Systems/Circuits

Optogenetic Control of Fly Optomotor Responses

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When confronted with a large-field stimulus rotating around the vertical body axis, flies display a following behavior called "optomotor response." As neural control elements, the large tangential horizontal system (HS) cells of the lobula plate have been prime candidates for long. Here, we applied optogenetic stimulation of HS cells to evaluate their behavioral role in *Drosophila*. To minimize interference of the optical activation of channelrhodopsin-2 with the visual perception of the flies, we used a bistable variant called ChR2-C128S. By applying pulses of blue and yellow light, we first demonstrate electrophysiologically that lobula plate tangential cells can be activated and deactivated repeatedly with no evident change in depolarization strength over trials. We next show that selective optogenetic activation of HS cells elicits robust yaw head movements and yaw turning responses in fixed and tethered flying flies, respectively.

Introduction

To safely navigate through space particularly fast-moving animals face the challenging need of constantly integrating changing information about the environment and self-motion. Flies are capable of impressively robust, precise, and fast flight maneuvers that largely depend on visual and mechanosensory information (Sherman and Dickinson, 2004; Budick et al., 2007).

Depending on the species, a bilateral set of 20–60 large neurons, termed lobula plate tangential cells (LPTCs), located in the posterior part of the optic lobes, are considered critical for vision-based estimation of self-motion. LPTCs are individually identifiable and have large receptive fields, sometimes covering more than one hemisphere of visual space (Hausen, 1982; Krapp and Hengstenberg, 1996; Krapp et al., 1998; Schnell et al., 2010). Their most distinguishing characteristic is their directional tuning to visual wide-field motion, termed optic flow (Borst et al., 2010).

Among the LPTCs, three horizontal system (HS) cells sensitive to horizontal wide-field motion, such as occurring during rotation about the vertical body axis, have long been prime candidates to control compensatory yaw head movements and body turns of the fly (Bishop and Keehn, 1967; Dvorak et al., 1975). This suggestive notion rests on the following observations: (1) Mutant fruit flies in which LPTCs are missing or defective show a strong reduction in their optomotor response (Heisenberg et al., 1978). (2) Cutting HS cell axons in flies (Hausen and Wehrhahn, 1983) or laser ablation (Geiger and Nässel, 1981) of HS/vertical system precursor cells in larvae significantly affects the optomotor response of adult flies. (3) Extracellular electrical stimulation of the lobula plate region where HS cells are located elicits yaw turning responses (Blondeau, 1981). While all of these findings support the idea that HS cells control optomotor responses, it has not been conclusively shown that activation of HS cells is sufficient to evoke yaw optomotor behavior.

We analyzed the role of HS cells for head movements and flight-turning responses by stimulating them optogenetically in *Drosophila* (Borst, 2009). When a fly is visually stimulated by a pattern rotating around the vertical axis, its HS cells depolarize on the side where motion progresses from front to back (Schnell et al., 2010), and the fly displays syndirectional head movements and flight turns (Duistermars et al., 2012). If HS cells control both these optomotor responses, unilateral optogenetic depolarization of HS cells alone should mimic these behaviors. To avoid behavioral artifacts due to activation of photoreceptors during optical stimulation of HS cells, we generated flies expressing the bistable channelrhodopsin-2 variant ChR2-C128S, a depolarizing cation channel that can be switched between an open and closed configuration with brief light pulses of different wavelengths (Berndt et al., 2009). Expressing ChR2-C128S in HS cells via a selective driver line enabled us to control the cells in a similar way as visual motion does. When we activated HS cells in one brain hemisphere, flies turned their heads toward the stimulated side. Likewise, tethered flying flies displayed unilateral wing beat changes indicative of flight-turning responses in the same direction (Götz, 1987). Our results strongly suggest that HS cell activity alone is sufficient to elicit yaw head movements and flight-turning responses in *Drosophila*.

Materials and Methods

Fly strains and genetics. The Gal4 driver line *R27B03-Gal4* (Pfeiffer et al., 2008; Chiappe et al., 2010) was used to genetically target all three HS cells. Additionally, *norpA*^{7} flies with dysfunctional phototransduction were used as a control (Hotta and Benzer, 1970). To generate *ChR2-C128S* flies, *ChR2-C128S-EYFP* DNA (kindly provided by K. Deisseroth, Stanford University, Standford, CA) was first amplified via PCR with additional EcoRI restriction sites and cloned into pUAST. For our experiments, we used a transgenic line with *ChR2-C128S-EYFP* on the

