pose a limitation for experiments that require bidirectional control of large numbers of cells where precise control of a single-cell activity or sub-cellular ion gradients is not so crucial. BiPOLES as a covalently linked fusion protein displays a fixed expression of both opsins at a 1:1 stoichiometry anywhere in the membrane and membrane trafficking or degradation of both opsins occur at identical rates, preserving excitatory and inhibitory currents at a fixed ratio in all expressing cells. A fixed stoichiometry anywhere in the cell membrane is important if local, subcellular activation of the opsins is required, such as during two-photon excitation or when a fixed ratio of cation and anion conductance is desired between different neurons or in particular neuronal compartments, such as single dendrites or dendritic spines.

Notably, BiPOLES employs an anion channel for optogenetic silencing and therefore relies on the extracellular and intracellular chloride concentration. In the case of a depolarized chloride Nernst potential, the opening of the anion channel may induce depolarizing currents, which can trigger action potentials or neurotransmitter release. Unlike for rhodopsin pumps, efficient silencing consequently requires low cytosolic chloride concentrations and is therefore limited in neurons or cellular compartments with a depolarized Nernst potential for chloride, such as immature neurons or axon terminals. Given these caveats, BiPOLES may not be suitable for bidirectional control of developing neurons or presynaptic boutons. In this case, silencing may be more efficient with rhodopsin pumps, despite their own limitations or with G-protein coupled rhodopsins. As with any optogenetic application, neurophysiological parameters need to be considered by the experimenter, guiding the appropriate choice of the tool suitable to address the specific experimental requirements.

Since BiPOLES can be used to spike or inhibit the same population of mature neurons in vivo, a number of previously inaccessible questions can be addressed. During extracellular recordings, BiPOLES may be useful for optogenetic identification (optotagging) with red light and optogenetic silencing of the same neurons. This will permit verification of the identity of silenced neurons by their spiking profiles. Moreover, in combination with a second, blue-light sensitive ChR, BiPOLES can be used to map local networks of spatially intermingled neurons. For example, expressed in distinct types of molecularly defined GABAergic neurons, connectivity of these neurons to a post-synaptic target cell can be evaluated. Additional applications of
BiPOLES may encompass bidirectional control of engram neurons33 to test both necessity and sufficiency of a particular set of neurons for memory retrieval or switching the valence of a particular experience by inhibiting or activating the same or even two distinct populations of neuromodulatory neurons. In principle, this could even be achieved with cellular resolution using two-photon holography. Due to its utility for a wide range of research questions, its versatile functionality, and its applicability in numerous model systems, as demonstrated in this study, BiPOLES fills an important gap in the optogenetic toolbox and might become the tool of choice to address a number of yet inaccessible problems in neuroscience.

Methods

Molecular biology. For HEK-cell expression, the coding sequences of Chrimson (KF992060.1), CaChrimson (K995863.2) from Chlamydomonas nontagata32, ChR2 from Rhodomonas lens although initially attributed to Tariansa fusca22,25 (Addgene #130997), bReaChES 20, Chlamydomonas noctigama (KF992060.1), CsChrimson (KJ995863.2) from Rhodomonas lens (Gt+), and ACR1 (KP171708) and ACR2 (KP171709) from Guillardia theta34, as well as the blue-shifted Arch3.0 mutant M128A/S151A/A225T herein described as ArchBlue26 were cloned together with mCerulean35 and a trafficking signal (ts) from the Kri.2 channel19 into a pCDNA3.1 vector containing the original opsin tandem cassette2 with a linker composed of eYFP and the first 105 N-terminal amino acids of the rat gastric H+/K+-ATPase beta subunit (BH, NM_012510.2), kindly provided by Sonja Kleinlogel (University of Bern, CH). For direct comparison also the bicistronic tool eNPA1C2.0—kindly provided by Karl Deisseroth (Stanford University, CA)—was cloned into the same backbone. Site-directed mutagenesis to introduce the f-Chrimson and v-f-Chrimson mutations Y261F, W251F, and C225F was realized using the QuickChange Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA) according to the manufacturers’ instructions.

