Optogenetic inhibition of behavior with anion channelrhodopsins

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Optogenetics uses light exposure to manipulate physiology in genetically modified organisms. Abundant tools for optogenetic excitation are available, but the limitations of current optogenetic inhibitors present an obstacle to demonstrating the necessity of neuronal circuits. Here we show that anion channelrhodopsins can be used to specifically and rapidly inhibit neural systems involved in *Drosophila* locomotion, wing expansion, memory retrieval and gustation, thus demonstrating their broad utility in the circuit analysis of behavior.

The ability to understand the neuronal control of behavior has been transformed by the advent of techniques enabling researchers to precisely manipulate neuronal activity in transgenic animals[1–6]. Optogenetics is one such technique, which uses controlled light exposure to reversibly modulate the activity of light-sensitive ion channels. Optogenetic actuators can be expressed in genetically modified organisms with circuit specificity[3] in behaving animals[7], thus enabling millisecond-resolution control[4]. However, although optogenetic activators are widely used, the toolkit of optogenetic inhibitors of neuronal activity is more limited, especially in *Drosophila*. Consequently, in a number of experimental systems, it is easier to show that a specific neural circuit is sufficient for any given function, whereas demonstrating the necessity of that circuit remains problematic. Suppression of neuronal excitability is based on an influx of anions or an efflux of cations; this suppression has previously been achieved through use of light-driven chloride[8] or proton pumps[9], but these techniques require high-density expression and intense light for effective inhibition, thus limiting their utility. Recently, a new class of inhibitory, anion-conducting, light-gated channels has been developed, including channelrhodopsins engineered to conduct chloride ions[10,11], and two naturally evolved anion channelrhodopsins have been cloned from an alga[12]. The algal *Guillardia theta* anion channelrhodopsins (GtACRs) possess several attractive features as optogenetic inhibitors: they have higher conductance than other inhibitory optogenetic tools, are rapidly responsive, require low light intensities for activation and comprise both a cyan-gated channel (GtACR1, maximal sensitivity at 515 nm) and a blue-gated channel (GtACR2, maximal sensitivity at 470 nm)[12]. Considering these properties, we hypothesized that the GtACRs might be used to effectively optogenetically inhibit neuronal circuits in a model organism. Here we show that both GtACR1 and GtACR2 potently, rapidly and reversibly inhibit a range of behaviors in *Drosophila* during controlled exposure to light.

We expressed genes encoding the GtACRs in cells that release the neurotransmitter acetylcholine[2]. *Drosophila* expressing the cyan-gated GtACR1 were allowed to climb a vertical surface and fell when they were exposed to only 1.3 μW/mm^2 green light (**Fig. 1a** and **Supplementary Video 1**). GtACR1 flies (*Cha>GtACR1*) were also susceptible to blue and red light (at ≥14 μW/mm^2 and 39 μW/mm^2, respectively). Flies expressing the blue-gated GtACR2 in cholinergic neurons fell in response to blue light at 14 μW/mm^2 (**Fig. 1b**) and in response to green light at 49 μW/mm^2. Flies expressing enhanced *Natronomonas pharaonis* halorhodopsin (eNpHR) did not fall when they were illuminated with 39 μW/mm^2 red light, and control flies were unaffected by light (**Supplementary Fig. 1a**).

Some of the fallen *Drosophila* moved during GtACR actuation (**Supplementary Video 1**), thus revealing incomplete inhibition of Cha$^+$ neurons. Examination of the motion of individual flies with a high-frame-rate camera revealed that green light with intensities ≥38 μW/mm^2 induced rapid and complete immobility in *Cha>GtACR1* flies (**Fig. 1c**,**f** and **Supplementary Fig. 1f**). At higher powers, immobilization was observed in a fraction of *UAS-GtACR1*/+ flies (*UAS*, upstream activating sequence; 36% (95% confidence interval (95CI) 12, 68) at 253 μW/mm^2, n = 11 flies; 13% (95CI 2, 31) at 119 μW/mm^2, n = 15; 0% at lower intensities, n = 12), possibly because of 'leaky' expression without a driver transgene. In *Cha>GtACR2* flies, 391 μW/mm^2 blue light induced motionless paralysis during the entire duration of light exposure (**Fig. 1d**,**i**,**j** and **Supplementary Fig. 1f**); the identical light level had no effect on control *UAS-GtACR2*/+ flies. Illumination of *Cha>eNpHR* flies with 495 μW/mm^2 amber light had no visible effects (**Fig. 1e** and **Supplementary Fig. 1f**). We also placed *Cha>eNpHR* animals 3 mm below an LED emitter (~1,900 μW/mm^2) and observed sporadic paralysis, thus confirming that these flies carried active eNpHR (**Supplementary Fig. 1d**,**e** and **Supplementary Video 2**). Video analysis indicated that *Cha>GtACR1* and *Cha>GtACR2* paralysis onset times were strongly dependent on light intensity, and millisecond-scale onsets occurred at powers ≥38 μW/mm^2 and ≥391 μW/mm^2, respectively (**Fig. 1f**,**i**). Recovery times were independent of power (**Fig. 1g**,**h**,**j**,**k** and **Supplementary Fig. 1b**,**c**). We further verified

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GtACR inhibition by comparison with paralysis induced by the channelrhodopsin variant CsChrimson, an effect that was distinct (**Supplementary Fig. 2** and **Supplementary Video 3**), and by electrophysiological recordings from a nerve, which showed that GtACR1 actuation inhibited action potentials (**Fig. 1l** and **Supplementary Figs. 3** and **4**).