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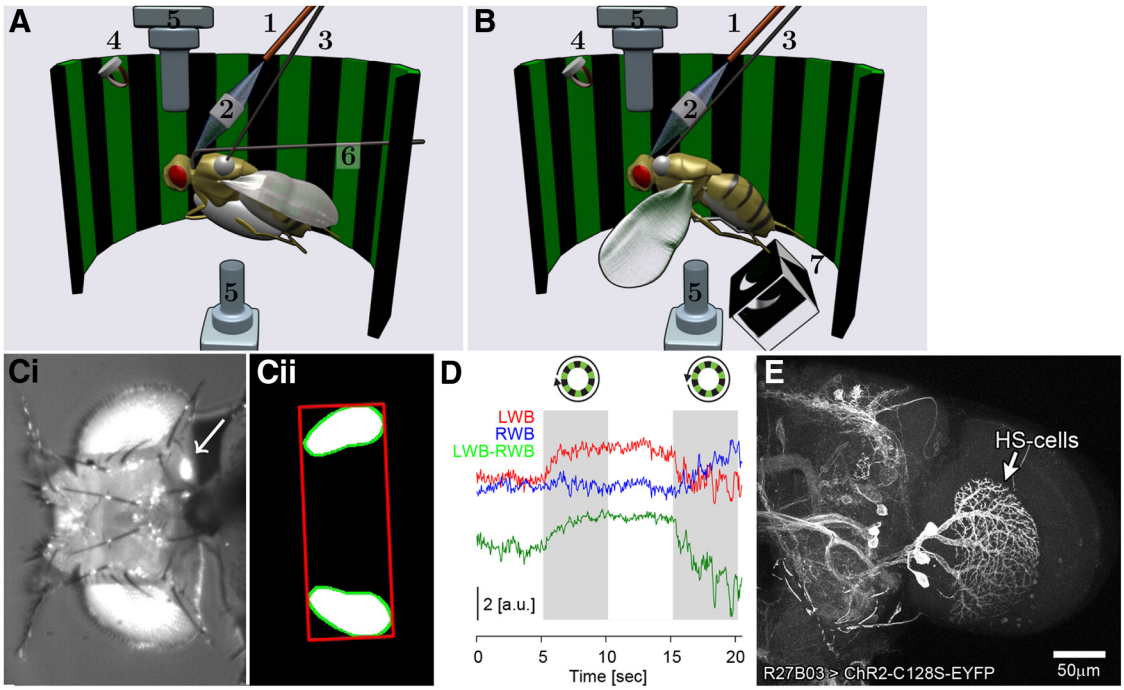

Figure 1. Schematic of the experimental approach and analysis procedures. ***A***, Setup used to analyze head movement. ***B***, Apparatus for tethered flight experiments. 1, Thin light fiber; 2, matched achromatic doublet pair; 3, tether; 4, infrared LED; 5, camera; 6, head positioner; 7, wing beat analyzer consisting of a mask, two prisms (white), and two photodetectors (not shown). ***Ci***, Head movement analysis from raw images of fly head. The light spot for unilateral optogenetic stimulation is indicated with an arrow. ***Cii***, A box is fitted around the contours (green) of the thresholded image shown in ***Ci***. ***D***, Example wing beat trace for clockwise and counter-clockwise visual rotation (stimulus time interval indicated by gray boxes). The LWB is in red, the RWB is in blue, and the LWB–RWB is in green. ***E***, ChR2–C128S-EYFP expression using *R27B03-Gal4*. The micrograph shows a collapsed confocal image stack of one brain hemisphere (posterior view). In the optic lobe, expression is largely confined to the three HS cells: HSN (top), HSE (middle), and HSS (bottom).

third chromosome. *R27B03-Gal4* and *ChR2-C128S-EYFP* flies were crossed into a CantonS wild-type background. To maximize the expression of ChR2-C128S, we used flies that were homozygous for *Gal4* and *ChR2-C128S* in all experiments, except for tethered flying $norpA^{7}$ flies. Flies were raised at 25°C and 60% humidity on standard cornmeal agar medium with a 12 h light/dark cycle.

Confocal imaging. To verify expression, we dissected brains from female flies (1–2 d after eclosion) homozygous for *R27B03-Gal4* and *UAS-ChR2-C128S-EYFP* (as used in behavioral experiments) in PBS, pH 7.4 (280 mOsm/kg). Brains were fixed at RT for 30 min in PBS/4% paraformaldehyde and an additional 10 min in PBS/4% paraformaldehyde/0.1% Triton X-100. After three and two washing steps in PBS/0.5% Triton X-100 and PBS, respectively, brains were mounted (Ibidi mounting medium) and optically sectioned from posterior to anterior with a Leica SP5 confocal laser scanning microscope using 514 nm excitation and a step size of 1 μm. For documentation, stacks encompassing entire optic lobes were collapsed in ImageJ 1.46r (NIH).

Electrophysiology. For our electrophysiological experiments, freshly eclosed female flies were kept at 25°C and fed (unless stated otherwise) for 1 d with yeast paste containing 1 mM all-*trans*-retinal (ATR; R2500; Sigma-Aldrich). Preparation and recording protocols were modified from the studies by Joesch et al. (2008) and Maimon et al. (2010). Flies were anesthetized on ice and attached to a Plexiglas holder with the head bent down using melted beeswax. The holder was placed underneath a recording chamber with a magnet so that the back of the fly's head was accessible through a 1 mm slit in the bottom of the chamber consisting of thin foil. The head was gently attached to the slit edges on one side with melted beeswax. In external solution, a window was cut into the head capsule on the other side with a hypodermic needle and muscle/fat tissue removed with forceps. Further dissection and recordings were performed under a Zeiss Axiotech Vario microscope equipped with polarized light contrast and epifluorescence. Under polarized light contrast using a long-pass optical filter (550 nm cutoff) to avoid chronic stimulation of ChR2-C128S-EYFP-expressing cells, the glial sheath was digested locally, and LPTC somata were exposed by applying a stream of 0.5 mg/ml collagenase IV (Invitrogen) through a cleaning micropipette (~5 μm opening). For whole-cell recordings, ChR2-C128S-EYFP-expressing HS cells were identified by epifluorescence with 472/30 nm light illumination kept at a minimum intensity and duration (~0.5–1 mW/mm^2 for 5–10 s total). Whole-cell recordings were established with patch electrodes of 5–8 MΩ resistance, and data were acquired using a BA-1S bridge amplifier (NPI Electronics) low-pass filtered at 3 kHz, and digitized at 10 kHz via a analog-to-digital converter (PCI-DAS6025; Measurement Computing) with Matlab (R2010b; MathWorks). External solution, carboxygenated (95% O_2/5% CO_2) and constantly perfused over the preparation (2 ml/min), contained the following (in mM): 103 NaCl, 3 KCl, 5 2-[(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)amino]ethanesulfonic acid 10 trehalose, 10 glucose, 7 sucrose, 26 $NaHCO_3$, 1 NaH_2PO_4, 1.5 $CaCl_2$, and 4 $MgCl_2$, pH 7.35, 290 mOsm/kg. Internal solution, adjusted to pH 7.26 with 1N KOH, contained the following (in mM): 140 K-aspartate, 10 HEPES, 4 Mg-ATP, 0.5 Na-GTP, 1 EGTA, and 1 KCl, 265 mOsm/kg.