For neuronal expression, the insertion consisting of GtACR2-ts-mCerulean36-JH-Chrision was cloned into an AAV2-backbone behind human synapsin (hSyn) promoter (pAAV-hSyn-BiPOLES-mCerulean; Addgene #154944). For neuronal expression, the insertion consisting of CHACR2-ts-mCerulean36-JH-Chrision was cloned into an AAV2-backbone behind the minimal Dlxs (dDlxs) promoter36 resulting in pAAV-dDlxs-BiPOLES-mCerulean (Addgene #154945). For expression in GABAergic neurons, BiPOLES was cloned into an AAV2-backbone behind the minimal Dlxs (dDlxs) promoter36 resulting in pAAV-dDlxs-BiPOLES-mCerulean (Addgene #154945). For expression in projection neurons, somBiPOLES was cloned into an AAV2-backbone behind the minimal CaMKII promoter36 resulting in pAAV-CaMKII somBiPOLES-mCerulean (Addgene #154945). For expression in GABAAergic neurons, BiPOLES was cloned into an AAV2-backbone behind the minimal Dlxs (dDlxs) promoter36 resulting in pAAV-dDlxs-BiPOLES-mCerulean (Addgene #154945).

Preparation of organotypic hippocampal slice cultures. All procedures were in agreement with the German national animal care guidelines and approved by the independent Hamburg state authority for animal welfare (Behörde für Justiz und Verbraucherschutz). They were performed in accordance with the guidelines of the German Animal Protection Law and the animal welfare officer of the University Medical Center Hamburg-Eppendorf (UMC).

Organotypic hippocampal slice cultures were prepared from Wistar rats or VIP-IRES-Cre mice of both sexes (Jackson-No. 031628) at post-natal days 5–7. Dissected hippocampi were cut into 350 μm slices with a tissue chopper and placed on a porous membrane (Millicell CM, Millipore). Cultures were maintained at 37 °C, 5% CO2 in a medium containing 80% MEM (Sigma M7278), 20% heat-inactivated horse serum (Sigma H1138) supplemented with 1 mM L-glutamine, 0.00125% ascorbic acid, 0.01 mg ml−1 insulin, 1.44 mM CaCl2, 2 mM MgSO4 and 13 mM D-glucose. No antibiotics were added to the culture medium.

Transgene delivery for single-photon experiments. For transgene delivery in organotypic slices, individual CA1 pyramidal cells were transfected by single-cell electroporation36 between DIV 14–16. Except for pAAV-hSyn-eNPA2.0, which was used at a final concentration of 20 ng μl−1, all other plasmids, namely pAAV-hSyn-BiPOLES-mCerulean, pAAV-hSyn-somBiPOLES-mCerulean, pAAV-hSyn-Chrison-mCerulean, and pAAV-hSyn-CACR2-mCerulean were used at a final concentration of 5 ng μl−1 in K-glucosate-based solution consisting of (in mM): 135 K-glucosate, 10 HEPS, 4 NaATP, 0.4 Na-GTP, 0.5 MgCl2, 3 ascorbate, 10 Na2-phosphocreatine (pH 7.2). A plasmid encoding hSyn-mKate2 or hSyn-mCerulean (both at 50 ng μl−1) was co-electroporated with the opsin-mCerulean or eNPA2.0 plasmids, respectively, and served as a morphology marker. An Axopotor 800 A (Molecular Devices) was used to deliver 50 hypopolarizing pulses per soma in a raster scan pattern at 5 μs inter-pulse interval at 1 kHz.

Table 1 List of recombinant adeno-associated viral vectors used for experiments in organotypic hippocampal slices.

| Recombinant adeno-associated virus (pAAV2/9) | Titer used for transduction of hippocampal organotypic slice cultures (vg/ml) | Addgene plasmid reference |
|---------------------------------------------|---------------------------------------------------------------|---------------------------|
| mDlxs-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-ChRiff-tsf-mScarlet-ER      | 8.15 × 10^{11}                                               | n.a.                      |
| mDlxs-H2B-EGFP                               | 2.8 × 10^{10}                                                | n.a.                      |
| CaMKIIa-Cre                                 | 3.0 × 10^{12}                                                | n.a.                      |

Viruses were transduced at the indicated titers. n.c.: not applicable.
pulses (~12 V, 0.5 ms) at 50 Hz. During electroporation, slices were maintained in pre-warmed (37 °C) HEPES-buffered solution (in mM): 143 NaCl, 10 HEPES, 25 D-glucose, 2.5 KCl, 2 CaCl2 and 2 MgCl2. After 400 μA current pulses for 10 to 15 ms, 3–5 μm wide diameter, two consecutive 30 μm wide diameter, two slices were delivered in current-clamp mode. In the case of BiPOLES and somBiPOLES the blue light ramp went up to 100 mW mm–2 to rule out that very high blue-light irradiation might still spike neurons. The irradiance value at the time of the first spike was defined as the irradiance threshold (in mW mm–2) needed to evoke action potential firing.

