

Supporting Information

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SI Materials and Methods

Molecular Biology and Genetics. Oocytes. cDNA of C-terminally truncated wild-type channelopsin-2 (*chop2*), encoding amino acids 1–315 (1), was cloned into oocyte expression vectors, based on the plasmid pGEMHE 22, a derivative of pGEM3z (Promega). Mutations were inserted via site-directed mutagenesis (Quik-Change kit; Stratagene). The red-activatable ChR (*ReaChR*) sequence was synthesized by combining two GeneArt Strings DNA Fragments (Life Technologies, Thermo Fisher Scientific), ordered according to the published amino acid sequence (2), with *Drosophila* codon use. The DNA was inserted into the pGEMHE vector between *Bam*HI and *Xho*I restriction sites with a YFP attached to its C-terminal end (*ReaChR::YFP*) and confirmed by sequencing. *Nhe*I-linearized plasmid DNA was used for the in vitro generation of cRNA with the AmpliCap-MaxT7 High Yield Message Maker Kit (Epicentre Biotechnologies).

Drosophila. ChR mutants for fly transgenesis were PCR-amplified from plasmid templates for oocyte expression [forward primer, 5'-TCTAGAatcaacatggattatggagcgcctgagtg-3'; reverse primer, 5'-ACCGGTttagtgccggccggctaccgcgcca-3' (restriction sites capitalized and Kozak sequence underlined)] and inserted via *Xba*I and *Age*I into the expression vector pJFRC7 (3), whose multiple cloning site was previously extended into *Not*I-*Stu*I-*Kpn*I-*Xho*I-*Xba*I-3xFlag-*Age*I. Inserts were verified by restriction analyses and DNA sequencing.

For expression in *Drosophila*, ChR2 variants were not fused to photoproteins. Previously published ChR2-wt flies (4) carried the truncated protein *UAS-chop2*³¹⁵ (1). Flies carrying *UAS-chop2-T159C* and *UAS-chop2-D156C* were generated by targeted PhiC31 recombinase-mediated insertion of either transgene into the genomic P[acman] landing site *attP-9A/VK18* carried on the second chromosome (5) at BestGene Inc. *UAS-ReaChR*^{VK5} (6) was obtained from the Bloomington *Drosophila* Stock Center (no. 53749). Animals were raised at 25 °C in the dark. For dietary supplementation with retinal, aliquots from a 50-mM stock (250 mM for olfactory learning) of *all-trans*-retinal (dissolved in ethanol) were mixed into the food slurry just before egg laying to yield a final concentration of 100 μM (250 μM for olfactory learning).

The following genotypes were used for expression in motor neurons: *ok6-GAL4/+; UAS-chop2*^{wt/+}, *UAS-chop2*^{T159C/ok6-GAL4}, *UAS-chop2*^{D156C/ok6-GAL4}, *ok6-GAL4/+; UAS-ReaChR/+*. The following were used in gustatory cells: *Gr5a-GAL4/+; UAS-chop2*^{wt/+}, *Gr5a-GAL4/ UAS-chop2*^{T159C}, *Gr5a-GAL4/ UAS-chop2*^{D156C}. The following were used in *fru* neurons: *UAS-chop2*^{D156C/+; fru-GAL4/+. The following were used in dopaminergic neurons: *UAS-chop2*^{T159C/+; TH-Gal4/+}, *UAS-chop2*^{D156C/+; TH-Gal4/+}; *UAS-chop2*^{T159C/+; UAS-chop2^{D156C/+; TH-Gal4/+}.}}

Electrophysiology. Oocytes. Oocytes were injected with 20 ng of RNA of *chop2* variants, or *ReaChR* C-terminally tagged with YFP. Oocytes were incubated in medium containing 10 μM *all-trans*-retinal, as indicated. Two electrode voltage-clamp recordings of photocurrents were made in Ringer's solution (pH 7.6) at a holding potential of –100 mV. Photocurrent amplitudes, half-saturating light intensities, and action spectra were taken from stationary currents. A diode pumped solid-state laser was used for longer illumination times (473 nm, 8 mW/mm², 2 × 10¹⁸ photons·cm⁻²·s⁻¹) and a pulsed laser for 5-ns flashes (473 nm, pulse energy density 13 mJ/mm²).