Preparation for behavioral assays. For behavioral experiments, female flies were selected 1–3 d after eclosion and fed (unless stated otherwise) with a yeast–water mix containing 1 mM ATR kept for 2 d in a dark vial. Flies were cooled down at 5°C and prepared under red light. For the automatic detection of head movements, a 130 μm × 76 mm tungsten wire (TW5-3; Science Products), reinforced with two hypodermic stainless steel tubings (843600 and 832000; Science Products) was waxed to the thorax. Legs and wings were waxed to the abdomen (Fig. 1*A*). For tethered flight, the wire was positioned between head and thorax and waxed to the thorax. A drop of nail polish on the tip of the wire prevented head movements during flight (Fig. 1*B*).

Visual stimulation. We used an arena engineered and modified based on open-source information from the Dickinson laboratory (Reiser and Dickinson, 2008). Dot matrix displays (29 × 8; TA08-81GWA, Kingbright), each (2 × 2 cm) having 8 × 8 individual green (568 nm) light-emitting diodes (LEDs) were arranged in a cylindrical form. The cylinder had a diameter of 19 cm and covered 348° visual space in azimuth (from −174 to 174°) and 80° in elevation (from −40 to 40°). The spatial reso-

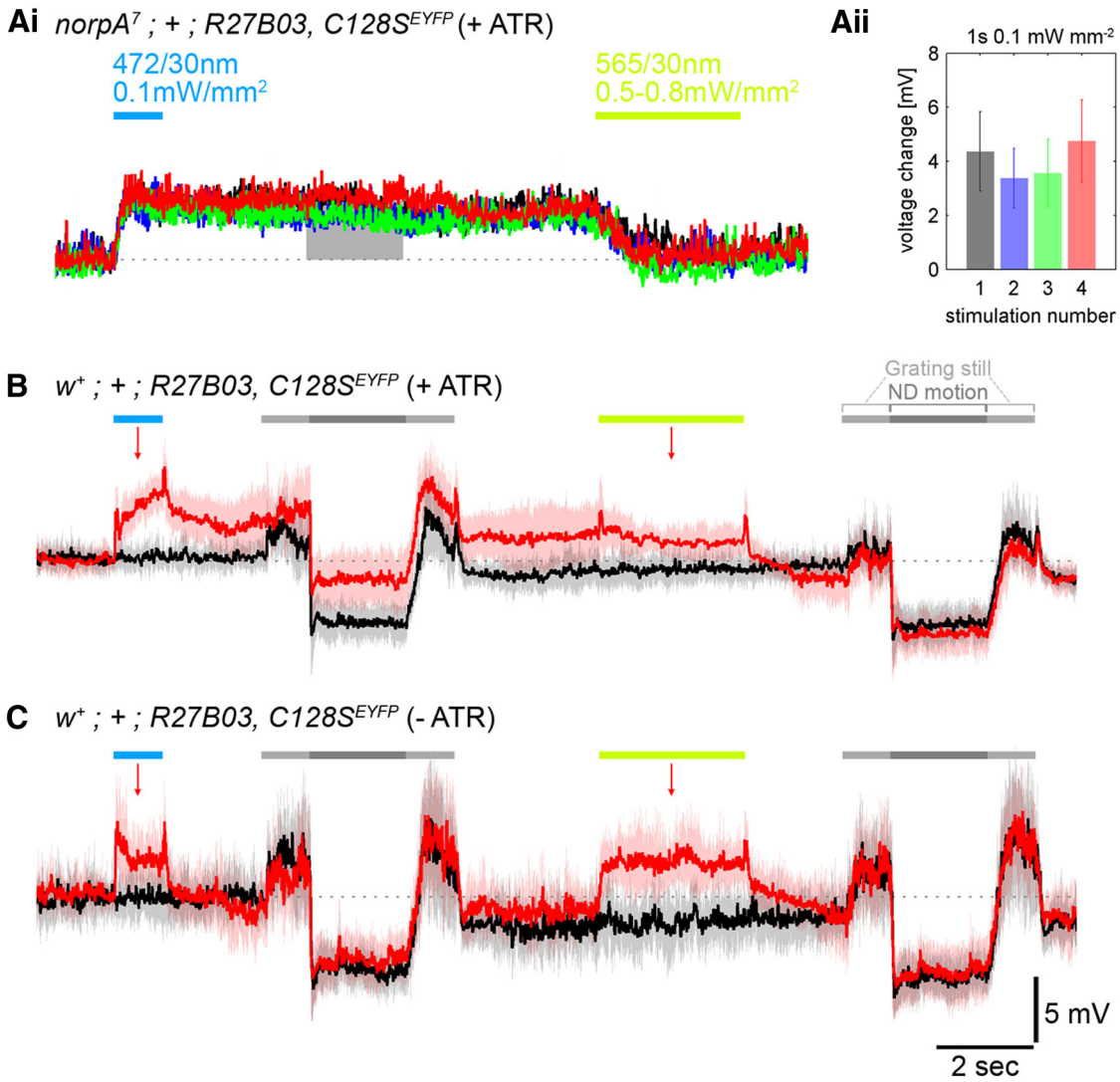

Figure 2. Electrophysiological recordings from HS cells expressing ChR2–C128S. ***Ai***, Four consecutive recordings (separated by ~2 min; each trace represents an average from 5 cells) of ChR2–C128S-expressing HS cells in blind *norpA*^{7} mutant flies. One second, 472 nm (30 nm bandpass) light elicits a prolonged depolarization that is terminated 9 s later by 3 s, 565/30 nm light. Amplitude and dynamics of the membrane potential changes remain comparable across trials. ***Aii***, Mean membrane potential changes ± SEM of the traces shown in ***Ai*** calculated from the integrated responses 3 to 5 s after stimulus offset (indicated by shaded area in ***Ai***) relative to the baseline (2 to 0 s before stimulus onset). ***B***, Averaged responses (4 cells with four traces each per condition) of combined optogenetic and visual HS cell stimulation in visually intact flies (red trace). Blue light pulses evoke transient visual and prolonged optogenetic depolarizing responses that are terminated by 3 s yellow–green light pulses. Presenting vertical still gratings to the fly (light gray bars) leads to depolarizing flicker responses, while gratings moving back to front (3 to 5 s after blue/yellow–green light offset; dark gray bars) leads to robust hyperpolarization in all conditions. Hence, HS cell photoactivation does not notably interfere with visual motion processing. The black trace shows the responses of the same cells to visual stimulation only. ***C***, Same experiments as in ***B*** performed on control flies raised without ATR. Blue and yellow–green light pulses lead to transient visual responses, but not prolonged depolarizing voltage changes as in the +ATR condition. Blue bars in all panels indicate optical stimulation with 1 s 472/30 nm light at ~0.1 mW/mm^{2}. Yellow–green bars indicate stimulation with 3 s 565/30 nm light at ~0.5–0.8 mW/mm^{2}. Light and dark gray bars in ***B*** and ***C*** indicate visual stimulation with still and moving square wave gratings in the HS cell null direction, respectively. Shaded areas (omitted in ***Ai*** and ***Aii*** for clarity) represent the SD.

lution at the equator amounted to 1.5°, thus going far beyond the interommatidial angle of ~5° (Götz, 1965). An arena controller controlled 232 microcontrollers (Atmega-168; Atmel). Combined with a line driver (ULN2804; Toshiba America) acting as a current sink, each of those microcontrollers was in charge of one dot matrix, allowing refresh rates of 550 Hz and 16 intensity levels. Visual stimuli consisted of square wave gratings (10–80 cd/m^{2}) with a wavelength of 24° and temporal frequency of 1.2 Hz. During optogenetic stimulation, the visual stimulation system was turned off.