To compare the irradiance threshold needed to spike CA1 cells with BiPOLES, somBiPOLES, eNPAC2.0, Chromion, and CheRiff across different wavelengths, 470–670 nm light ramps (500 ms) were applied. Alternatively, 2.1), and a combination of these two (onset of blue light 0 mW mm−2) were delivered in current-clamp mode. In the case of BiPOLES and somBiPOLES the blue light ramp went up to 100 mW mm–2 to rule out that very high blue-light irradiation might still spike neurons. The irradiance value at the time of the first spike was defined as the irradiance threshold (in mW mm–2) needed to evoke action potential firing.

To measure the ability of BiPOLES, somBiPOLES, and somGtACr2 to shift the rheobase upon blue-light illumination, depolarizing current ramps (from 0–100 to 0–900 pA) were injected into CA1 neurons in the dark and during illumination with blue light at irradiance values ranging from 0.001 to 0.02 mW mm–2. 2. Light traces were median-filtered to remove orange/red-light-mediated spikes and reveal the slow change in membrane voltage during illumination.

To assess and compare the performance of BiPOLES and somBiPOLES across different wavelengths, light–optical coupling of the membrane potential was achieved by tuning a single wavelength between 385 and 660 nm (2 s light pulses, 0.1 mW mm–2). Voltage traces were median-filtered to remove orange/red-light-mediated spikes and reveal the slow change in membrane voltage during illumination.

To compare the irradiance threshold needed to spike CA1 cells with BiPOLES, somBiPOLES, eNPAC2.0, Chromion, and CheRiff across different wavelengths, 470–670 nm light ramps (500 ms) were applied. Alternatively, 2.1), and a combination of these two (onset of blue light 0 mW mm−2) were delivered in current-clamp mode. In the case of BiPOLES and somBiPOLES the blue light ramp went up to 100 mW mm–2 to rule out that very high blue-light irradiation might still spike neurons. The irradiance value at the time of the first spike was defined as the irradiance threshold (in mW mm–2) needed to evoke action potential firing.

To measure the ability of BiPOLES, somBiPOLES, and somGtACr2 to shift the rheobase upon blue-light illumination, depolarizing current ramps (from 0–100 to 0–900 pA) were injected into CA1 neurons in the dark and during illumination with blue light at irradiance values ranging from 0.001 to 0.02 mW mm–2. 2. Light traces were median-filtered to remove orange/red-light-mediated spikes and reveal the slow change in membrane voltage during illumination.

To assess and compare the performance of BiPOLES and somBiPOLES across different wavelengths, light–optical coupling of the membrane potential was achieved by tuning a single wavelength between 385 and 660 nm (2 s light pulses, 0.1 mW mm–2). Voltage traces were median-filtered to remove orange/red-light-mediated spikes and reveal the slow change in membrane voltage during illumination.

To compare the irradiance threshold needed to spike CA1 cells with BiPOLES, somBiPOLES, eNPAC2.0, Chromion, and CheRiff across different wavelengths, 470–670 nm light ramps (500 ms) were applied. Alternatively, 2.1), and a combination of these two (onset of blue light 0 mW mm−2) were delivered in current-clamp mode. In the case of BiPOLES and somBiPOLES the blue light ramp went up to 100 mW mm–2 to rule out that very high blue-light irradiation might still spike neurons. The irradiance value at the time of the first spike was defined as the irradiance threshold (in mW mm–2) needed to evoke action potential firing.

To measure the ability of BiPOLES, somBiPOLES, and somGtACr2 to shift the rheobase upon blue-light illumination, depolarizing current ramps (from 0–100 to 0–900 pA) were injected into CA1 neurons in the dark and during illumination with blue light at irradiance values ranging from 0.001 to 0.02 mW mm–2. 2. Light traces were median-filtered to remove orange/red-light-mediated spikes and reveal the slow change in membrane voltage during illumination.