For action spectra of ChR2-XXL (ChR2 extra high expression and long open state) and *ReaChR* (*n* = 3 each), light of different wavelengths was obtained by narrow bandwidth interference filters (Edmund Optics) together with a PhotoFluor II light source (89

North). The wavelength was further confirmed with a spectrometer (Ocean Optics). Equal photon flux was set for each wavelength. The action spectrum of ChR2-XXL was normalized to photostimulation at 480 nm. Because of some “rundown” in *ReaChR*-expressing cells (maximally to 50% during one measurement), these recordings were made at –60 mV, and 517 nm light stimulation was measured before every single wavelength as a reference and for normalization.

Drosophila NMJ. Semi-intact preparations and data acquisition were performed essentially as previously reported (7). In brief, current-clamp recordings were made from ventral longitudinal muscle 6 in abdominal segments A3 and A4 at room temperature using a microelectrode (filled with 3M KCl) in combination with an Axoclamp 900A amplifier (Axon Instruments/Molecular Devices). The extracellular solution (HL3.1) (8) contained 70 mM NaCl, 5 mM KCl, 5 mM MgCl₂, 10 mM NaHCO₃, 5 mM D-(+)-trehalose, 115 mM sucrose, 5 mM Hepes, 1.5 mM CaCl₂; pH was adjusted to 7.2. Excitatory postsynaptic potentials (EPSPs) were evoked with 0.5-s light pulses using the following intensities (μW/mm² at 460 nm): 5, 16, 29, 60 (ChR2-T159C); 5 (ChR2-XXL). The decay of EPSP frequencies was measured using a 5-s sliding window (Fig. 3C).

Imaging. Oocytes. For documentation of ChR2 expression in oocytes, 12-bit images were acquired under identical settings with a confocal laser-scanning microscope (LSM 5 Pascal; Carl Zeiss) equipped with a Zeiss Plan-Neofluar 20×/0.5 objective. Fluorescence signal intensity was determined by quantification of mean gray levels across the entire image of a confocal slice. The mean gray value of an untransfected oocyte in the same dish was subtracted from each measurement. Image analysis was performed using the ImageJ software package (<http://imagej.nih.gov/ij/index.html>).

Drosophila. For imaging, larvae of all genotypes were fed 100 μM retinal. ChR2 expression was coimaged with HRP (goat anti-HRP-Cy3, 1:250) as previously described (7). In brief, larvae expressing ChR2-wt, ChR2-T159C, or ChR2-XXL were stained in the same vial under identical conditions using a commercially available antibody (mouse anti-ChR2 supernatant 1:1, 15E2, mfd Diagnostics; secondary antibody Alexa 488 goat anti-mouse 1:250, Invitrogen), and confocal imaging was performed using equal settings. In the example images, a Gaussian blur (1 px radius) was applied following background subtraction and before normalizing fluorescence intensities.

Light stimulation. Larval immobilization. Blue light was used to stimulate late third instar larvae (light from a mercury lamp passed through a GFP excitation band-pass filter), and a red LED (peak 623 nm) was used for *ReaChR*. Measurements reflect the time between light-induced immobilization of crawling larvae and resumed movement (defined as anterior displacement of posterior end) during ongoing irradiation (*n* ≥ 5 animals per genotype and light intensity). No visible response was scored as <1 s of immobilization. In several examples, long-term photostimulation of larvae was performed: with ChR2-T159C, an individual was immobilized for approximately 4 h (0.1 mW/mm²) and resumed crawling after several seconds following the end of the light stimulus; with ChR2-XXL, two larvae were immobilized for approximately 6 h (50 μW/mm²) and resumed crawling a few minutes after the end of the light stimulus.

Adult immobilization. Five to 10 flies (1–15 d after eclosion) were placed in a vertically positioned Petri dish (8 cm diameter), which was homogeneously irradiated with light from a mercury lamp

(filtered appropriately) for 1 min. For ReaChR, high-power LEDs were used to provide sufficient light intensity (at 626 nm and without retinal addition, on average five ReaChR flies were placed in a 500- μ L PCR tube to ensure homogeneous light application). The flies were then tapped down, and the immobilized individuals were counted. Flies were kept in the dark for 5 min before switching to the next filter, and at least five groups were measured for each data point. For action spectra, irradiance was adjusted to deliver $7.8\text{--}8.4 \times 10^{17}$ photons \cdot cm $^{-2}$ \cdot s $^{-1}$ at each wavelength, and measurements proceeded from short to long wavelengths.