Optogenetic stimulation. During electrophysiological recordings, light pulses for optogenetic stimulation were delivered via the epifluorescence light path of the microscope through a 40×/0.8 NA water-immersion objective (LUMPlan FI; Olympus). As a light source, a Lambda DG-4 wavelength switcher (Sutter Instrument) with a 300 mW xenon arc lamp was connected to the illumination port of the microscope via a liquid light guide. Attenuating the output of the DG-4 was achieved by offsetting the output galvanometer. The output for each setting was measured with a light meter under the 40× objective in air. Taking into account the field of illumination under water immersion, the light intensity per area on the specimen was estimated, as given in the results section and Figure 2.

For behavioral experiments, optogenetic light stimulation was accomplished by a monochromator (Polychrome IV; TILL Photonics) equipped with a glass fiber. Wavelength and duration of light pulses were controlled using a microcontroller (Atmega-128; Atmel; monochromator controller) and a digital-to-analog converter (LTC1592ACG; Linear Technology) with a ±10 V output. To synchronize optogenetic light stimulation with the visual stimuli of the arena, we controlled the monochromator controller with the arena controller. The light from the monochromator glass fiber was passed through a shutter, controlled

with a single-channel shutter driver (VCM-D1; Uniblitz) and the monochromator controller. Using a matched achromatic doublet pair (MAP051919-A; Thorlabs), the light was guided into a second fiber with a 50 μm core (M14L01; Thorlabs). Another matched achromatic doublet pair (MAP051919-A; Thorlabs) was positioned between the head and a 50 μm fiber to focus the outcoming light of the thin optical fiber onto the fly's head (resulting light spot is indicated in Fig. 1*Ci*, arrow). To align lenses and optic fibers, we maximized the light intensity measured with a power and energy meter console (PM100D; Thorlabs). Light intensities at the head for blue and yellow light were 10 and 6 μW, respectively. Spectral wavelengths were measured with a compact spectrometer (CCS200; Thorlabs).

Head movement detection. Flies were positioned in the center of the arena using a camera (Dragonfly; PointGrey) with an InfiniStix (1.0× primary magnification, 94 mm working distance) video lens (NT55-359; Edmund Optics) positioned beneath the fly (Fig. 1*A*). The fly's head was illuminated with an infrared 870 nm LED (JET-800-10; Roithner Lasertechink). Using another camera (Firefly MV; PointGrey) with an InfiniStix (2.0× primary magnification, 44 mm working distance) video lens (NT55-355; Edmund Optics), images of the head were taken at 27.8 frames per second. The head was pushed slightly forward using a 130 μm × 76 mm tungsten wire (TW5-3; Science Products) with one end bent to an L shape. The camera was configured to use an external trigger supplied by the arena controller, to output a strobe signal and to use embedded image timestamps, which served to detect whether images were lost during trials. Together with the strobe of the camera images, direction of the visual stimuli and optogenetic stimulation could be aligned. Each trial was analyzed off-line using OpenCV (Open Source Computer Vision) and Python software. To automatically measure the head angle, we divided the image in half, each containing one eye, and applied a threshold to these. After thresholding, the eyes of the fly appeared white and were identified as the two biggest white areas of the thresholded image. Contours were taken of these, and a box was fitted around them, where the angle of the box corresponded to the angle of the head (Fig. 1*Cii*). During photoactivation of ChR2-C128S, we removed the light spot from the image using a mask, calculated as the difference of the first image with a light spot and the previous image.