To assess and compare the performance of BiPOLES and somBiPOLES across different wavelengths, light–optical coupling of the membrane potential was achieved by tuning a single wavelength between 385 and 660 nm (2 s light pulses, 0.1 mW mm–2). Voltage traces were median-filtered to remove orange/red-light-mediated spikes and reveal the slow change in membrane voltage during illumination.

To compare the irradiance threshold needed to spike CA1 cells with BiPOLES, somBiPOLES, eNPAC2.0, Chromion, and CheRiff across different wavelengths, 470–670 nm light ramps (500 ms) were applied. Alternatively, 2.1), and a combination of these two (onset of blue light 0 mW mm−2) were delivered in current-clamp mode. In the case of BiPOLES and somBiPOLES the blue light ramp went up to 100 mW mm–2 to rule out that very high blue-light irradiation might still spike neurons. The irradiance value at the time of the first spike was defined as the irradiance threshold (in mW mm–2) needed to evoke action potential firing.

To measure the ability of BiPOLES, somBiPOLES, and somGtACr2 to shift the rheobase upon blue-light illumination, depolarizing current ramps (from 0–100 to 0–900 pA) were injected into CA1 neurons in the dark and during illumination with blue light at irradiance values ranging from 0.001 to 0.02 mW mm–2. 2. Light traces were median-filtered to remove orange/red-light-mediated spikes and reveal the slow change in membrane voltage during illumination.

To assess and compare the performance of BiPOLES and somBiPOLES across different wavelengths, light–optical coupling of the membrane potential was achieved by tuning a single wavelength between 385 and 660 nm (2 s light pulses, 0.1 mW mm–2). Voltage traces were median-filtered to remove orange/red-light-mediated spikes and reveal the slow change in membrane voltage during illumination.
Slice culture immunohistochemistry and confocal imaging. The subcellular localization of BiPOLES and somBiPOLES in hippocampal neurons was assessed 20 days after viral transduction (AAV9-AsIO2::mCherry, CaMKIIα-Cre, and CaMKIIα(0.4)-somBiPOLES-mCerulean, respectively. See Table 1 for details). Hippocampal organotypic slice cultures were fixed in a solution of 4% (v/v) paraformaldehyde (PFA) in PBS for 30 min at room temperature (RT). Next, slices were washed in PBS (3 × 10 min), blocked for 2 h at RT (10% [v/v] normal goat serum [NGS]) in 0.3% (v/v) Triton X-100 containing PBS and subsequently incubated for 48 h at 4 °C with a primary antibody against GFP to amplify the mCerulean signal (chicken, anti-GFP, Invitrogen, A10262, Lot 11039, 1:1000). Slices were washed again, transferred onto glass slides, and mounted for visualization with Shandon Immu-Mount (Thermo Scientific; 9990402).

Confocal images were acquired using a laser-scanning microscope (Zeiss, LSM 900) equipped with a ×40 oil-immersion objective lens (Zeiss EC Plan-Neofluar ×40/1.3 oil). Excitation/emission filters were appropriately selected for Alexa 488 using the dye selection function of the Zen software. The image acquisition settings were optimized once and kept constant for all images within an experimental data set. Z-stack images were obtained using a 1 μm z-step at a 1024 × 1024-pixel resolution scanning at 8 μs per pixel. Fiji was used to quantify fluorescence intensity values along a line perpendicular to the cell equator and spanning the cell diameter. For each cell, gray values above 80% of the maximum intensity were distributed in 10 bins according to their location along the line.

Slice culture two-photon imaging. Neurons in organotypic slice cultures (DIV 19–21) were imaged with two-photon microscopy to check for the live expression of IsyN-DIO-somBiPOLES-mCerulean, CaMKIIα(0.4)-DO-ChemRiff-tsf-mScarlet, or mCherry, and CaMKIIα(0.4)-somBiPOLES-mCerulean. The custom-built two-photon imaging setup was based on an Olympus BX-51WI upright microscope equipped with a multiphoton laser (Mira 900, Coherent), a laser scanning head containing a multi-mode fiber (Fiber LUTE, Sutter Instrument), and controlled by ScanImage 2017b software (Vidrio Technologies). Fluorescence was detected through the objective (Leica HC FLUOTAR L 25x/0.95 VR VISIR) and through the oil immersion condenser (numerical aperture 1.4, Olympus) by two pairs of GaSP photomultiplier tubes (Hamamatsu, H11706-40). Dichroic mirrors (560 DCXR, Chroma Technology) and emission filters (ET525/70m-2P, ET605/70m-2P, Chroma Technology) were used to separate cyan and red fluorescence. Excitation light was blocked by short-pass filters (ET700SP-2P, Chroma Technology). A tunable Ti:Sapphire laser (Chameleon Vision-S, Coherent) was set to 810 nm to excite mCerulean on BiPOLES and somBiPOLES.

