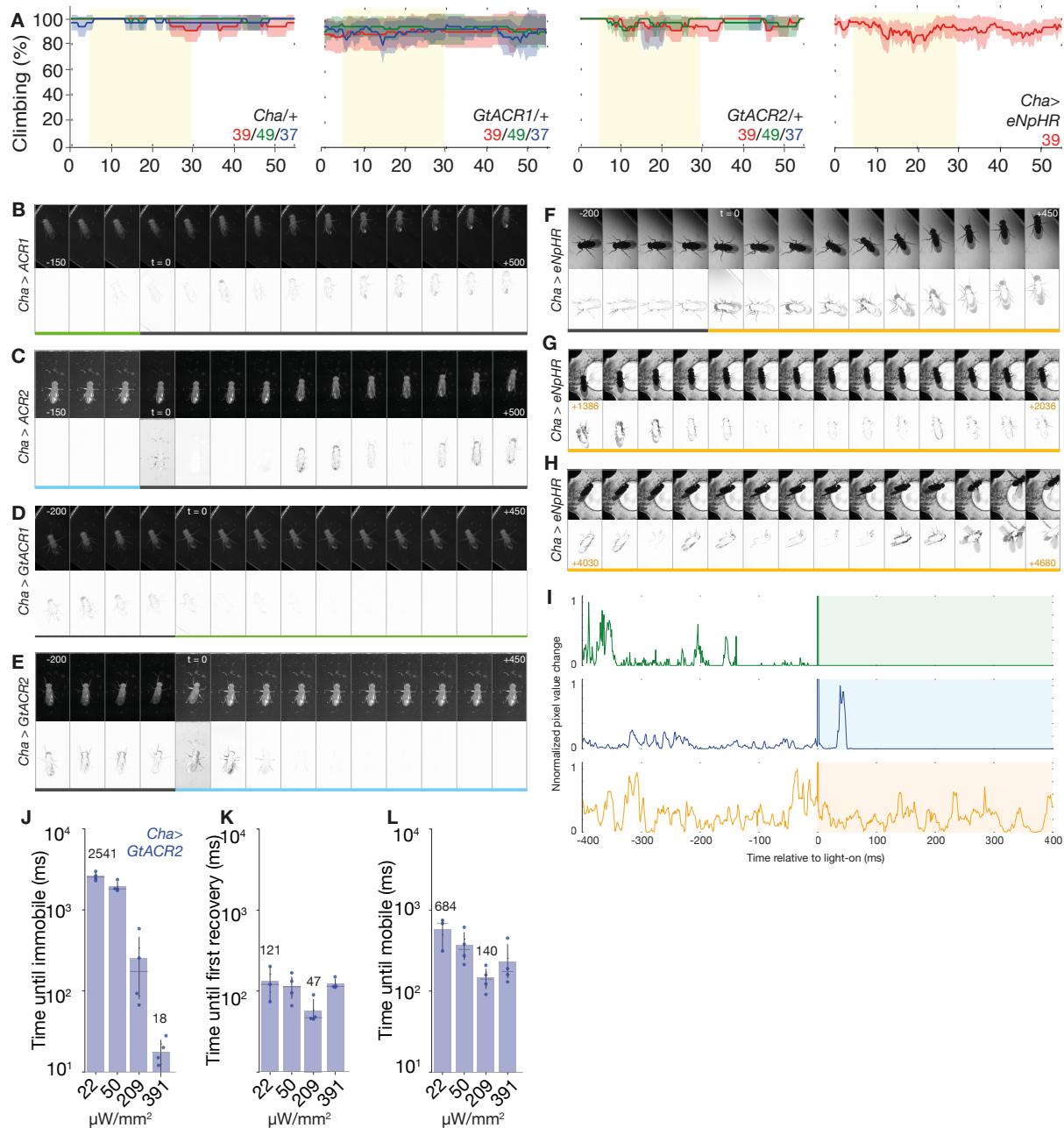


*Supplementary Information for*

**Optogenetic inhibition of behavior with anion channelrhodopsins**



**Supplementary Figure 1. Anion channelrhodopsin action in the *Drosophila* cholinergic system.**

**A.** Control flies carrying either the driver or GtACR transgenes alone did not fall when illuminated with different wavelengths and intensities of light. The indicated colored light intensities are shown in  $\mu\text{W}/\text{mm}^2$ . Similarly, the climbing performance of *Drosophila* expressing eNpHR in cholinergic cells remained largely unimpaired when the flies were illuminated with  $39 \mu\text{W}/\text{mm}^2$  of red light.

**B.** Muybridge series (top row) from a high-speed video (1000 frames per second) before and after light onset ( $t=0 \text{ ms}$ ) of a fly expressing GtACR1 in cholinergic neurons. Illumination with green light (peak 525 nm,  $38 \mu\text{W}/\text{mm}^2$ ). Frames are spaced at 50 ms intervals, numbers indicate time relative to illumination start in ms.

Difference images between consecutive frames (bottom row) map the motion. Coloured bar indicates frames where the fly is illuminated. Series is representative for four flies.

**C.** Muybridge series as in (B) for a *Cha-Gal4>UAS-GtACR2* fly. Illumination with  $253 \mu\text{W}/\text{mm}^2$  of blue light.

**D.** Muybridge series illustrates the recovery of a *Cha-Gal4>UAS-GtACR1* fly after light-off. Motion in the difference series prior to lights-off indicates that the fly was immobile, though not motionless. Inter-frame steps are 50 ms.