Wing beat measurements. A "wing beat analyzer" (Götz, 1987) was added to the head detection setup for tethered flight experiments (Fig. 1*B*). Using the infrared light source, the shadow of the wings was casted onto a mask with two crescent-shaped apertures. Beneath this mask, two prisms (BRP-25.4; Newport) were placed and reflected the light onto two photodetectors (UDT-555D; Osi Optoelectronics). Depending on the wing beat amplitude, different portions of light were blocked by the wings, turning the measured photocurrent at each detector into a voltage signal inversely proportional to the wing beat amplitude. To obtain the wing beat angles, we normalized these signals to their maximum. After detecting the peak of each wing stroke, the mean over every 0.05 s was calculated. By subtracting the values of the right (RWB) from the left wing beat (LWB) analyzer, we obtained a value largely proportional to yaw torque and indicative of the turning behavior of the fly (Götz, 1968; Götz et al., 1979; Fig. 1*D*).

Data analysis. Total numbers of flies (N) and trials (n) used for analyses are given below for each behavioral assay and genotype in parentheses: head-turning assay, w^+;+;*R27B03-C128S* ($N = 44$, $n = 116$), w^+;+;*R27B03-C128S* no ATR ($N = 34$, $n = 102$), w^+;+;*R27B03* ($N = 34$, $n = 100$), *norpA*^{7};+;*R27B03-C128S* ($N = 15$, $n = 33$), *norpA*^{7};+;*R27B03-C128S* no ATR ($N = 19$, $n = 65$), *norpA*^{7};+;*R27B03* ($N = 15$, $n = 47$); tethered flight assay, w^+;+;*R27B03-C128S* ($N = 46$, $n = 235$), w^+;+;*R27B03-C128S* no ATR ($N = 33$, $n = 165$), w^+;+;*R27B03* ($N = 35$, $n = 200$), *norpA*^{7};+;*R27B03-C128S/TM6b* ($N = 29$, $n = 94$), *norpA*^{7};+;*R27B03-C128S/TM6b* no ATR ($N = 31$, $n = 84$), *norpA*^{7};+;*R27B03/TM6b* ($N = 29$, $n = 101$). Trials obtained from single flies were averaged, and the mean was used as the fly's optomotor behavior. The response to wide-field pattern rotation was calculated as the mean turning behavior during rotation minus the mean turning behavior during the last second before rotation onset. Responses for blue light activation were defined as the mean signal (head angle or turning behavior) between the onset of blue light and the onset of yellow light, minus the mean of the signal during the last second before blue light onset. All data are given as means ± SEM. The significances of differences between data sets are given as p values of a two sided Mann–Whitney rank test.

Results

Switchable optogenetic depolarization of HS cells using ChR2-C128S

Optogenetic stimulation has become the method of choice to specifically activate genetically defined neuron types to study their roles in behavior. However, applying optical stimulation in the *Drosophila* visual system will also activate photoreceptors and hence produce undesired artifacts interfering with visual processing. To overcome this problem, we explored the possibility of using the bi-stable ChR2 variant C128S (Berndt et al., 2009) with minute-scale deactivation kinetics. ChR2-C128S channel opening and closure can be gated by brief blue and yellow light pulses, respectively, and leaves neurons in a depolarized state in between.

We generated a transgenic fly strain carrying *ChR2-C128S-EYFP* under control of *upstream activating sequences* (*UAS*) and performed patch-clamp recordings from HS cells expressing this construct using *R27B03-Gal4* (Fig. 1*E*). To avoid potential network artifacts from stimulating photoreceptors, we first conducted experiments in *norpA* mutant flies with dysfunctional phototransduction, which do not show any visual responses at the level of LPTCs. Stimulation of HS cells expressing ChR2-C128S with 1 s 472 nm (30 nm bandpass) light pulses (0.1 mW/mm^2) led to depolarizations of 4.4 mV ± 1.5 on average, as measured 3 to 5 s after the offset of the optogenetic stimulus ($n = 5$; Fig. 2*Ai*,*Aii*). This value is well in the range of visually evoked motion responses (Schnell et al., 2010). One concern was a possible photocurrent loss over consecutive stimulations as has been published for hippocampal pyramidal cells expressing ChR2-C128S and related ChR2 variants (Schoenenberger et al., 2009). However, our results show that in the *Drosophila* visual system, ChR2-C128S-mediated membrane depolarizations do not significantly attenuate. This was shown for at least four consecutive blue light pulses, which were interleaved by longer-wavelength light flashes after 9 s to terminate the conducting state of the channel (Fig. 2*Ai*,*Aii*).