Drosophila extend their proboscises toward sweet liquids in a response called the proboscis-extension reflex (PER), which depends on neuronal expression of Gustatory receptor 64f (Gr64f)[13]. Optogenetic activation with CsChrimson is sufficient to elicit PER in the absence of sugar[14]. Under green light, flies expressing *UAS-GtACR1* in *Gr64f-Gal4* neurons exhibited decreased PER when they were presented with a sucrose solution (**Supplementary Fig. 5a**,**b**). Flies expressing eNpHR in Gr64f cells showed decreased PER relative to controls under strong amber light (**Supplementary Fig. 5a**,**b**). Expression of Kir2.1 in Gr64f cells decreased PER by ΔPER = −0.96 (95CI 0.92, −1.0) (**Supplementary Fig. 5a**,**b**). The potent suppression of PER by illuminated GtACR1—the opposite effect from that of CsChrimson—verified that the channel effectively inhibited the Gr64f cells. Actuation of GtACRs in bursicon cells in the nerve cord (**Supplementary Fig. 6**) and in mushroom-body cells (**Supplementary Fig. 7**) verified that these channels inhibit cells in the central nervous system, which is less accessible to light than more peripherally located neurons.

We examined behavioral responses to optogenetic activation and inhibition of the bitter-sensing Gustatory receptor 66a (Gr66a) neurons as well as the sweet-sensing Gustatory receptor 64f (Gr64f) neurons (**Fig. 2a,b**). We tested flies in linear chambers illuminated with two bands of either red or green light (**Fig. 2c,d**). Red-light actuation of CsChrimson in the bitter-sensing neurons was aversive (**Fig. 2e**), in agreement with previous results[15] However, *Gr66a>GtACR1* flies were indifferent to green light over a range of intensities known to have effects in other behaviors (**Fig. 2f**), thus suggesting that the bitter-sensing system is quiet in the absence of a stimulus. Sated flies bearing CsChrimson in