Proboscis extension reflex. Flies (2-4 d after eclosion) were fixed on a glass slide using nail polish and left for 1 h in the dark before the experiment. ChR2-T159C was activated with blue light (mercury lamp), and ChR2-XXL was activated with white LEDs (for white-light sources, the intensity at 460 nm was measured after passing light through a GFP excitation bandpass filter). In between light stimulation, ChR2-T159C and ChR2-XXL were exposed to only very low light intensities or kept in the dark, respectively. To analyze the dependence of photostimulated proboscis extension reflex (PER) on light intensity, four to five light pulses (each lasting \sim 1 s) were applied at each intensity and separated by 20 s (ChR2-T159C) or 5 min (ChR2-XXL). For ChR2-T159C, light intensities were (in mW/mm 2 at 460 nm): 0.01, 0.02, 0.06, 0.12, 0.24, and 0.32 (5 min rest in between different intensities, $n = 5$ flies); for ChR2-XXL (in μ W/mm 2): 0.02, 0.09, 0.23, 0.94, 8.58 (10 min rest in between different intensities, $n = 6$ flies).

For measurements of PER frequency with ChR2-T159C, partial proboscis extensions were also scored. Partial proboscis extensions were never observed with ChR2-XXL. Four to five light pulses (\sim 1-s duration) were applied at each frequency. For ChR2-T159C (0.32 mW/mm 2 , $n = 8$ flies), pulse intervals were 1 s, 2 s, 3 s, 4 s, 9 s, and 14 s (5 min rest between intervals); for ChR2-XXL (8.58 μ W/mm 2 , $n = 10$ flies): 29 s, 59 s, 119 s, 239 s, 359 s, 479 s, and 599 s (10 min rest between intervals).

Courtship. Males and females were collected \leq 6 h after eclosion and aged for at least 3 d in isolation or in groups, respectively. Individual flies were placed in an arena and left in the dark for 30 min before photostimulation with an \sim 2-s light pulse (30 μ W/mm 2 at 460 nm; light from a mercury lamp passed through a GFP excitation bandpass filter). Videos were recorded using red light for offline analysis of courtship behaviors. Abdomen bending was defined as a male curling its abdomen beyond 90 degrees.

Courtship songs were amplified, digitized, and recorded in Audacity (<http://audacity.sourceforge.net>) at a sampling frequency of 44.1 kHz. Audio traces were low-pass filtered at 1 kHz and high-pass filtered at 90 Hz (9). Photostimulation of individual flies in

a compact, soundproofed recording arena was performed with an LED (\sim 2-s light pulse; white light 2 μ W/mm 2 at 460 nm for males and blue light 30 μ W/mm 2 for females). Wing vibration was visually inspected during audio recordings. Photostimulation of sine wave song components was observed in only 1 out of 13 ChR2-XXL-expressing flies.

Associative learning. For associative olfactory learning, groups of \sim 100 flies (4-7 d old) were trained as described by Tully and Quinn (10) with modifications: i.e., four experiments were performed simultaneously in a modified learning apparatus (11). A constant airflow of \sim 167 mL/min in each training tube assured a constant odor flow inside the training tubes. The relative humidity was 65–75%, and experiments were carried out at 25 $^{\circ}$ C and diffuse red light conditions. Ten minutes before each experiment, flies were transferred to empty fly culture vials. The odorants 4-methylcyclohexanol (CAS 589-91-3; Sigma,) and 3-octanol (CAS 589-98-0; Sigma), diluted in mineral oil (CAS 8042-47-5; Sigma) at a ratio of 1:750 or 1:500, respectively, were used. The odors were applied using plastic cups of 5-mm diameter that contained 60 μ L of the diluted odorants. Training started 1 min after transferring the flies into the training tubes. Each odor was presented for 1 min with a 1-min break between two odor applications. One odor [conditioned stimulus + (CS+)] was temporally paired with 12 electric shocks of 90 V (1.25-s shock and 3.75-s interpulse interval, DC) applied through an electrifiable grid covering the inside of the tubes. The second odor [conditioned stimulus – (CS–)] was presented without shocks.