Next, we aimed to establish whether ChR2-C128S-mediated HS cell depolarization can be combined with simultaneous probing of visual responses. To this end, we repeated the same optogenetic stimulus protocol with visually intact flies and additionally presented square wave gratings moving in the HS cells' null direction (ND; back to front) over the ipsilateral eye (Fig. 2*B*). As in blind flies, blue light exposure elevated the membrane potential of HS cells persisting until channel closure was gated with longer-wavelength light. ND motion stimulation 3 s after optogenetically gated depolarization and repolarization (Fig. 2*B*, red trace) hyperpolarized HS cells by amplitudes comparable to trials where optogenetic stimulation was omitted (Fig. 2*B*, black trace). In contrast, control flies raised without the necessary ChR2 cofactor ATR did not show prolonged depolarizations in response to blue light while displaying normal visually evoked activity (Fig. 2*C*).

Together, ChR2-C128S permits the prolonged and repetitive activation of HS cells with blue light pulses and simultaneous probing of visual responses.

Unilateral HS cell activation elicits yaw head movements

Having confirmed that application of short light pulses to LPTCs expressing ChR2-C128S leads to a prolonged depolarization of the cells, we next aimed to test the effect of this depolarization on head movements. In blow flies, a possible optomotor pathway has been described previously (Strausfeld et al., 1987; Huston and Krapp, 2008; Haag et al., 2010): three muscles controlling yaw

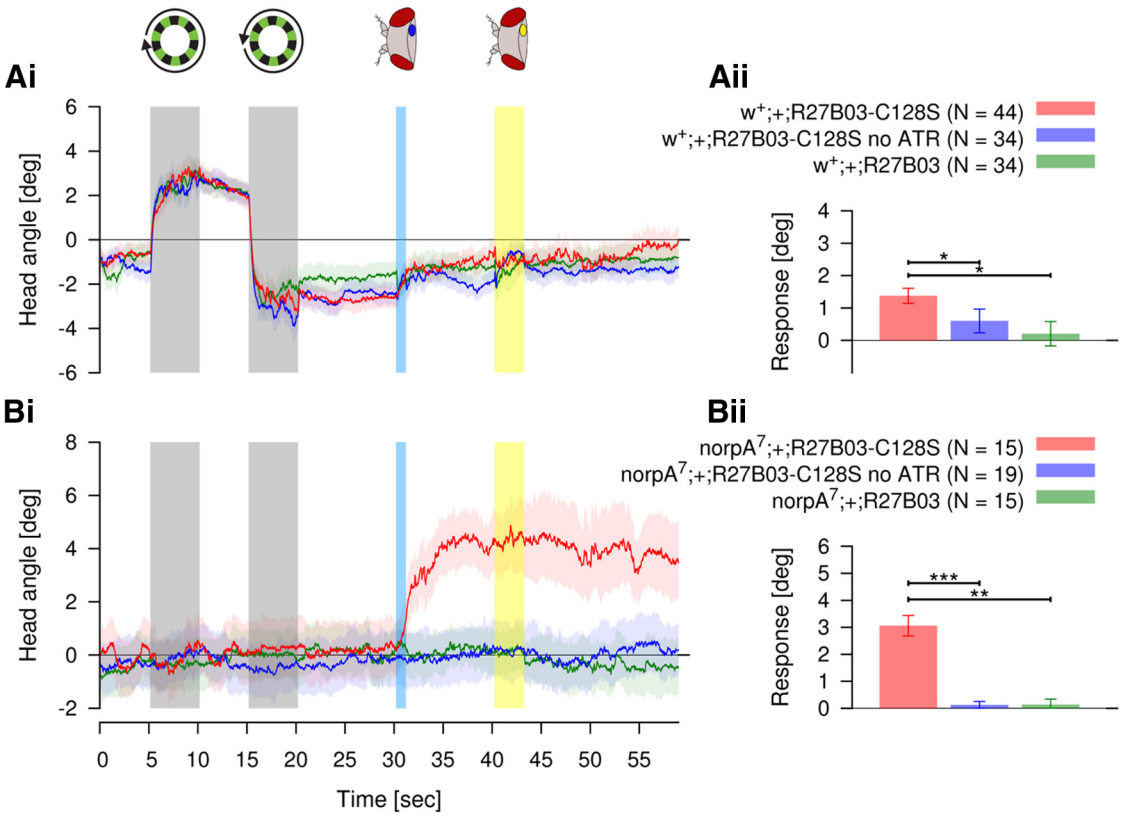

Figure 3. Head movement analysis. ***Ai***, ***Bi***, Mean traces ± SEM. Gray boxes indicate the time of visual stimulation, and blue and yellow boxes show timing of optogenetic light stimulation. ***Aii***, ***Bii***, Mean ± SEM response to the blue light pulses for *R27B03-Gal4* (***Aii***) and *norpA7;+;R27B03-Gal4* (***Bii***) experimental and control flies. Experimental flies expressed ChR2–C128S and were fed with ATR (red traces). Control flies had either the same genotype, but were not fed ATR (blue traces) or did not carry the ChR2–C128S transgene (green trace). *$p < 0.01$; **$p < 0.001$; ***$p < 0.0001$.

head movements receive input from the ventral cervical nerve, containing the axons of three motor neurons, which in turn receive visual input from two of the three HS cells [equatorial and northern HS cells (HSE and HSN)].