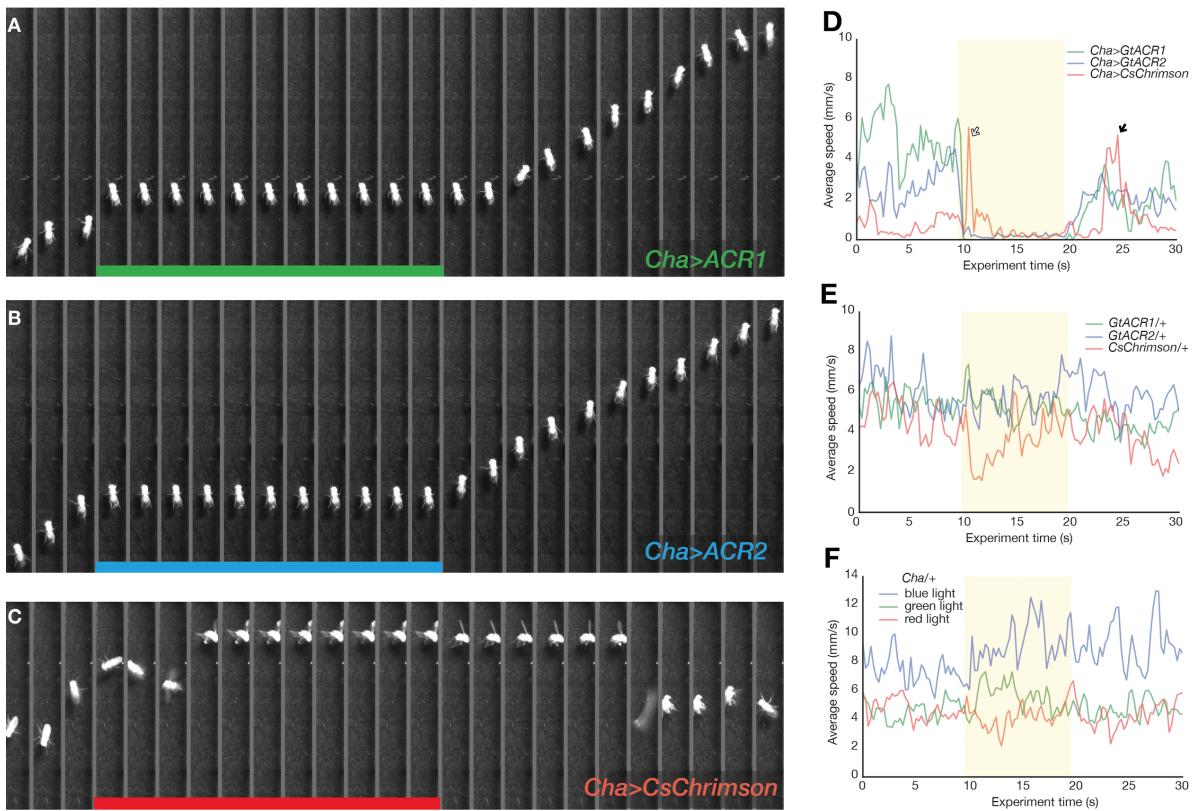
**E.** Recovery of a *Cha-Gal4>UAS-GtACR2* fly over a 500 ms interval after light-off.

**F.** A representative series of a *Cha-Gal4>UAS-eNpHR* fly with amber illumination (peak 591 nm,  $495 \mu\text{W}/\text{mm}^2$ ).

**G–H.** A fly with a *Cha-Gal4>UAS-eNpHR* genotype exposed to  $1900 \mu\text{W}/\text{mm}^2$  illumination (i.e. sitting 3 mm above the LED) was only sporadically immobilized.

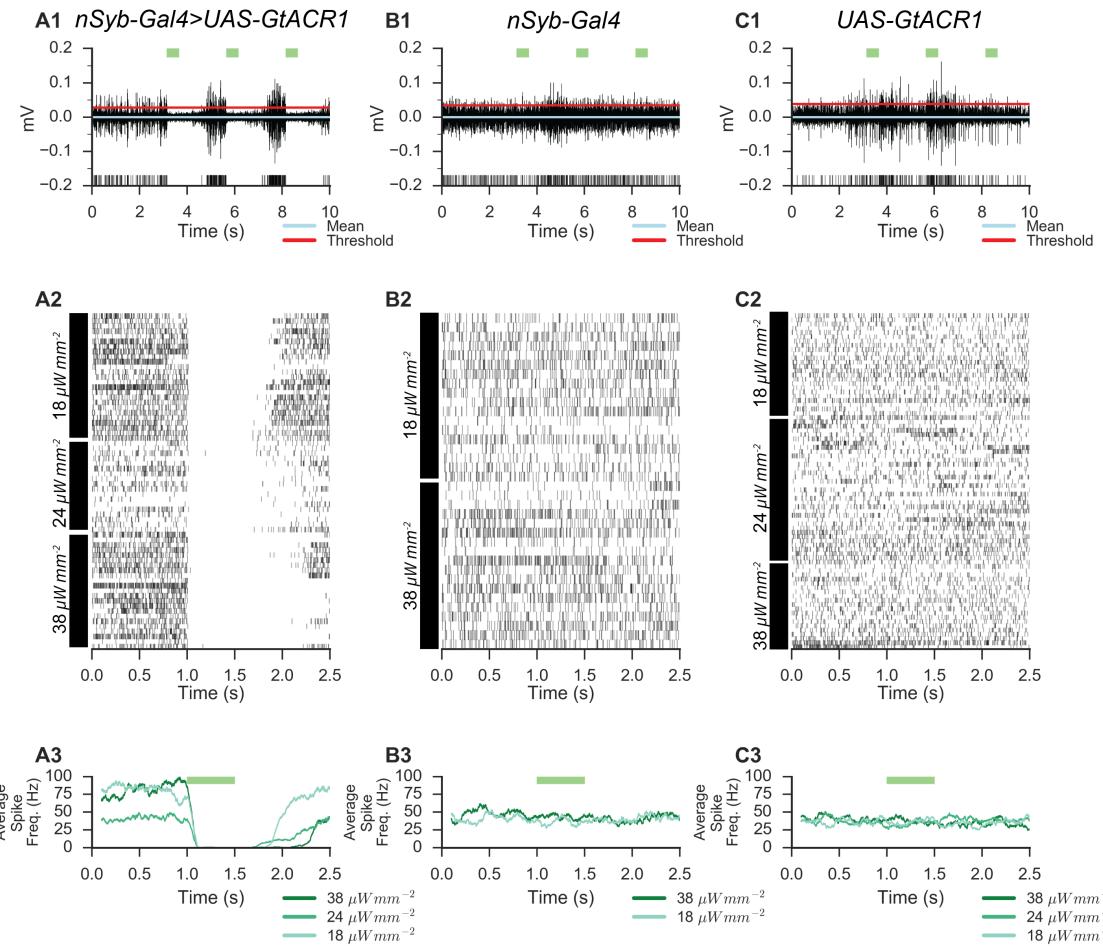
**I.** Difference images from the videos in panels B, C, F were used to quantify motion. Plots show the normalized sum of pixel value differences across a region of interest that contained the moving fly. *Cha-Gal4>GtACR* flies were motionless within 100 ms, while illuminated *Cha-Gal4>eNpHR* flies showed similar pixel changes before and during light exposure. The pixel difference spike observed at light onset is due to the change in lighting.