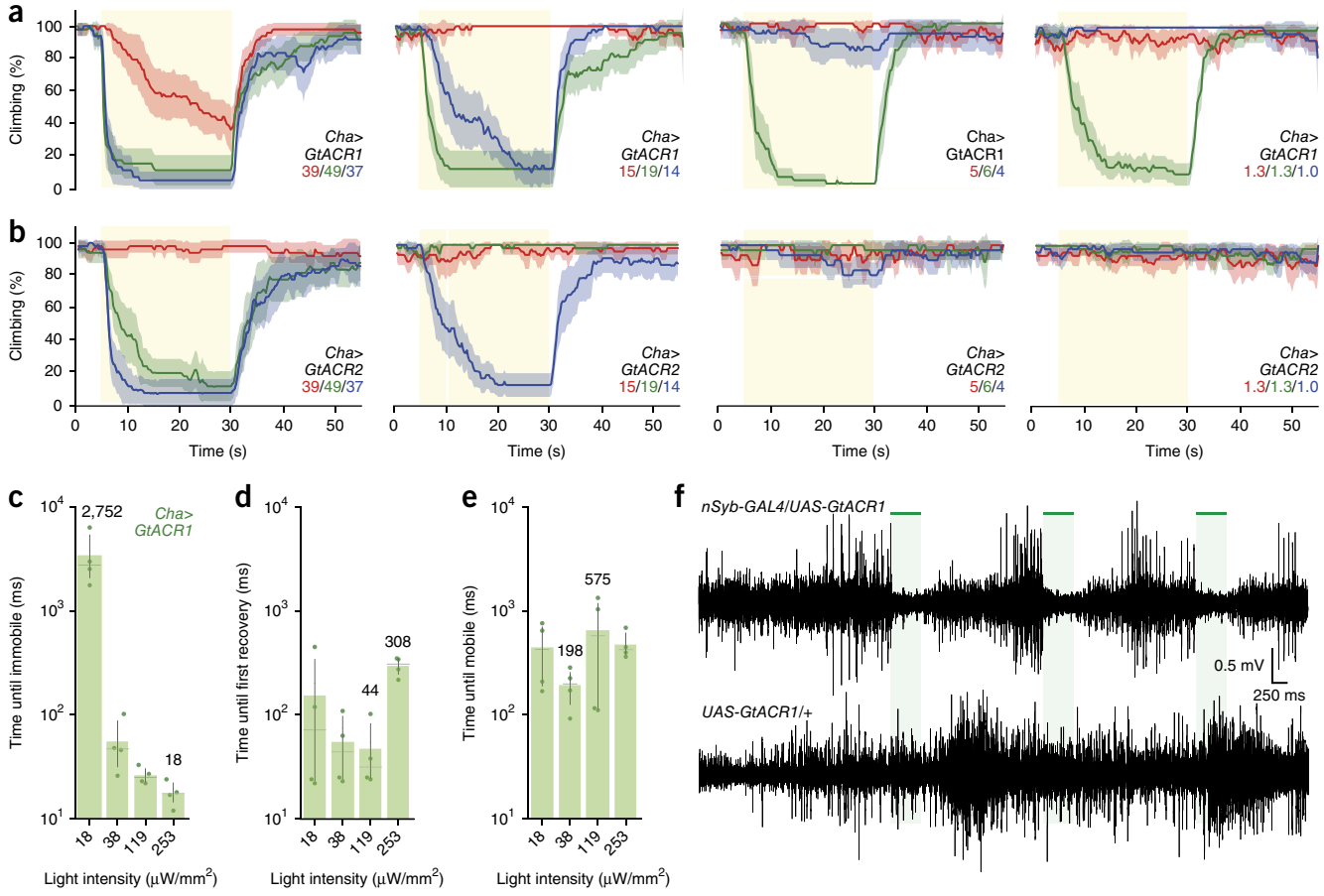

Figure 1 | *G. theta* anion channelrhodopsins are inhibitors of motor function and neuronal spiking. (**a**) Climbing assay with flies expressing GtACR1 in cholinergic neurons (*Cha-Gal4>UAS-GtACR1*). Yellow shading indicates the time when flies were illuminated. Light intensities (in μW/mm^2) are indicated. The proportion of climbing flies is shown as a percentage of flies outside the floor area of the chamber. Each solid trace is the mean of values from 3 experiments (48 flies each). Line color corresponds to the color of illumination from a projector. Error ribbons are 95% confidence intervals. (**b**) Climbing assay as in **a**, with flies expressing GtACR2 (*Cha>GtACR2*). (**c**) Time to complete immobilization after onset of green light (peak 525 nm) for *Cha-Gal4>UAS-GtACR1* flies. The horizontal axis indicates the four light intensities tested. Bars indicate means; horizontal lines indicate medians; dots indicate time measured from a single fly. The numerals above the bars indicate the medians of the fastest and slowest responses. Horizontal lines indicate medians; dots indicate time measured from a single fly. Error bars, 95% confidence intervals (n = 4 flies). (**d**,**e**) Times between cessation of illumination and the first sign of recovery (**d**) and times to full mobility (**e**), over four light intensities. (**f**) Representative recordings from larval segmental nerves. Pulses of green light (500 ms, 24 μW/mm^2, green lines and shading) were delivered to nerves expressing GtACR1 (*nSyb>GtACR1*, pan-neuronal expression) or to control larvae (*UAS-GtACR1/+*).

their Gr64f sweet-taste cells were mildly attracted to dim red light and were minimally averted by stronger red light (**Fig. 2g**). Sated *Gr64f>GtACR1* flies avoided several green-light intensities and exhibited a particularly strong aversion to 92 μW/mm^2 (**Fig. 2h**). Hungry *Gr64f>CsChrimson* flies avoided higher red-light intensities slightly more (**Fig. 2i**).However, hungry *Gr64f>GtACR1* flies were indifferent to green light (**Fig. 2j**). These results confirmed that responses to anion channelrhodopsin conductance in gustatory cells are distinct from responses to optogenetic activators, thus verifying that GtACR1 inhibits neuronal activity and demonstrating the utility of an optogenetic inhibitor in elucidating how tonic activity and quiescence contribute to behavior.

GtACR expression and actuation produced no major deleterious effects on neuronal morphology, eye morphology or life span (**Supplementary Fig. 8**).

The *G. theta* anion-conducting channelrhodopsins are effective tools to inhibit behavioral circuits in freely moving animals. The present data reveal that the GtACRs have potency comparable to