To optogenetically substitute the electric shocks with blue light of \sim 1 μ W/mm 2 , transparent training tubes were used that were equipped with 12 blue-light diodes (peak wavelength 468 nm) evenly inserted into the tube surface. To apply high light intensities of \sim 0.3 mW/mm 2 , four powerful blue-light diodes were positioned around the transparent training tube, and heat was dissipated using cooling grids. Equivalent to the electric shock training, 12 pulses of illumination (1.25-s illumination and 3.75-s interpulse intervals) were applied. After another minute of either training, the flies were transferred to the T-maze part of the apparatus with both odors presented from each side, and flies were tested for odor preference for 2 min. Subsequently, the flies were counted and a preference index was calculated by subtracting the number of flies on the side of the CS– from the number of flies on the side of the CS+, divided by the total number of flies. Learning indices were calculated by averaging two reciprocal experiments in which each odor was used as CS+ or CS–, respectively. In Fig. 6 and Fig. S3, box plots of learning indices show medians and interquartile ranges and whiskers show minimum and maximum values.

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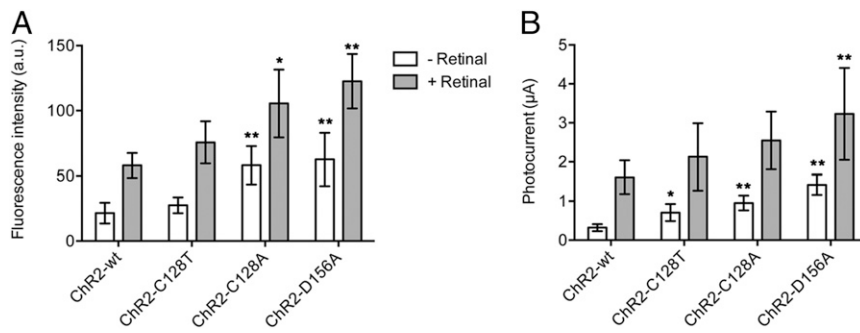


Fig. S1. Expression and photocurrents of slow ChR2 mutants in oocytes. (A) Measuring fluorescence intensities of YFP-tagged ChR2 variants describes increased expression of C128A and D156A compared to wt (Mann–Whitney rank sum test vs. wt no added retinal: C128T, $P = 0.222$; C128A, $P = 0.008$; D156A, $P = 0.008$; vs. wt plus retinal: C128T, $P = 0.056$; C128A, $P = 0.032$; D156A, $P = 0.008$). (B) Steady-state photocurrent amplitudes of slow variants are slightly increased over wt, scaling roughly with expression levels (Mann–Whitney rank sum test vs. wt no added retinal: C128T, $P = 0.016$; C128A, $P = 0.008$; D156A, $P = 0.008$; vs. wt plus retinal: C128T, $P = 0.31$; C128A, $P = 0.056$; D156A, $P = 0.008$). Asterisks denote significance to ChR2-wt at equal retinal concentrations ($*P \leq 0.05$; $**P \leq 0.01$). Data ($n = 5$ for each group) are presented as mean \pm SD.

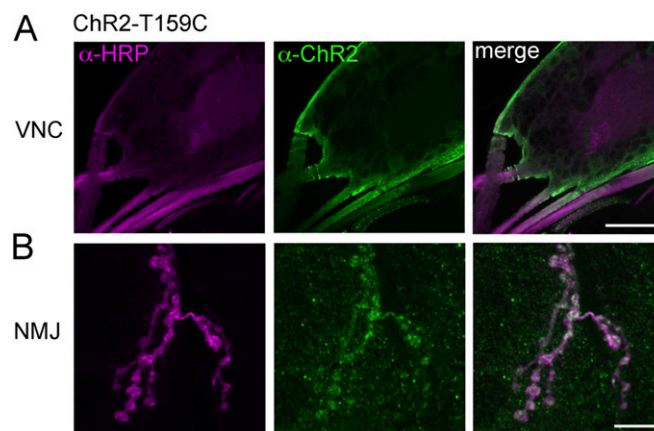


Fig. S2. Localization of ChR2-T159C in larval motor neurons. Antibody staining against ChR2 (green) and HRP (magenta). (A) ChR2-T159C was present in motor neuron axons leaving the ventral nerve chord (VNC), and (B) could be detected at the neuromuscular junction (NMJ) (shown is a single optical slice). (Scale bars: A, 30 μ m; B, 10 μ m.)