**J–L.** Times of immobility (J), onset (K), and recovery (L) of *Cha-Gal4>UAS-GtACR2* flies over a range of intensities. Bars indicate mean, horizontal lines indicate medians, dots indicate time measured from a single fly ( $N_{flies} = 4$ ).



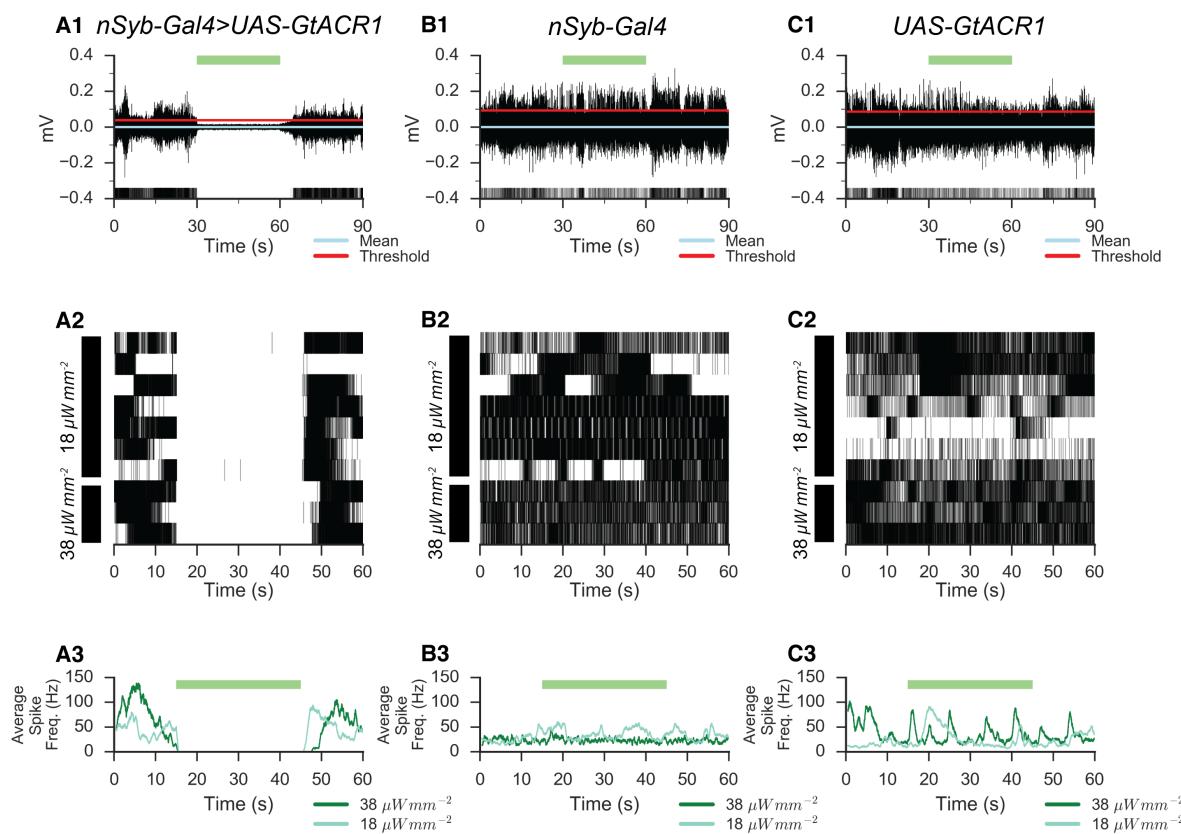
**Supplementary Figure 2. Differences between GtACR-induced cholinergic paralysis and CsChrimson-induced seizure**

**A.** A *Cha>GtACR1* fly entered into a static paralysis upon illumination with green projector light ( $92 \mu\text{W}/\text{mm}^2$ ). Frames are separated by 1 s intervals. **B.** A *Cha>GtACR2* fly underwent static immobilization during exposure to blue light ( $67 \mu\text{W}/\text{mm}^2$ ), though retained some leg movement. **C.** A *Cha>CsChrimson* fly underwent an active convulsion for several seconds upon illumination with red light ( $70 \mu\text{W}/\text{mm}^2$ ). During paralysis, the wings were extended; after light-off, seizure continued for ~6 seconds and was followed by another active convolution before the fly regained a standing pose (last frame). **D.** Average speed of the three strains illustrated in F-H, before during and after exposure to projector light (*Cha>GtACR1*, green  $92 \mu\text{W}/\text{mm}^2$ ; *Cha>GtACR2*, blue  $67 \mu\text{W}/\text{mm}^2$ ; *Cha>CsChrimson*, red  $70 \mu\text{W}/\text{mm}^2$ ). *Cha>GtACR* flies rapidly entered a static paralysis and retained their pre-light pose (see also Supplementary Video 3). Lines are the mean speed of seven flies of each genotype. The GtACR flies are rapidly immobilized; the CsChrimson flies had increased speed after light-on that is related to convulsions (white arrow). The *Cha>CsChrimson* flies also convulse during recovery (black arrow). **E.** The walking speed of responder control flies is largely unchanged by light exposure,  $N = 15, 15, 15$ . **F.** The driver control (*Cha-Gal4/+*) line is unresponsive to projector illumination of any color,  $N = 15, 15, 15$ .