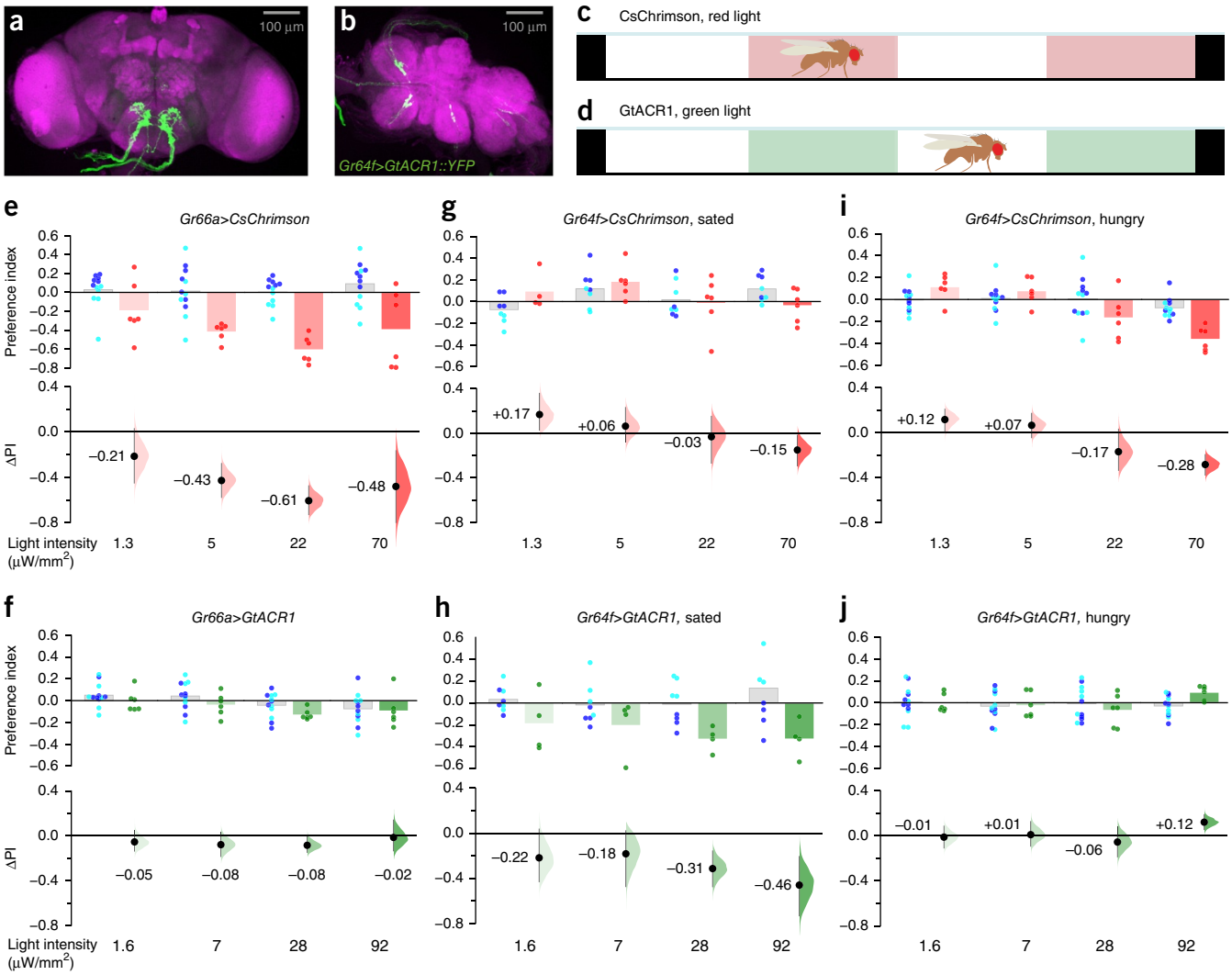

Figure 2 | Flies avoid optogenetic suppression of sweet-taste receptors. (**a**,**b**) The morphology of *Gr64f>GtACR1::YFP* neurites in the brain and ventral nerve cord. Gr64f neurites project to the subesophageal zone (**a**) and the ventral nerve cord (**b**). Magenta indicates neuropil stained with anti-DLG; green indicates YFP fluorescence. (**c**,**d**) To examine neuronal activation preference (valence) in CsChrimson-bearing flies, a chamber was illuminated with two bands of red light. Diagram not to scale. Valence responses of GtACR1-bearing flies were examined in a chamber illuminated with bands of green light. (**e**) Flies expressing CsChrimson in their bitter-taste neurons (*Gr66a>CsChrimson*, red dots) were tested for their preference for red light (*Gr66a/+*, blue dots; *UAS-CsChrimson/+*, cyan dots), measured as a preference index (PI). The projector light intensities were: 1.3, 5, 22 and 70 μW/mm^2 from left to right. Each dot indicates an experimental iteration; i.e., $n \geq 4$ iterations, with 15 flies per iteration. Bottom, preference relative to control animals (ΔPI), calculated as the mean difference between experimental and control PI scores. At 22 μW/mm^2, ΔPI = −0.61 (95CI −0.47, −0.73), $P = 0.0009$ (related to two-tailed Student's *t* statistics here and throughout figure; sample sizes in **Supplementary Fig. 5**). (**f**) Flies expressing *GtACR1* in their bitter-taste neurons (*Gr66a>GtACR1*) were tested for green-light preference at intensities of 1.6, 7, 28 and 92 μW/mm^2 (left to right). (**g**) Red-light preference of sated flies expressing CsChrimson in sweet-taste neurons (*Gr66a>GtACR1*). At 1.3 μW/mm^2 red light, ΔPI = +0.17 (95CI 0.03, +0.37), $P = 0.10$. At 70 μW/mm^2 red light, ΔPI = −0.15 (95CI +0.02, −0.29), $P = 0.11$. (**h**) Responses to green light at a variety of intensities in flies expressing *GtACR1* in sweet-taste neurons (*Gr64f>GtACR1*). ΔPI = −0.46 (95CI −0.19, −0.73), $P = 0.05$. (**i**) Responses of hungry flies expressing CsChrimson in sweet-taste neurons (*Gr64f>CsChrimson*). Illumination as in **g**. (**j**) Responses to green light in hungry flies expressing GtACR1. At the highest intensity. ΔPI = +0.12 (95CI +0.05, +0.19), $P = 0.01$.