Table S1. Biophysical properties of channelrhodopsin variants

Variant	Photocurrent amplitude (rel. to wt)	EC ₅₀ , mW/mm ²	λ _{max} , nm	τ _{on} , ms*	τ _{off}	Source
ChR2-XXL	~19–60× [†]	0.003	480	5	76 s	This study (1)
ChR2-wt	—	0.7	470	0.2	10 ms	
Slow mutants						
C128T	Similar	0.03	480	5.7	2 s	Refs. 2 and 3 and this study
C128A	~0.3–1.6×	0.01	480	7.9	39–52 s	
C128S	~0.3×	0.01	480	13	28–106 s	
D156A	~1–2×	0.01	480	5	>150 s	
ChR2-T159C (TC)	~2–10×	Similar to wt	470* [‡]	~2× slower than wt [§]	20 ms	(4, 5)
ChETA (E123T; ET)	Similar [‡]	Less sensitive than wt [¶]	500	~2.5× faster than wt [§]	5 ms	(6)
ChR2-ET/TC	~2.5×	Similar to wt [¶]	505* [‡]	Similar to wt [§]	8 ms	(4)
CatCh	~3×	0.7	474	0.6	16 ms	(7)
ChR2-H134R (HR)	~3×	Similar to wt	450 –470* [‡]	Similar to wt [§]	15 ms	(4, 8, 9)
ChR1–2 chimera						
ChEF	~4×	~2× more sensitive than wt	490	Similar to wt	~2× longer than wt	(9)
ChIEF	~3×	Similar to wt	450	Similar to wt	Similar to wt	(9)
Red-shifted ChRs						
ReaChR	>HR, similar to ChIEF [‡]	~10× more sensitive than wt [¶]	530** and 630	~30× longer than wt ^{††}	137 ms	(10)
Chrimson [‡]	<wt	Similar to wt [¶]	590	1.2× slower than wt [§]	Similar to wt	(11)

Values refer to stationary photocurrents in oocytes under respective experimental conditions unless indicated otherwise (7).

*Related to photocurrent peak.

[†]Depending on retinal concentration.

[‡]Measurements from neurons.

[§]Flash-to-peak current time.

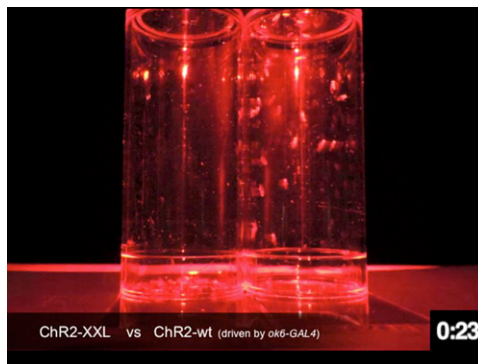
[¶]Approximation based on τ_{off}.

^{||}Measurements from HEK293 cells.

**Primary and secondary steady-state spectral peaks; 530 nm delivers a stronger response in flies (Fig. 4A) (12) and in cultured neurons (11).

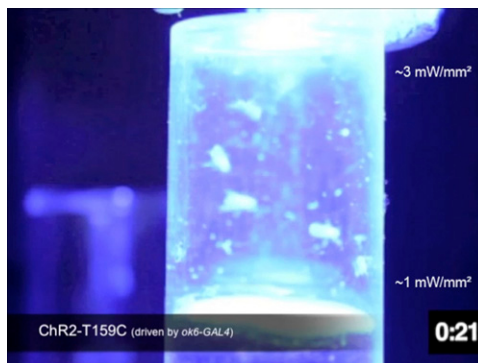
^{††}Approximation based on comparison with ChR2-wt in ref. 9.

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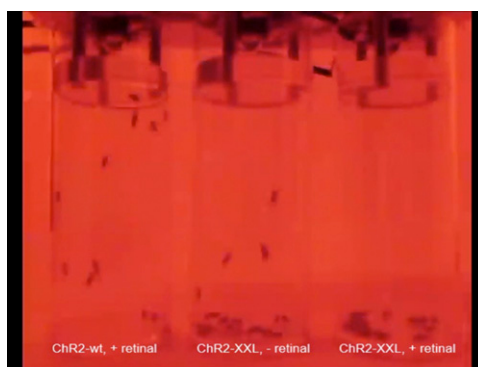
Movie S1. Light-induced stimulation of motor neurons in adult flies with ChR2-XXL. Activation of ChR2-XXL in motor neurons (driven by *ok6-GAL4*, plus retinal; *Left*) with white light LEDs ($2.5 \mu\text{W}/\text{mm}^2$ at 460 nm) induced reversible immobilization of adult flies. In contrast, flies expressing ChR2-wt (plus retinal; *Right*) displayed no response.