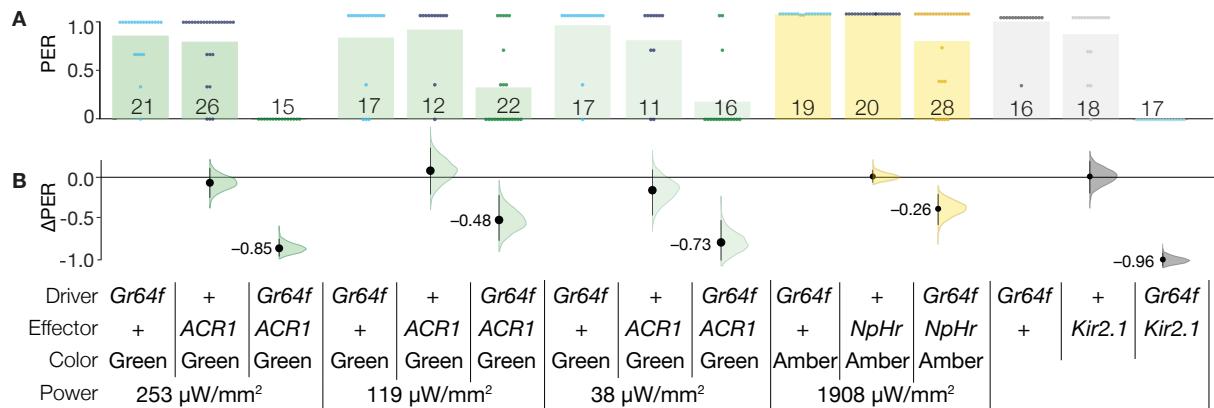
**Supplementary Figure 3. Actuated GtACR1 rapidly and reversibly silences action potentials in a *Drosophila* larval nerve.**

Photo-actuation of GtACR1 expressed in larval abdominal nerves (*nSyb>GtACR1*, pan-neuronal expression) produced reductions in spiking frequency, as seen in representative recordings from larval segmental nerves. **A.** A representative trace (A1) from an extracellular recording from a larval abdominal 3 nerve showing the inhibitory effect of three 500 ms pulses of green light (38  $\mu\text{W}/\text{mm}^2$ ) in a *nSyb>GtACR1* larva. The red line indicates the voltage threshold used to detect spikes. The blue line indicates the average mV across the trace recording. Rasters of spike timings (A2) from several light intensities (38  $\mu\text{W}/\text{mm}^2$ , 24  $\mu\text{W}/\text{mm}^2$ , and 18  $\mu\text{W}/\text{mm}^2$ ) and a spike frequency plot (A3) indicate that the time of full activity recovery is longer with more intense illumination ( $N_{\text{pulses}} = 93$ ,  $N_{\text{flies}} = 3$ ). With 500 ms pulses of 38  $\mu\text{W}/\text{mm}^2$  green light, spiking in nerves from *nSyb>GtACR1* larvae was suppressed by  $\Delta = -98.5\%$  [95CI -99.2, -97.6],  $P = 3.05 \times 10^{-39}$ ,  $N_{\text{pulses}} = 23$ ,  $N_{\text{flies}} = 3$ . With 500 ms pulses of 24  $\mu\text{W}/\text{mm}^2$  green light, the spiking frequency exhibited a decrease of -99.0% [95CI -99.7, -97.7],  $P = 3.415 \times 10^{-35}$ ,  $N_{\text{pulses}} = 21$ ,  $N_{\text{flies}} = 3$ . 500 ms pulses of 18  $\mu\text{W}/\text{mm}^2$  green light suppressed the spiking frequency of *nSyb>GtACR1* larval nerves by  $\Delta = -97.8\%$  relative to the preceding activity [95CI -96.0, -98.7],  $P = 3.62 \times 10^{-33}$ ,  $N_{\text{pulses}} = 22$ ,  $N_{\text{flies}} = 3$ . **B.** Across several light intensities and pulse durations tested, we did not observe any decrease in the spiking frequency of control larvae during green light illumination (*nSyb-Ga4/+* and *UAS-GtACR1/+*). Illumination of *nSyb-Ga4/+* control flies with green light (38 and 18  $\mu\text{W}/\text{mm}^2$ ) for 500 ms had no detectable effect on the rate of identifiable spikes ( $N_{\text{pulses}} = 63$ ,  $N_{\text{flies}} = 3$ ). **C.** Illumination of *UAS-GtACR1/+* control flies for 500 ms with green light (38, 24, and 18  $\mu\text{W}/\text{mm}^2$ ) had no effect on nerve firing ( $N_{\text{pulses}} = 106$ ,  $N_{\text{flies}} = 3$ ). For example, 500 ms pulses of 38  $\mu\text{W}/\text{mm}^2$  green light did not substantially alter the spiking frequency of *UAS-GtACR1/+* larval nerves ( $\Delta = -0.24\%$  [95CI -11.6, +15.5],  $P = 0.97$ ,  $N_{\text{pulses}} = 25$ ,  $N_{\text{flies}} = 3$ ).



**Supplementary Figure 4. Actuated GtACR1 silences action potentials for 30 seconds.**

Longer actuation (30 s) of GtACR1 was able to almost completely silence action potentials in a nerve. **A.** A representative recording (A1), raster plot (A2) and frequency plot (A3) of *nSyb>GtACR1* nerves indicate that firing is almost completely suppressed during a 30 s illumination epoch. Firing recovers in <5 s at 18  $\mu\text{W}/\text{mm}^2$  and ~10 s at 38  $\mu\text{W}/\text{mm}^2$  ( $N_{\text{pulses}} = 10$ ,  $N_{\text{flies}} = 3$ ). At 18  $\mu\text{W}/\text{mm}^2$ , 30 s pulses decreased the spiking frequency by -99.8% [95CI -99.4, -100],  $P = 3.83 \times 10^{-16}$ ,  $N_{\text{pulses}} = 7$ ,  $N_{\text{flies}} = 3$ . 30 s pulses of 38  $\mu\text{W}/\text{mm}^2$  light completely silenced large spikes,  $\Delta = -100\%$  [95CI -100, -100],  $P = 0$ ,  $N_{\text{pulses}} = 10$ ,  $N_{\text{flies}} = 3$ . **B.** Illumination of *nSyb-Gal4/+* controls with green light for 30 s had no effect on the spike rate ( $N_{\text{pulses}} = 10$ ,  $N_{\text{flies}} = 3$ ). **C.** Green illumination of *UAS-GtACR1/+* controls for 30 s had no effect on firing ( $N_{\text{pulses}} = 10$ ,  $N_{\text{flies}} = 3$ ).