those of widely used constitutive transgenic and thermogenetic inhibitors and temporal precision similar to that of optogenetic activators. The GtACRs have comparable efficacy to that of *shi*^{ts} but are faster[2], and they are more potent than the chloride pump eNpHR, which requires strong light intensities[16,17]. Indeed, GtACR1 is so effective that intensities as low as 119 μW/mm^2 elicited immobilization in a fraction of *UAS-GtACR1*/+ control flies, thus necessitating light power calibration. We obtained complete millisecond-scale paralysis in *Cha>GtACR1* flies at powers as low as 38 μW/mm^2 (**Fig. 1f**), whereas we observed no immobilization in *UAS-GtACR1*/+ with light as strong as 92 μW/mm^2 (**Supplementary Fig. 2e**). Therefore, we recommend the light range of 38–92 μW/mm^2 for experiments with GtACR1 in adult *Drosophila*. For GtACR2, we recommend ~391 μW/mm^2, a blue-light power that had no visible effect on control *UAS-GtACR2*/+ flies but elicited fast and complete cholinergic paralysis in *Cha>GtACR2* flies (**Fig. 1i**). In practical terms, GtACR1 can be actuated with a projector, whereas GtACR2 requires LED arrays for most freely moving adult *Drosophila* applications.

The GtACRs inhibit action potentials in several mammalian cell types[12,18], though chloride conductance is known to have various effects on membrane potential (hyperpolarization, depolarization and shunting), depending on a cell's chloride-reversal potential[19,20]. Some neurons with high chloride concentrations[19] can be activated by GtACR conductance[18,20]; however, our experiments suggest that this scenario is relevant for a minority of neurons, at most. The GtACRs provide an improvement over existing methods; they are orders of magnitude faster than thermogenetic tools and more potent than eNpHR. Algal anion channelrhodopsins should be an important addition to the optogenetic toolkit and should make neural activity necessity tests as accessible as sufficiency tests are currently.

METHODS

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

Conceptualization, F.M. and A.C.-C.; methodology, F.M., J.C.S. and A.C.-C.; software, J.C.S. (CRITTA, LabView) and J.H. (Python); investigation, F.M. (transgenic design, genetics, falling, walking immobilization, wing expansion, eye toxicity, valence, PER and neuroanatomy), J.C.S. (falling and high-frame rate paralysis), S.O. (learning and survival), J.Y.C. (brain dissection, immunohistochemistry and microscopy), K.C. (PER) and T.-W.K. (electrophysiology); resources, J.C.S. (instrumentation); data analysis, J.H. (valence, electrophysiology), K.C. (PER), J.C.S. (paralysis, falling and walking immobilization), S.O. (STM) and F.M. (paralysis, valence and anatomy); writing original draft, F.M. and A.C.-C. with contributions from all authors; writing revision, F.M., S.O., J.C.S. and A.C.-C.; visualization, F.M., J.H., J.C.S., S.O., K.C. and A.C.-C.; supervision, A.C.-C.; project administration, A.C.-C.; funding acquisition, A.C.-C.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Transgenes, fly strains and rearing conditions. *Drosophila melanogaster* flies were used in all experiments. *ChAT-Gal4.7.4* (BL 6798)[21], *Burs-Gal4* (BL 40972)[22], *20x-UAS-eNpHr3* (BL 36350)[23], *20x-UAS-CsChrimson* (BL 55134)[14], *Gr64f-Gal4* (BL 57699) and *Gr66a-Gal4* (BL 57670)[24] were obtained from the Bloomington *Drosophila* Stock Center. *OK107-Gal4* (DGRC 106098)[25] was obtained from the Kyoto Stock center. GMR-Gal4 flies[26] were a gift from G. Rubin (Howard Hughes Medical Institute). *Crz-Gal4*[27] was a gift from J. Park (University of Tennessee, Knoxville). w^{1118} flies were used as wild-type controls in all experiments. To generate *UAS-GtACR1* and *UAS-GtACR2* transgenic lines, *Drosophila*-codon-optimized sequences of *GtACR1* and *GtACR2* (ref. 12) were synthesized *de novo* (GenScript) as EYFP fusions and cloned into *pJFRC7-20XUAS-IVS-mCD8::GFP*, acquired from Addgene (plasmid no. 26220)[28], by replacing the *mCD8:GFP* fragment with *GtACR1-EYFP* or *GtACR2-EYFP* fragments and performing sequence verification (GenScript). Constructs were injected into an *attP2* insertion site on the third chromosome, and the transgenic progeny were balanced (BestGene). Expression of the GtACR constructs was verified by YFP fluorescence. For experiments on adult flies, 3- or 4-d-old male flies were fed with 1 mM all-*trans*-retinal (ATR) (Sigma) for 2 to 3 d at 25 °C in the dark. A stock solution of all-*trans*-retinal was prepared in 95% ethanol (wt/vol) and mixed into warm liquefied fly food. Each vial was covered with aluminum foil and placed in the dark. No statistical method was used to predetermine sample size, except for the valence and MOT experiments, for which sample sizes were selected to provide a power of at least 80%, for an effect size of 0.5 and $\alpha = 0.05$. The experiments were not randomized and were not performed with blinding, except for the PER experiments which were done with blinding.