An Ytterbium-doped 1070-nm pulsed laser (Fidelity-2, Coherent) was used at a pulse rate of 1 kHz to excite mCerulean on somBiPOLES. Two-photon photostimulation was performed using a tunable femtosecond laser (Coherent Discovery, 80 MHz, 100 fs, tuned between 850 and 1100 nm). A schematic diagram of the experimental setup is presented in Supplementary Fig. 10. A 10-Hz train of 15 ms pulses of 1100 nm light was used to excite a train of action potentials which were shunted using a continuous 200 ms pulse of 920 nm light.

Electrophysiology for two-photon photostimulation of somBiPOLES. At DIV 13–17, whole-cell patch-clamp recordings of somBiPOLES-infected excitatory neurons were performed at room temperature (21–23 °C). An upright microscope (Scientifica, Eclipse E400, equipped with a multiphoton laser (Mira 900, Coherent), and controlled by Motion Control Link 2.2.7 software) was used to view the soma of each patch-clamped neuron. Two-photon photostimulation was performed using a two-photon laser with a fixed wavelength (Spark Alcor, 80 MHz, 100 fs, 920 nm) which was combined to modulate the maximum power of the beam. Holograms designed to generate 12 μm holographic spots at the focal plane of the microscope were computed using an iterative Gerchberg-Saxton algorithm. The zeroth diffraction order from the SLM was removed using a physical beam block. The modulated field was relayed and de-magnified using a pair of telescopes (formed of lenses L3 (Thorlabs, AC508-750-B), L4 (Thorlabs, AC508-750-B), L5 (Thorlabs, AC508-500-B) and L6 (Thorlabs, AC508-300-B)) to fill the back-aperture of the microscope objective (Nikon, CF1 APO NIR, ×40, 0.8 NA) which projected the holograms onto the focal plane. Phase masks were calculated such that these holographic spots for the light of different wavelengths overlapped laterally and axially. The zeroth diffraction order of each pair of holographic spots was used to form the beam. A 10-Hz train of 15 ms pulses of 1100 nm light was used to excite a train of action potentials which were shunted using a continuous 200 ms pulse of 920 nm light. Data presented in Fig. 7d–g was acquired in current-clamp experiments. Where necessary, the current was injected to maintain neurons at the resting membrane potential or 20 mV.

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

Sustained neuronal silencing by two-photon excitation of somBiPOLES under 920 nm illumination was characterized by continuously injecting current above the rheobase for 1 s. The protocol was repeated with 200 ms co-incidental illumination using a 920 nm holographic spot (power densities between 0.05 and 0.3 mW μm⁻²).

Two-photon, bidirectional, control of single neurons was demonstrated by co-incidental illumination of targeted 920 nm and 1100 nm light. A 10-Hz train of 15 ms pulses of 1100 nm light was used to evoke a train of action potentials which were shunted using a continuous 200 ms pulse of 920 nm light.
In vivo recordings from ferret visual cortex. Data were collected from 3 adult female ferrets (Mustela putorius). All experiments were approved by the independent Hamburg state authority for animal welfare (Behörde für Justiz und Verbraucherschutz) and were performed in accordance with the guidelines of the German Animal Protection Law and the animal welfare officer of the University Medical Center Hamburg-Eppendorf.