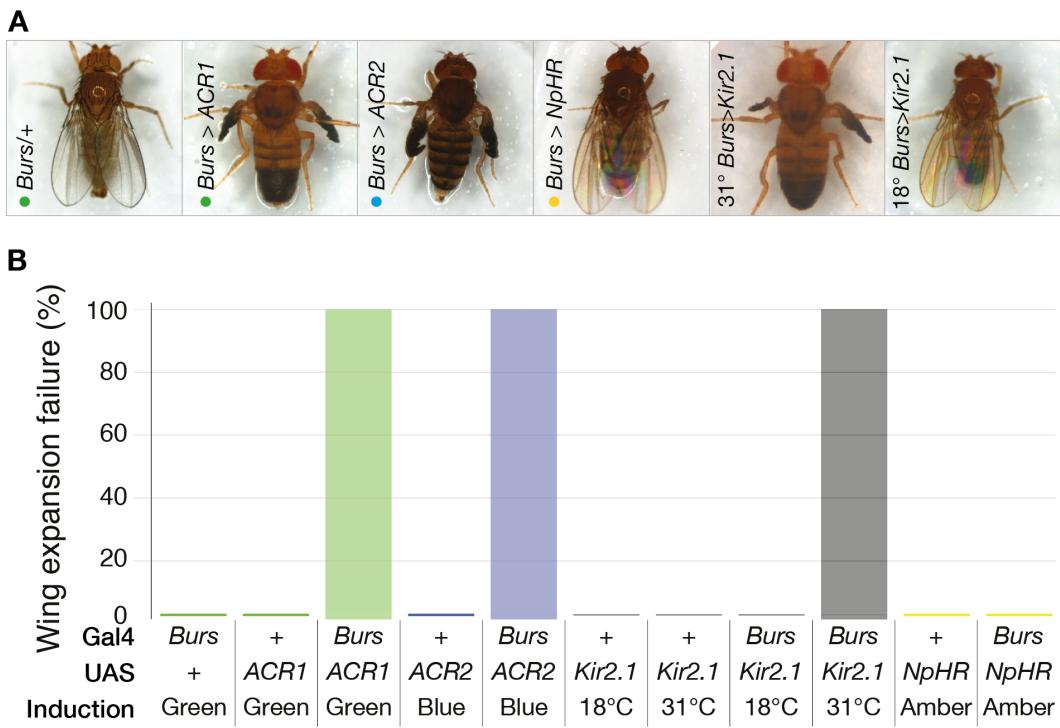
**Supplementary Figure 5. Optogenetic inactivation of taste neurons suppresses proboscis extension reflexes.**

**A.** Proboscis extension responses of transgenic flies exposed to a range of protocols aimed at silencing the Gr64f cells. Dots show individual animal response probabilities, bars represent mean response PER probabilities, numerals indicate sample sizes. Genotypes and conditions are given in key.

**B.** Changes in responses ( $\Delta\text{PER}$ ) relative to *Gr64f-Gal4/+* controls. The mean differences are as follows:

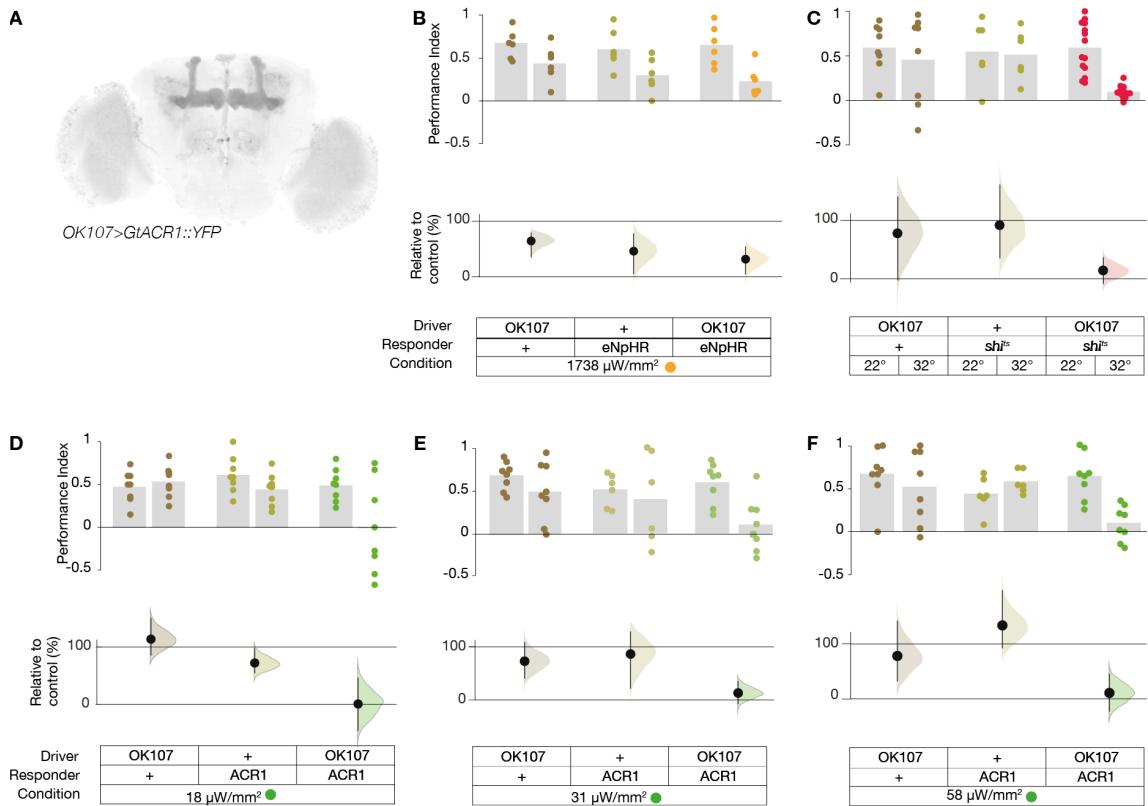
*Gr64f>GtACR1* at 253  $\mu\text{W}/\text{mm}^2$   $\Delta\text{PER} = -0.85$  [95CI -1.0, -0.64],  $P = 3.5 \times 10^{-8}$ ; 119  $\mu\text{W}/\text{mm}^2$   $\Delta\text{PER} = -0.48$  [95CI -0.21, -0.71],  $P = 1.9 \times 10^{-3}$ ; 38  $\mu\text{W}/\text{mm}^2$   $\Delta\text{PER} = -0.73$  [95CI -0.49, -0.94],  $P = 1.7 \times 10^{-5}$ ; *Gr64f>eNpHr*  $\Delta\text{PER} = -0.26$  [95CI -0.42, -0.12],  $P = 0.054$  and *Gr64f>Kir2.1*  $\Delta\text{PER} = -0.96$  [95CI -1.0, -0.92],  $P = 2.7 \times 10^{-8}$ .