We used the Gal4 driver line R27B03 to target ChR2-C128S-YFP to all three HS cells in the optic lobes (Fig. 1*E*). Confocal image stacks reveal robust expression in the three HS cells in a slightly graded fashion from dorsal to ventral, with strongest signals in HSN. It should be noted that in homozygous flies ChR2-C128S expression can also be detected in central brain neurons. Optic lobes, however, were largely devoid of unspecific expression other than occasional faint activity in few lobula and medulla cells. Since we were able to restrict illumination to the optic lobes predominantly exposing posteriorly located strongly expressing HS cells to the stimulus, we are confident that we largely excluded unidentified neurons from optogenetic stimulation. In the beginning of each trial, flies were visually stimulated by a square wave pattern rotating for 5 s first in the clockwise and then in the counterclockwise direction. On average, flies responded to these stimuli by head movements with amplitudes of about 3° in the direction of the pattern motion (Fig. 3*Ai*, left). After the clockwise rotation had stopped, the flies' heads remained in the position until the counterclockwise rotation started. When we depolarized HS cells in the right hemisphere of experimental flies (*R27B03-C128S* fed with ATR) by means of a 1 s pulse of blue light, flies on average showed a subtle head movement in the clockwise direction starting at the onset of blue light (Fig. 3*Ai*, right, red trace). In contrast, although control flies (same genotype and no ATR fed or Gal4 driver only) displayed some behavioral fluctuations in response to visually perceived blue light, these did not lead to a consistent change in yaw head angle (Fig. 3*Ai*, right, blue and green traces). Quantifying these responses as the difference between the mean head angle between blue light and yellow light onsets and the mean head angle within the last second before blue light onset (see Materials and Methods), we found that the responses of the experimental flies were significantly different compared to both groups of control flies (Fig. 3*Aii*).

To further investigate the role of HS cells in head-turning responses, we used mutant flies defective in phototransduction (*norpA*^{7}) and combined them with the R27B03 driver line to express ChR2-C128S in HS cells. As expected, we did not observe any responses to visual stimulation (Fig. 3*Bi*, left). In contrast, a blue light pulse elicited strong head turning toward the illuminated side in experimental flies, whereas both groups of control flies did not show any consistent reaction (Fig. 3*Bi*, right). This response was again significantly different (Fig. 3*Bii*) from controls, and provides further support for the notion that activation of HS cells in one hemisphere leads to head movements toward the activated side. Longer-wavelength light to gate ChR2-C128S closure did not reestablish baseline head angles.

Depolarization of HS cells elicits yaw optomotor responses in tethered flying *Drosophila*

Previous studies on *Drosophila* and *Calliphora* suggest that HS cell output is not limited to the neck motor system, but serves a more general purpose to also control thoracic motor circuits, which for instance underlie flight steering maneuvers (Heisenberg et al., 1978; Blondeau, 1981; Geiger and Nässel, 1981; Hausen and Wehrhahn, 1983). To produce torque during flight, flies reduce their wing beat amplitude on the side they turn to

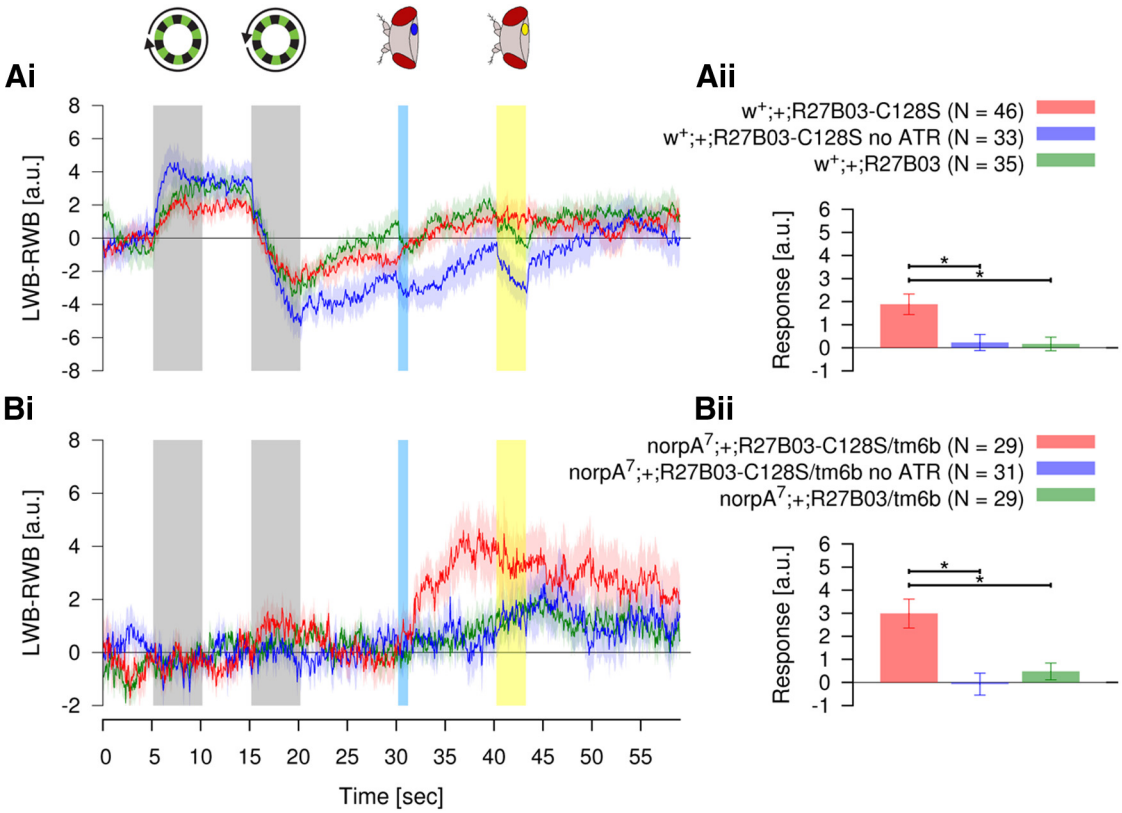

Figure 4. Wing beat analysis. ***Ai***, ***Bi***, Mean traces ± SEM. Gray boxes indicate the time of visual stimulation, blue and yellow boxes show timing of optogenetic light stimulation. ***Aii***, ***Bii***, Mean ± SEM for *R27B03-Gal4* (***Aii***) and *norpA*^{7}*;+;R27B03-Gal4* (***Bii***) experimental and control flies. Experimental flies expressed ChR2–C128S and were fed with ATR (red traces). Control flies had either the same genotype, but were not fed ATR (blue traces) or did not carry the ChR2–C128S transgene (green trace). *$p < 0.01$.

while enlarging it on the opposite side (Götz, 1968; Götz et al., 1979). HS cells are known to synapse onto descending neurons that connect to the motor centers in the thoracic ganglion (Gronenberg and Strausfeld, 1990), which in turn might impinge on muscles controlling the wing beat (Egelhaaf, 1989; Heide and Götz, 1996).