Viruses

Forty-eight hours before surgery, ferrets were placed on a heating pad to maintain body temperature, fixed in a stereotactic frame, and eye ointment (Vidisic; Bausch + Lomb, Germany) was applied to prevent drying of the eyes. To bilaterally access the LC, an incision (~1 cm) was made along the midline of the scalp, the skull was cleaned, and small craniotomies were drilled (~5.4 mm posterior and ±1 mm lateral to Bregma). 0.4 μl of virus suspension was injected into each LC (~3.6 mm relative to Bregma) at a speed of ~100–200 nl min−1 using a custom-made air pressure system connected to a glass micropipette. After each injection, the micropipette was left in place for a minimum of 3 min before removing. After virus injection, the incision was closed with two ferrule-coupled optical fibers (200 μm core diameter, 0.37 NA, 4 mm length) spaced 2 mm apart (TFC_200/245-0.37_4mm_TLS20_FL; DORI Lenses, Canada) were inserted just above the injection site to a depth of ~3.5 mm relative to Bregma using a stereotactic micromanipulator. The implant, as well as a headpost for animal fixation during the experiment, were fixed to the roughened skull using cyanoacrylate glue (Pattex; Henkel, Germany) and dental cement (Super Bond C&B; Sun Medical, Japan). The incised skin was glued to the cement to close the wound. Anesthesia was antagonized by intraperitoneally injecting a cocktail of atipamezole/ilumazenil/buprenorphine (2.5/0.5/0.1 mg kg−1, diluted in NaCl).
For injection of rAAV2/9 viral particles encoding mDlx-BiPOLES-mCerulean (see Table 2) animals were anesthetized with an injection of ketamine (15 mg kg\(^{-1}\)), medetomidine (0.02 mg kg\(^{-1}\)), midazolam (0.5 mg kg\(^{-1}\)) and atropine (0.15 mg kg\(^{-1}\)). Subsequently, they were intubated and respiration with a mixture of 70:30 N\(_2\)O/O\(_2\); and 1–1.5% isoflurane. A cannula was inserted into the femoral vein to deliver a bolus injection of enrofloxacin (15 mg kg\(^{-1}\)) and Rimadyl (4 mg kg\(^{-1}\)) and, subsequently, continuous infusion of 0.9% NaCl and fentanyl (0.01 mg kg\(^{-1}\)h\(^{-1}\)). Body temperature, heart rate, and end-tidal CO\(_2\) were constantly monitored throughout the surgery. Before fixing the animal’s head in the stereotaxic frame, a local anesthetic (lidocaine, 1%) was applied to the external auditory canal. The temporalis muscle was folded back, such that a small craniotomy (ø: 2.5 mm) could be performed over the left posterior cortex and the viral construct was slowly (0.1 μl min\(^{-1}\)) injected into the secondary visual cortex (area 18). The excised piece of bone was put back in place and the headpost was manually advanced via a micromanipulator (David Kopf Instruments) under visual inspection until the optic nerve was visible. Animals received preventive analgesics (Metacam, 0.1 mg) and antibiotics (Enrofloxacine, 5 mg kg\(^{-1}\)) after the completion of experiments using MATLAB scripts (MathWorks).

**Table 2 Photon flux given as a number of photons s\(^{-1}\) m\(^{-2}\).**

| Wavelength (nm) | 0.001 | 0.01 | 0.1 | 1 | 10 | 100 |
|-----------------|-------|------|-----|--|----|-----|
| 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 |
| 425             | 2.14E +18 | 2.19E +19 | 2.19E +20 | 2.19E +21 | 2.19E +22 | 2.19E +23 |
| 445             | 2.35E +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             | 3.13E +18 | 3.13E +19 | 3.13E +20 | 3.13E +21 | 3.13E +22 | 3.13E +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 |

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

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

Received: 2 May 2021; Accepted: 29 June 2021; Published online: 26 July 2021

**References**

1. Chen, I. W., Papagiakoumou, E. & Emiliani, V. Towards circuit optogenetics. *Curr. Opin. Neurobiol.* 50, 179–189 (2018).

2. Kleinlogel, S. et al. A gene-fusion strategy for stoichiometric and co-localized expression of light-gated membrane proteins. *Nat. Methods* 8, 1083–1088 (2011).

3. Tang, W. et al. Faithful expression of multiple proteins via 2A-peptide self-processing: a versatile and reliable method for manipulating brain circuits. *J. Neurosci.* 29, 8621–8629 (2009).

4. Gradinaru, V. et al. Molecular and cellular approaches for diversifying and extending optogenetics. *Cell* 141, 154–165 (2010).