Error bars are confidence intervals of the mean, curve is the bootstrap distribution of the mean.



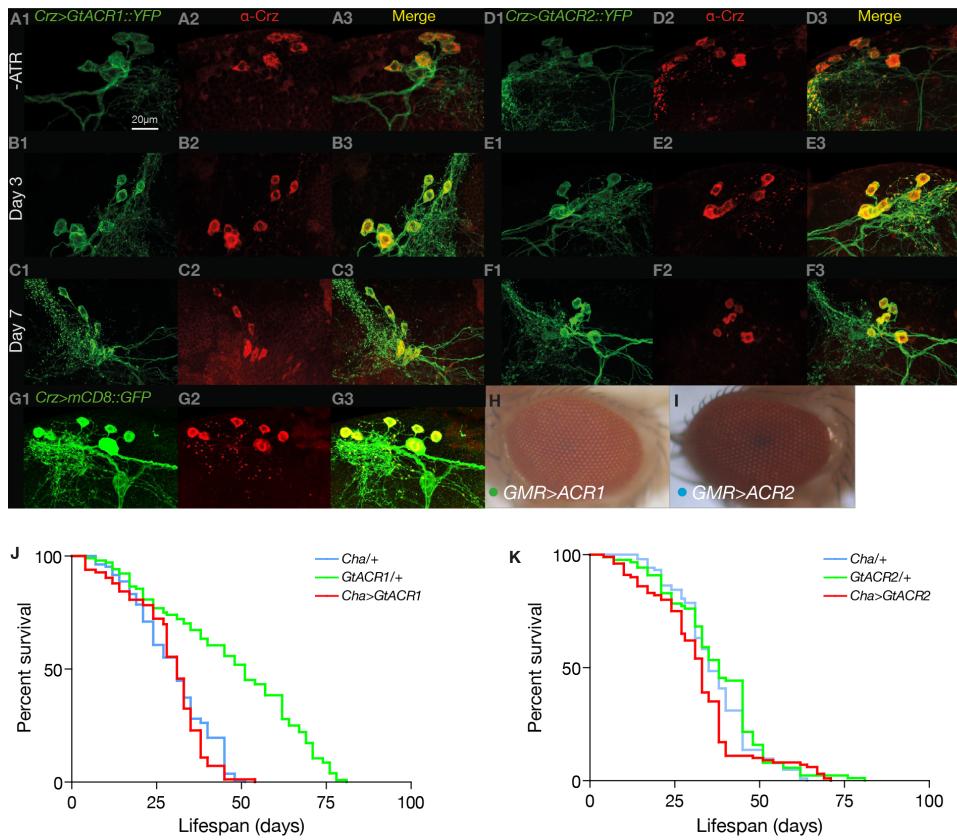
**Supplementary Figure 6. Illuminated anion channelrhodopsin expressed in *Bursicon* cells inhibit wing expansion.**

Inhibition of bursicon neurohormone-releasing cells had lethal effects during development and prevented normal wing expansion in surviving flies (Peabody, N.C. et al. *J. Neurosci.* **28**, 14379-14391 (2008)). A. Flies expressing GtACRs under the control of a bursicon promoter (*Burs-Gal4*) were illuminated from 1-2 days after puparium formation (APF) until 9-10 APF before wing expansion was scored. The majority of illuminated *Burs>GtACR* flies died during development (129 out of 249), while the survivors failed to expand their wings. Illumination of flies expressing GtACRs in bursicon-releasing cells (*Burs-Gal4; UAS-GtACR1/2*) inhibited wing expansion. Flies expressing GtACR1 or GtACR2 in bursicon-releasing cells failed to expand their wings following exposure to green light at 46  $\mu\text{W}/\text{mm}^2$  or blue light at 106  $\mu\text{W}/\text{mm}^2$ , respectively. Expression of an inward rectifying potassium channel (Kir2.1) (Baines, R.A. et al. *J. Neurosci.* **21**, 1523-1531 (2001)) resulted in total wing expansion failure as previously reported (Peabody, N.C. et al. *J. Neurosci.* **28**, 14379-14391 (2008)). Control flies were unaffected by illumination. Amber-illuminated *Burs>eNpHr* flies displayed normal wings (amber light at 106  $\mu\text{W}/\text{mm}^2$ ). B. Quantification of *Burs-Gal4* results. All light-exposed flies expressing GtACR1 or GtACR2 in bursicon-releasing cells failed to expand their wings; controls were unaffected (green at 46  $\mu\text{W}/\text{mm}^2$ , blue at 106  $\mu\text{W}/\text{mm}^2$  for GtACR1 and GtACR2 respectively). Induction of Kir2.1 at 31°C in *Burs-Gal4>UAS-Kir2.1, Tub-Gal80<sup>ts</sup>* flies produced a 100% failure in expansion. Illumination of *Burs-Gal4>UAS-eNpHr* flies with 106  $\mu\text{W}/\text{mm}^2$  amber light had no effect on wing expansion. *Burs/+* ( $N = 145$ ), *UAS-GtACR1/+* ( $N = 60$ ), *Burs-Gal4; UAS-GtACR1* ( $N = 114$ ), *UAS-GtACR2/+* ( $N = 60$ ), *Burs-Gal4; UAS-GtACR1* ( $N = 135$ ), *UAS-Kir2.1/+* (18°C,  $N = 58$ ), *UAS-Kir2.1/+* (31°C,  $N = 61$ ), *Burs-Gal4; UAS-Kir2.1* (18°C,  $N = 65$ ), *Burs-Gal4; UAS-Kir2.1* (31 °C,  $N = 63$ ), *UAS-NpHr/+* ( $N = 60$ ), *Burs-Gal4; UAS-NpHr* ( $N = 78$ )