Optogenetic stimulation. Green, blue, red and amber LEDs (Luxeon Rebel LEDs on a SinkPAD-II 10-mm Square Base available from http://www.luxeonstar.com (green SP-05-G4, peak emission 525 nm; blue SP-05-B4, peak 460 nm; red-orange SP-05-E4, peak 617 nm; and pc-amber SP-05-A5, peak 591 nm)) or an LED microprojector (Optoma ML750) was placed near behavioral arenas to provide an illumination source. LEDs were powered at maximum brightness by a 700-mA BuckPuck driver, and illumination intensity was controlled by varying the distance between the source and subject. A PC-controlled LED microprojector was used to project images consisting of entirely red (peak 617 nm), green (peak 530 nm) or blue (peak 459 nm) backgrounds onto the arena. The projector used a Digital Light Processing (DLP, Texas Instruments) micromirror device with a pulse frequency of 240 Hz. For a consistent environment, the illuminatory and behavioral monitoring system was placed inside the incubator and maintained at 25 °C throughout the experiment.

Video acquisition. Videos were captured at 25–30 frames per second with an AVT Guppy F-046B CCD camera (Stemmer Imaging) equipped with a 12-mm CCTV-type lens and connected to a computer via an IEEE 1394 cable. Experiments were conducted under infrared (IR) light; an IR long-pass filter (Edmund Optics, Singapore) was used to decrease detection of the microprojector/LED light. The custom-built image-acquisition software CRITTA, written in LabView (National Instruments), was used to track the movements of animals in the behavioral arenas. The designs of the video capture, illumination and behavior apparatuses are diagrammed in **Supplementary Figure 9**.

Light-intensity and temperature measurements. The light intensities of the projector and LED illumination were measured for all wavelengths for every configuration used in the experiments: number of LEDs, distance from chamber, type of lens and projector DLP intensity values. A thermal power sensor (Thorlabs S310C) connected to a power and energy-meter console (Thorlabs PM100D) was used to measure power in a dark room. The meter was zeroed before each set of measurements, and a cardboard shield with a 20-mm-diameter cutout was used to ensure that light struck only the sensor's absorbent surface. Temperature measurements were made from all the behavioral apparati under conditions identical to those used in the experiments, with a Phidgets K-Type thermocouple (P/N 3109, Phidgets) placed inside the behavioral arena, and data were captured with a Phidgets Temperature Sensor (P/N 1048) device and custom scripts in LabView (**Supplementary Fig. 10**).

Falling assay. Fly climbing and locomotion performance were monitored in a custom acrylic arena. Four ATR-treated flies, cooled on ice, were transferred into each arena. The climbing was performed in a rectangular arena (70 × 11 mm), and four such arenas were cut into a 1.5-mm-thick transparent acrylic sheet that was incorporated into a transparent acrylic 'sandwich' (**Supplementary Fig. 9**). Climbing behavior was recorded under IR backlighting. The arena sandwich was illuminated from the front with a DLP projector. Flies were allowed to freely explore the arena during the test session. The behavior of flies illuminated with IR, green, blue and red light was recorded. For higher intensity, the projector was placed closer to the behavioral arena. During the duration of the test, flies were individually tracked with a monochrome camera connected to CRITTA tracking software, which also controlled the timing, hue and intensity of the illumination by driving the projected image. As well as recording the vertical position of each fly, the software divided the chamber into two zones, the lower 5.5% and the upper 94.5%, and logged the number of flies occupying each zone. Fully paralyzed flies fell to the bottom of the chamber, occupied the lower zone and were scored as fallen.

Walking paralysis. A Guppy-046B camera was used to record fly behavior at 25 frames per second in stadium-shaped arenas (55 × 4 × 1.5 mm); 15 such arenas were cut from acrylic. *Cha>CsChrimson*, *Cha>GtACR1*, *Cha>GtACR2* and control flies were illuminated with a DLP projector (**Supplementary Fig. 9**). CRITTA and Python scripts were used to analyze walking speeds and to generate the Muybridge series.

High-frame-rate video analysis of walking paralysis. High-frame-rate video was recorded at 1,000 frames per second with a Photron FASTCAM MC2 Camera to quantify the onset of inhibition and recovery with high temporal accuracy. For each genotype studied, four flies were anesthetized on ice and loaded into four separate clear acrylic chambers measuring 9 × 9 × 1.5 mm and covered by a microscope cover slip (**Supplementary Fig. 9**). For GtACR recordings, the chamber was front-illuminated by a

red-filtered fiber-optic light source, and for eNpHR, which is known to be sensitive to red light, IR backlighting was used. Before recording, fly locomotion was elicited by shaking the chamber. Red, green, blue or amber LEDs, set at varying distances from 3 mm to 330 mm from the subject, were turned on manually when each fly was freely walking and were turned off for several seconds after paralysis occurred. The video buffer was limited to 16 s, and the illumination duration was typically 2 s, although the duration was prolonged to a maximum of 30 s if no effect was seen. Videos were analyzed offline through visual inspection to determine the frame in which flies were first immobilized, which was defined as the time when forward locomotion was abated (although some movement sometimes occurred because of momentum or gravity); the frame containing the first sign of recovery, for example, the movement of a leg; and the first frame in which the fly had recovered, had become fully mobile and began to walk away. In addition, the frames in which the light was turned on and off were marked, and then the following times were calculated: time from light onset to immobilization, time from light-off to first recovery and time from light-off to remobilization. Pixel difference data were analyzed with custom scripts in LabView and Python.