[Movie S1](#)



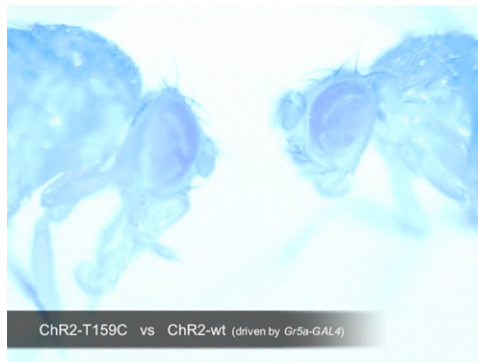
Movie S2. Inefficiency of ChR2-T159C in motor neurons of adult flies. Even with high light intensities ($\sim 3 \text{ mW}/\text{mm}^2$ at the top and $\sim 1 \text{ mW}/\text{mm}^2$ at the bottom of the vial), flies expressing ChR2-T159C in motor neurons (driven by *ok6-GAL4*, plus retinal) displayed no discernible response to photostimulation.

[Movie S2](#)



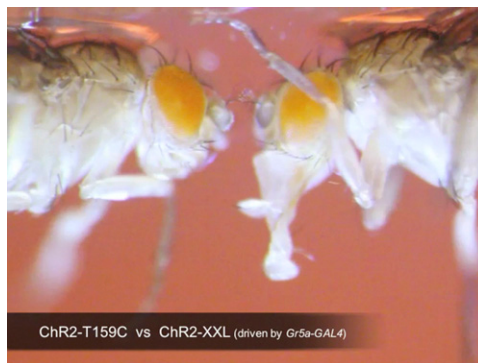
Movie S3. Accelerated recovery from photostimulation when retinal is not supplemented. The video is displayed at low resolution and sped up ten times. It shows adult flies expressing ChR2 variants in motor neurons (driven by *ok6-GAL4*). (*Left* vial) ChR2-wild type, plus retinal; (*Center*) ChR2-XXL, no retinal supplementation; (*Right*) ChR2-XXL, plus retinal. A weak blue light pulse of $\sim 2 \text{ s}$ ($4 \mu\text{W}/\text{mm}^2$) elicits no clear response from flies whereas $\sim 5\text{-s}$ -long light application has a drastic effect on ChR2-XXL-expressing flies, which are reversibly immobilized. Flies without retinal supplementation recover more rapidly, and even increasing irradiance duration and intensity (about 40-fold; $\sim 140 \mu\text{W}/\text{mm}^2$) fails to immobilize flies via ChR-wt.

[Movie S3](#)



Movie S4. Photostimulation of the PER with ChR2-T159C. Light from a mercury lamp was passed through a GFP excitation band-pass filter (~ 1 s, 0.2 mW/mm² at 460 nm) to activate ChR2-T159C (driven by *Gr5a-GAL4*; plus retinal; *Left*). For comparison, a fly expressing ChR2-wt (plus retinal; *Right*) shows no response.

[Movie S4](#)



Movie S5. Photostimulation of the PER with ChR2-XXL. White light LEDs (~ 1 s, 2 μ W/mm² at 460 nm) activated ChR2-XXL (driven by *Gr5a-GAL4*; plus retinal; *Right*). In contrast, a fly expressing ChR2-T159C (plus retinal; *Left*) shows no response.

[Movie S5](#)



Movie S6. Light-triggered courtship behaviors. Example of a male fly expressing ChR2-XXL in *fru* neurons (plus retinal). A mirror was used to inspect the fly from different angles. Examples of behavioral modules are indicated when they appeared for the first time. An ~ 2 -s light pulse (20 μ W/mm² at 460 nm) seemed to evoke a reversed courtship ritual, beginning with forceful abdomen bending and ending with unilateral wing extension.

[Movie S6](#)