**Supplementary Figure 7. Inhibition of the mushroom body with GtACR1 suppresses short term memory performance.**

Synaptic transmission from the mushroom body is required for normal olfactory short term memory (STM) (McGuire, S.E. *et al. Science* **293**, 1330-1333 (2001), Dubnau, J. *et al. Nature* **411**, 476-480 (2001), Yildizoglu, T. *et al. PLoS Genet.* **11**, e1005718 (2015)). Initial training cycles (left columns) were compared with a second training cycle (right columns). A. The expression pattern of *UAS-GtACR1::YFP* driven with mushroom body driver *OK107-Gal4*. Grey indicates YFP fluorescence. B. Flies expressing eNpHR in the mushroom body cells (*OK107>eNpHR*) had a reduction in STM during strong amber illumination (1.7 mW/mm<sup>2</sup>),  $\Delta\text{PI} = -0.43$  [95CI -0.71, -0.14],  $P = 0.005$ . However, STM reduction was only modestly stronger than in control animals, suggesting the effect was primarily due to the light alone. Genotype key is at the bottom, colored dots represent the average of two half PIs from 6 animals each,  $N = 8$  experiments as indicated by the dots. Lower axis: The memory effects of illumination as a percentage of the same animals' scores under infrared light. Error bars are confidence intervals of the mean; curve is the bootstrap distribution of the mean. C. Heat treatment of flies had only trivial effects on controls, but inhibition of *OK107* cells with *UAS-shits* at 32°C led to an almost complete block of STM,  $\Delta\text{PI} = -0.5$  [95CI -0.67, -0.33],  $P = 1.4 \times 10^{-5}$ . D. Illumination with green light produced a negligible increase (*OK107/+*) or decrease (*UAS-GtACR1/+*) STM performance in control animals. However, green light illumination at 18  $\mu\text{W/mm}^2$  reduced STM performance in flies expressing *OK107-Gal4>UAS-GtACR1*.  $\Delta\text{PI} = -0.50$  [95CI -0.97, -0.1],  $P = 0.03$ . E. Illumination of *OK107>GtACR1* flies with 31  $\mu\text{W/mm}^2$  green light also inhibited the expression of STM  $\Delta\text{PI} = -0.5$  [95CI -0.33, -0.66],  $P < 0.001$ . F. Illumination of *OK107>GtACR1* flies with 58  $\mu\text{W/mm}^2$  light inhibited conditioned avoidance  $\Delta\text{PI} = -0.55$  [95CI -0.31, -0.76],  $P = 0.003$ .



**Supplementary Figure 8. The GtACRs are minimally toxic.**

**A1.** Central brain cells were observed to express yellow fluorescent protein-tagged GtACR1 (green, endogenous fluorescence) in *Crz>GtACR1* fly brains prior to ATR feeding.

**A2–3.** Antibody staining against Crz protein ( $\alpha$ -Crz, red) confirmed the GtACR1-expressing cells were Crz<sup>+</sup>.

**B1–3.** The expression of GtACRs in *corazonin* neurons had little to no effect on neuronal morphology. Crz<sup>+</sup> cell morphology was normal in *Crz>GtACR1* flies after ATR feeding and one day of illumination with 19  $\mu$ W/mm<sup>2</sup> green light.

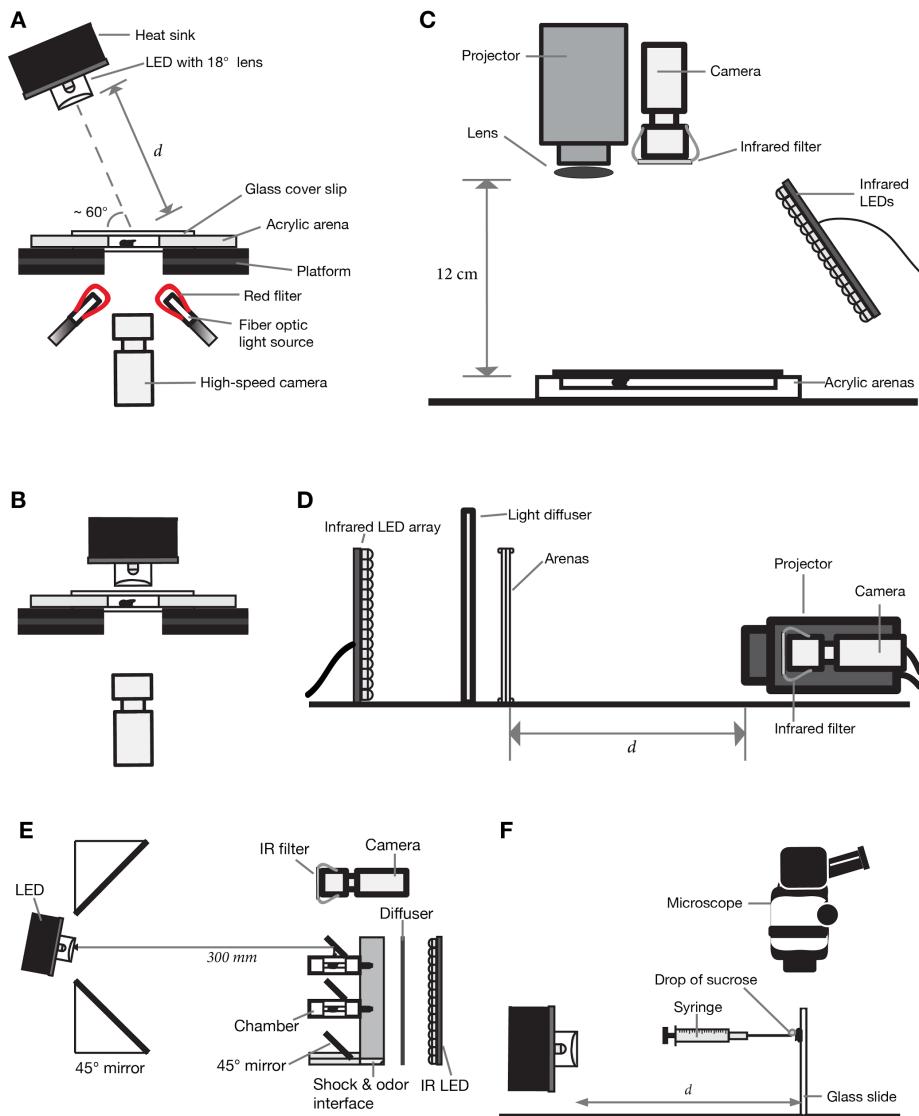
**C1–3.** Crz<sup>+</sup> cell morphology was normal in *Crz>GtACR1* flies after ATR feeding and 6 days of illumination.