Wing expansion assay. For wing expansion experiments, larvae were fed with 200 μM ATR throughout larval development. At 1 to 2 d after puparium formation (APF), pupae were transferred to green, blue or red light conditions at 25 °C. Flies remained under this light until 9 to 10 d APF, when wing expansion was scored under a dissection microscope. For Kir2.1, all flies were raised at 18 °C until 1 to 2 d APF, at which point pupae were transferred to either 18 °C or 31 °C for *Tub-Gal80^{ts}* derepression of Kir2.1 expression. Wing expansion was scored under a dissection microscope until 9 to 10 d APF.

Olfactory short-term memory. Aversive olfactory conditioning of *Drosophila* was performed in custom-built chambers modified from a previously described single-fly olfactory trainer apparatus[29]. To allow light penetration and video monitoring of multiple flies, windows were cut into the top and bottom of each chamber. The floor and ceiling of each chamber was a glass slide printed with transparent indium tin oxide (ITO) electrode boards (Visiontek)[30]. Each side of the ITO board was sealed by a gasketed lid that formed a seal around the gap between the ITO board and the chamber wall. The internal behavioral arena was 50 mm long, 5 mm wide and 1.3 mm high. Mirrors were aligned at a 45° angle and placed into holders on top of each chamber (**Supplementary Fig. 9**). Facilitated by carrier air, odors entered each end of the chamber via two entry pipes and left the chamber through two vents located in the middle of the chamber. Flies were conditioned with electric shocks (12 electric shocks at 60 V) that were delivered through the circuit boards. At the start of each conditioning experiment, the flies were cooled on ice in darkness and loaded into the chambers, with six flies per chamber. Experiments were performed with four chambers simultaneously, which were plugged into a rack in a 2 × 2 manner. Throughout the conditioning cycle, *Drosophila* were entrained to either avoid 3-octanol (OCT) or 4-methylcyclohexanol (MCH). Conditioned performance was tested by exposing one half of the chamber to the punished odor and the other half to the unpunished odor. A performance index (PI) was calculated by counting flies in individual video frames over the final 30 s of assessment, with a formula described previously[31]. We subjected *Drosophila* expressing a neuronal silencing factor under the control of a mushroom-body-specific driver (*OK107-Gal4*) to aversive olfactory conditioning. After the initial conditioning cycle, the same organisms were tested a second time using a similar protocol, except that green (λ 525 nm) or amber light (λ 591 nm) was turned on during memory retrieval. In *shi^{ts}* experiments, all flies were initially conditioned at 22 °C and subsequently incubated for 30 min at 32 °C to inactivate endocytosis. After the incubation period, the flies were conditioned against the same odor a second time at 22 °C. Finally, the fly performance between the first and second conditioning cycles was compared.

Optogenetic valence assay. Each arena had a 55 × 4 mm stadium layout; 15 such arenas were cut from 1.5-mm-thick transparent acrylic. During an experiment, all arenas were covered with a transparent acrylic lid. Ice-anesthetized flies were loaded into each chamber in the dark, and the whole arena was kept under infrared light at 25 °C for 2–3 min before the assays were started; behavior was recorded under IR lighting (**Supplementary Fig. 9**). The arena was illuminated from the top with visible light from a miniprojector (Optoma ML750). For CsChrimson experiments, four red-light intensities were used; for *ACR* experiments, four green-light intensities were used. The colored-light intensity was varied by changing the levels of the digital color components of the projection. For each batch of experiments, two light-test sessions were conducted, separated by 10 s. For the first test session, the arenas were illuminated for 60 s with stripes consisting of two light and two dark zones, all equally sized. For the second 60-s test session, the locations of the light and dark zones were reversed. For the duration of the test, the positions of the flies were individually tracked with a Guppy F-046B camera with an IR band-pass filter connected to CRITTA tracking software, which also controlled the timing, hue and intensity of the illumination by driving the projected image, as well as by counting the number of flies in each zone. Fly preference for light was calculated as a preference index by subtracting the total number of flies in the dark from the total number of flies in the light, and dividing this number by the total number of flies in the experiment, for each of the four light intensities.

Proboscis extension reflex assay. In all experiments, 4- to 5-d-old, 30-h wet-starved (0.5% agarose) male flies were used. The back of each fly was affixed onto a glass slide with nail polish, and the flies were then placed in a vial with a water-soaked tissue and allowed to recover for 1–2 h. The PER response was tested by manually presenting a drop of 1 M sucrose solution (Sigma) to the forelegs for up to 5 s, with a 1 ml syringe, in the presence of red, green or amber light (**Supplementary Fig. 9**). When the fly extended its proboscis, the syringe was immediately withdrawn to prevent drinking. GtACR flies were tested with red, green and blue light; eNpHR was tested with high-intensity amber light. Flies were given water before the experiment and after each light change. The light color was randomized for each fly. Each presentation was recorded manually. Fly responses were counted offline manually. Each fly was tested three times, and responses were counted as either 0 or 1 (for the absence or presence of PER, respectively): the mean outcome of three presentations denoted the fly performance.