5. Gradinaru, V. et al. Targeting and readout strategies for fast optical neural control in vitro and in vivo. *J. Neurosci.* 27, 14231–14238 (2007).

6. Carus-Cadavieco, M. et al. Gamma oscillations organize top-down signalling to hypothalamus and enable food seeking. *Nature* 542, 232–236 (2017).

7. Rashid, A. J. et al. Competition between engrams in the hypothalamus and enable food seeking. *Science* 353, 383–387 (2016).

8. Vesuna, S. et al. Deep postero medial cortical rhythm in dissociation. *Nature* 586, 87–94 (2020).

9. Heikenfeld, C. et al. Prefrontal subthalamic pathway supports action selection in a spatial working memory task. *Sci. Rep.* 10, 10497 (2020).

10. Mohammad, F. et al. Optogenetic inhibition of behavior with anion channelrhodopsins. *Nat. Methods* 14, 271–274 (2017).

11. Wietek, J. et al. Anion-conducting channelrhodopsins with tuned spectra and modified kinetics engineered for optogenetic manipulation of behavior. *Sci. Rep.* 7, 14957 (2017).

12. Kläpoeke, N. C. et al. Independent optical excitation of distinct neural populations. *Nat. Methods* 11, 338–346 (2014).

13. Yizhar, O. et al. Neocortical excitation/inhibition balance in information processing and social dysfunction. *Nature* 477, 171–178 (2011).

14. Akerboom, J. et al. Genetically encoded calcium indicators for multi-color neural activity imaging and combination with optogenetics. *Front. Mol. Neurosci.* 6, 2 (2013).

15. Eghbali, K., Peigle, M., Schneider, F., Hegemann, P. & Gotschalk, A. Bidirectional activation of different neuron classes with the spectrally red-shifted channelrhodopsin chimera C1V1 in Caenorhabditis elegans. *PLoS ONE* 7, e48827 (2012).
16. Stujenske, J. M., Spellman, T. & Gordon, J. A. Modeling the spatiotemporal dynamics of light and heat propagation for in vivo optogenetics. Cell Rep. 12, 525–534 (2015).

17. Yizhar, O., Fenno, L. E., Davidson, T. J., Mogri, M. & Deisseroth, K. Optogenetics in neural systems. Neuron 71, 9–34 (2011).

18. Govorunova, E. G., Sineshchekov, O. A., Janz, R., Liu, X. & Spudich, J. L. NEUROSCIENCE. Natural light-gated anion channels: A family of microbial rhodopsins forming cation-selective photoreceptors. Science 365, 467–465 (2019).

19. Berndt, A. et al. Structural foundations of optogenetic determinants of channelrhodopsin ion selectivity. Proc. Natl Acad. Sci. USA 113, 822–829 (2016).

20. Rajasekharapathy, P. et al. Projections from neocortex mediate top-down control of memory retrieval. Nature 526, 653–659 (2015).

21. Mayer, T. et al. High frequency neural spiking and auditory signaling by ultrafast (red-shifted) optogenetics. Nat. Commun. 9, 1750 (2018).

22. Marshel, J. H. et al. Cortical layer-specific critical dynamics triggering perception. Science 365, https://doi.org/10.1126/science.aaw5202 (2019).

23. Batahlay, S., Cervenka, G., Ha, J. H., Kim, Y. T. & Mohanty, S. Broad-band activatable white-opsin. PLoS ONE 10, e0136958 (2015).

24. Bansal, H., Gupta, N. & Roy, S. Theoretical analysis of low-power bidirectional optogenetic control of high-frequency neural codes with single spike resolution. Neuroscience 449, 165–188 (2020).

25. Sineshchekov, O. A. et al. Conductance mechanisms of rapidly desensitizing cation channelrhodopsins from cryptophyte algae. mBio 11, https://doi.org/10.1128/mBio.00657-20 (2020).

26. Diao, Y. et al. Artificially shifted light-driven proton pump for neural silencing. J. Biol. Chem. 288, 20624–20632 (2013).

27. Lim, S. T., Antonucci, D. E., Scannevin, R. H. & Trimner, J. S. A novel targeting signal for proximal clustering of the Kv2.1 K⁺ channel in hippocampal neurons. Neuron 25, 385–397 (2000).

28. Mahn, M. et al. High-efficiency optogenetic silencing with soma-targeted channelrhodopsin 2. Nat. Commun. 9, 1750 (2018).