**D–F.** Flies expressing GtACR2::YFP in Crz cells had normal morphology after 6 days of GtACR2 activation with 25  $\mu$ W/mm<sup>2</sup> blue light.

**G.** The morphology of cells carrying a fluorescent protein (mCD8::GFP) but no photochannel. The mCD8::GFP was visualized with anti-GFP antibody, thus appears brighter.

**H–I.** Expression of GtACRs in fly ommatidia with light exposure throughout metamorphosis had no effect on eye morphology: flies expressing either GtACR1 or GtACR2 with the *GMR-Gal4* driver had normal eye morphology after being illuminated during metamorphosis.

**J.** Broad expression of GtACRs in cholinergic neurons with intermittent light exposure did not dramatically shorten lifespans relative to controls. The lifespans of flies bearing *Cha>GtACR1* transgenic expression (median = 31 days [95CI 28, 33]) were similar to *Cha-Gal4/+* controls (median = 35 days [95CI 33, 40]), and shorter than the lifespans of *GtACR1/+* animals (median = 51 days [95CI 45, 57]). All flies were subjected to intermittent *ad hoc* light exposure. **K.** The lifespans of flies with *Cha>GtACR2* transgenic expression (median = 31 days [95CI 28, 33]) were similar to *Cha-Gal4/+* (median = 31 days [95CI 27, 33]) and *GtACR2/+* (median = 38 days [95CI 33, 45]) controls.



**Supplementary Figure 9. Designs of the behavior recording apparatuses.**

A. Apparatus for recording high-frame rate video. Light intensity was varied by setting the distance ( $d = 65, 110, 220, 330\text{ mm}$ ) between the LED and the behavior chamber. Flies were visualized from below with a red filtered light source.

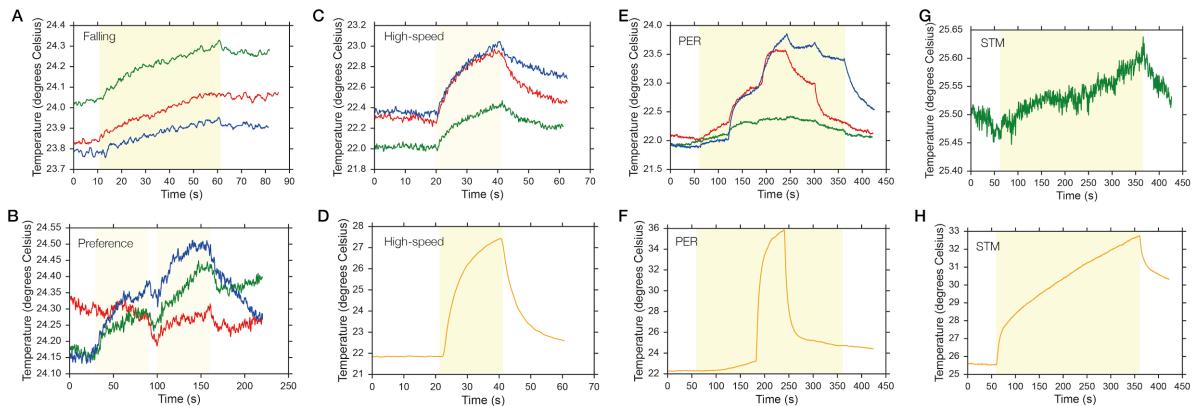
B. The configuration that was used to record *Cha>eNpHr* behavior, with the LED only 3 mm above the behavior chamber.

C. Apparatus to measure pacing and preference behaviors. The projector was used to illuminate either the entire chamber (pacing assay) or two bands (preference assay). The chambers were illuminated with an infrared LED panel; video was captured with an Allied Vision Guppy-046 B camera.

D. The configuration used to measure fly falling. The transparent chamber was back-lit with an infrared LED panel; video was captured with a Point Grey BFLY-U3-13S2M-CS camera. Projector distance was  $d = 300\text{mm}$  for all but the highest intensity experiments where  $d = 170\text{mm}$

E. Lighting configuration in the multi-fly olfactory trainer: the LED arrays were placed 300 mm in front of the conditioning chambers. Diffused infrared backlighting was used to image the flies with an IR-pass filtered video camera.

F. For the PER assay, flies were fixed to a glass slide and presented with sucrose solution. Responses were visualized with a dissection microscope. Light intensity from an LED was varied by varying the distance ( $d = 65, 110, 220\text{ mm}$ ) from the flies.



**Supplementary Figure 10. Illumination appropriate for GtACR actuation has minor effects on temperature.**

- A. The projector illumination used to actuate the GtACRs in the falling chamber had a minor heating effect: a maximum increase of 0.3°C in green light.
- B. Projector illumination of the flat preference and pacing arena produced only small increases in temperature, +0.35°C or less.
- C. In the high frame rate camera rig, illumination with GtACR-actuating LED light produced minor heat increases: a maximal increase of +0.7°C in 20 s.
- D. In the high-speed camera rig, bringing the amber LED close enough to observe sporadic immobilization of *Cha<eNpHr* flies produced a +5°C increase in 20 s, at an intensity of 1900  $\mu\text{W}/\text{mm}^2$ .
- E. The strongest green LED light (253  $\mu\text{W}/\text{mm}^2$ ) used to actuate flies in the PER experiments heated the flies' location by +0.5°C. To allow sequential exposure during the PER experiment, 5 flies were mounted on each slide. To simulate the temperature exposure of a typical fly, the thermocouple was placed at the equivalent position of the central position (third fly) and the slide was moved through the LED beam as would occur during an experiment, allowing one minute for each fly position: this accounts for the ramp-up/ramp-down structure in the temperature curves.
- F. Amber LED illumination of the PER slide at 1900  $\mu\text{W}/\text{mm}^2$  elicited an approximately +14°C maximal increase.
- G. In the olfactory conditioning apparatus, the green LED light (58  $\mu\text{W}/\text{mm}^2$ ) used to actuate *OK107>GtACR1* flies produced a +0.15°C increase in temperature over 300 s.
- H. In the MOT apparatus, 1740  $\mu\text{W}/\text{mm}^2$  amber LED light produced a +7.2°C temperature increase over five minutes.