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ΔPER values for each condition were calculated by subtracting the PER performance of the driver controls from the PER response of responder control and experimental animals.

Survival analysis and toxicity assays. We performed survival assays of flies expressing GtACRs in all cholinergic neurons with *Cha-Gal4* after treatment with ATR for 4 d. Animals were briefly exposed to light at days 4, 7 and 12 after eclosure, during transfer between vials and in their use in paralysis experiments. Throughout the assay, *Drosophila* were transferred into new food vials every third day, and any deaths were recorded; longevity was monitored until all flies were dead. To examine GtACR cellular toxicity, we expressed GtACRs in retinal cells with the Glass Multimer Reporter *GMR-Gal4* driver and examined the offspring for rough-eye phenotypes[32]. Larvae were kept in the dark on ATR food throughout development. Pupae were exposed to light from 2 to 3 d after pupal formation until eclosion, and eyes were examined 5 d after eclosion. To monitor toxicity in central brain cells, we expressed GtACR::YFP in *Corazonin* (*Crz*) cells with *Crz-Gal4*, which expresses in six to eight neurons[27]. After eclosion, flies were given ATR-treated food for 2–3 d. Brains were dissected at three stages: before ATR treatment, 1 d after ATR treatment with light exposure and 6 d after ATR with light exposure.

Immunohistochemistry. Adult brains were dissected in PBS and fixed in 4% paraformaldehyde for 20 min at room temperature. Samples were washed three times in PBT (phosphate-buffered saline with 1% Triton X-100, pH 7.2) and blocked with 5% normal goat serum for 1 h. Samples were then incubated with primary antibodies overnight at 4 °C. After being washed three additional times with PBT, samples were incubated with a secondary antibody overnight at 4 °C. Stained brains were mounted in Vectashield (Vector Laboratories) and recorded with a confocal fluorescence laser scanning microscope (Zeiss). Anti-Crz (gift from J.A. Veenstra (Université de Bordeaux)[33], 1:1,000) and goat anti-rabbit Alexa Fluor 568 (A-11011, Molecular Probes, 1:200 dilution) were used. GtACR1::YFP and GtACR2::YFP were visualized without antibody staining. Control mCD8::GFP expression was visualized with anti-GFP (ab13970, Abcam, validation data for immunohistochemistry in *Drosophila* and references available on manufacturer's website) and anti-chicken Alexa Fluor 488 staining. OK107>GtACR1 brains were visualized with mouse anti-DLG1 (1:200, Developmental Studies Hybridoma Bank; validation references available on manufacturer's website)[34].

Electrophysiology. Third-instar larvae were dissected in HL3 solution modified from a previous recipe as follows: 110 mM NaCl, 5 mM KCl, 5 mM HEPES, 10 mM $NaHCO_3$, 5 mM trehalose, 30 mM sucrose, 1.5 mM $CaCl_2$ and 4 mM $MgCl_2$ (ref. 35). An individual abdominal nerve (A 3/4) was drawn into a fire-polished-glass suction electrode as described previously[36]. Extracellular recordings of action potentials from both sensory and motor neurons were performed with a DAM-50 Differential Amplifier (World Precision Instruments) in AC mode at 1,000× gain and band-pass filtered at 100 Hz–1.5 kHz. Optogenetic silencing of the segmental nerve was achieved with 500-ms and 30-s pulses of light from a green LED triggered from Clampex software (Molecular Devices) during recordings. Spiking activity occurred in bouts separated by intervals of quiescence; after recording, if no spiking was observed before light onset, that epoch's data were excluded from the analysis. Spike detection was performed with a window discriminator[37]. A spike was defined as an upward signal that peaked within 50 ms and had an amplitude threshold of 2.58 s.d. from the mean amplitude. The spiking frequency of each nerve was calculated with a rolling window of 100 ms and 500 ms, for 500-ms and 30-s illumination pulses, respectively.

Statistics and analysis. Estimation statistical methods were used to analyze and interpret quantitative data[38–40]. For each olfactory STM experiment, the mean difference in PI for actuating light (relative to IR light) was computed (ΔPI). For each PER experiment, the mean difference in PER scores relative to driver controls was computed (ΔPER). Data are presented as mean difference contrast plots[40,41]. Bootstrap methods[42] were used to calculate 95% confidence intervals for the mean difference between control and experimental groups. Confidence intervals were bias-corrected and accelerated[43], and are displayed with the bootstrap distribution of the mean; resampling was performed 2,000 times. All reported *P* values are related to two-tailed Student's *t* statistics. Data analysis was performed and visualized in LabView, in Matlab, and in Python with Jupyter and the scikits-bootstrap, seaborn and SciPy packages.

Data availability. The data that support the findings of this study are available from the Zenodo data repository at https://doi.org/10.5281/zenodo. 208424. The DNA sequences of the synthetic genes used in this study are available from GenBank under accession codes KY385291 and KY385292.

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