29. Messier, J. E., Chen, H., Cai, Z. L. & Xue, M. Targeting light-gated chloride channels to neuronal somatodendritic domain reduces their excitatory effect in the axon. eLife 7, https://doi.org/10.7554/eLife.38506 (2018).

30. Hochbaum, D. R. et al. All-optical electrophysiology in mammalian neurons using engineered microbial rhodopsins. Nat. Methods 11, 823–833 (2014).

31. Mardirian, A. R. et al. Precise multimodal optical control of neural ensemble inhibition. Nat. Neurosci. 20, 1067–1069 (2017).

32. Zhang, F. et al. Multimodal fast optical interrogaion of neural circuitry. Nature 446, 633–639 (2007).

33. Hu, C. et al. Sensory integration and neuromodulatory feedback facilitate Drosophila mecanonceptive behavior. Nat. Neurosci. 20, 1085–1095 (2017).

34. Breto-Prevoncher, V. & Sur, M. Active control of arousal by a locus coeruleus GABAergic circuit. Nat. Neurosci. 22, 218–228 (2019).

35. Ramírez, S. et al. Creating a false memory in the hippocampus. PLoS ONE 10, e014193 (2015).

36. Dittgen, T. et al. Lentivirus-based genetic manipulations of cortical neurons and their optical and electrophysiological monitoring in vivo. Proc. Natl Acad. Sci. USA 101, 18206–18211 (2004).

37. Grimm, C., Vierock, J., Hegemann, P. & Wietek, J. Whole-cell patch-clamp recordings for electrophysiological determination of ion selectivity in Cholinergic neurons. J. Vis. Exp. https://doi.org/10.3792/jove.54549 (2017).

38. Stierl, M. et al. Light modulation of cellular cAMP by a small bacterial channelrhodopsin. Neuron 2673–2680 (1986).

39. Schindelin, J. et al. Fiji: an open-source platform for biological-image analysis. Nat. Methods 9, 676–682 (2012).

40. Lutz, C. et al. Holographic photocaging of caged neurotransmitters. Nat. Methods 5, 821–827 (2008).

41. Gerchberg, R. W. & Saxton, W. O. Practical algorithm for determination of phase image and diffraction plane pictures. Optik 35, 237+ (1972).

42. Fire, A. Integrative transformation of Caenorhabditis elegans. Embo J 5, 2633–2680 (1986).

43. Liewald, J. F. et al. Optogenetic analysis of synaptic function. Nat. Methods 5, 821–827 (2008).

44. Stephens, G. J., Johnson-Kerner, B., Baekle, W. & Ryu, W. S. Dimensionality and dynamics in the behavior of C. elegans. PLoS Comput. Biol. 4, e1000208 (2008).

45. Pfeiffer, B. D. et al. Refined tools of targeted gene expression in Drosophila. Genetics 186, 735–755 (2010).

46. Broth, A. C., Fish, M., Nuß, R. & Calos, M. P. Construction of transgenic Drosophila by using the site-specific integrase from phage phiC31. Genetics 166, 1775–1782 (2004).

47. Risse, B. et al. FLIM, a novel FTIR-based imaging method for high throughput locomotion analysis. PLoS ONE 8, e53963 (2013).

48. Savitz, J. M., Jiang, S. S., Mu, W., Dawson, V. L. & Dawson, T. M. Bcl-x is required for proper development of the mouse substantia nigra. J. Neurosci. 25, 6721–6728 (2005).

49. McGinley, M. J., David, S. V. & McCormick, D. A. Cortical membrane potential signature of optimal states for sensory signal detection. Neuron 87, 179–192 (2015).

50. Quirvoa, R. Q., Nadasy, Z. & Ben-Shaul, Y. Unsupervised spike detection and sorting with wavelets and superparamagnetic clustering. Neural Comput. 16, 1661–1687 (2004).
Funding
Open Access funding enabled and organized by Projekt DEAL.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41467-021-24759-5.

Correspondence and requests for materials should be addressed to J.S.W.

Peer review information Nature Communications thanks Ute Hochgeschwender, Adam Packer and the other, anonymous, reviewer(s) for their contribution to the peer review of this work. Peer reviewer reports are available.

Reprints and permission information is available at http://www.nature.com/reprints

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2021