To test this, we used flies in a tethered flight assay of the same genotypes as above, i.e., expressing ChR2-C128S in HS cells using *R27B03-Gal4*, and the same stimulus protocol as for the analysis of head movements. We had the flies' heads fixed to the thoraxes and studied their flight-turning behavior with a wing beat analyzer using the difference between left and right wing beat amplitude as our measure (see Materials and Methods). Flies showed a reliable wing response to the visual stimuli that would lead to a body turn in the direction of visual motion (Fig. 4*Ai*, left). Following delivery of a blue light pulse to the right hemisphere, experimental flies showed consistent turning behavior toward the stimulated side (Fig. 4*Ai*, right, red trace). In contrast, both types of control flies had no consistent directional bias in their responses, but rather displayed behavioral fluctuation of both polarities (Fig. 4*Ai*, right). Comparing quantified turning behavior between experimental and control flies (difference of yaw torque between blue and yellow light onsets and during 1 s before blue light onset) revealed a significant difference (Fig. 4*Aii*).

As done previously for the study of head movements, we repeated the experiments using flies with defective phototransduction, i.e., a *norpA*^{7} background combined with the R27B03 driver. During clockwise and counterclockwise rotations of the visual panorama, no responses were detectable (Fig. 4*Bi*, left). In contrast, upon delivery of blue light pulses to one hemisphere, only those flies that expressed ChR2-C128S in HS cells and were fed with ATR showed a turning behavior toward the stimulated side (Fig. 4*Bi*, right, *Bii*). Both groups of control flies revealed no consistent unilateral response in their wing beat amplitudes (Fig. 4*Bi*, right, *Bii*).

Together, these findings strongly suggest that HS cells, in addition to head movements, also contribute to the flight-turning behavior of flies. As in the head-turning assay, gating ChR2-C128S inactivation with yellow light did not produce clear immediate changes in wing beat amplitude.

Discussion

Lobula plate tangential cells of flies have been an important research focus to explore how visual features are extracted by neural circuits and converted into appropriate motor outputs (Borst et al., 2010). In *Drosophila*, at least two of the three individually identifiable HS cells are assumed to mediate compensatory yaw movement because their activity profiles match optomotor responses to wide-field horizontal visual motion. Here, by optogenetic stimulation, we provide strong evidence that HS cell activity is sufficient to elicit optomotor behavior in *Drosophila*.

In recent years, optogenetic methods have been successfully applied in a number of studies, involving different species such as *Caenorhabditis elegans* (Nagel et al., 2005), zebrafish (Douglass et al., 2008; Arrenberg et al., 2010; Schoonheim et al., 2010), and mice (Arenkiel et al., 2007; Matyas et al., 2010). Nonetheless, its use in *Drosophila* (Zimmermann et al., 2009; Yaksi and Wilson, 2010) has been rather limited, in particular for analyzing visual circuits, perhaps in part because light delivery to activate light-sensitive ion channels inevitably leads to direct stimulation of the

photoreceptors (Fig. 2*B*,*C*, red traces) and thus interferes with the visual processing to be studied. To avoid this problem, optogenetic stimulation has been performed in blind flies (de Vries and Clandinin, 2012). This strategy, however, raises potential concerns as to whether the circuits are compromised by visual deprivation. Moreover, it precludes the simultaneous visual probing of the system. In this respect, the development of switchable, bistable channelrhodopsin-2 variants (Berndt et al., 2009) offers a powerful solution. As we have shown, one of these variants (ChR2-C128S) allows for prolonged and repeated excitation of large motion-sensitive neurons in the *Drosophila* visual system by delivery of pulses of light with the appropriate wavelength.

As a first application of this method, we aimed to revise the role of lobula plate HS cells. While previous work in *Drosophila* and *Calliphora* has already put forward the idea that HS cells control yaw optomotor behavior (Heisenberg et al., 1978; Blondeau, 1981; Geiger and Nässel, 1981; Hausen and Wehrhahn, 1983), well in agreement with their visual response properties (Hausen and Wehrhahn, 1989), only Hausen and Wehrhahn (1983) actually tested their requirement directly by lesioning HS cell axons unilaterally and recording the yaw torque response to visual motion. The resulting behavioral phenotype nicely supports the assumed role of HS cells. Nonetheless, Hausen and Wehrhahn (1983) acknowledged that axons of other lobula plate output neurons could have also been severed. Our complementary experiments indicate that unilateral optogenetic stimulation of the three HS cells is sufficient to evoke both yaw head movements as well as flight-turning responses. While light pulses were visually perceived by flies and led to transient physiological and behavioral artifacts, consistent long-lasting responses were only observed in animals expressing functional ChR2-C128S, in agreement with the bistable nature of this channel. The direction of the behavioral responses toward the stimulated side can be readily interpreted such that the flies attempt to counteract perceived unintended yaw body rotations. The weaker response levels in visually intact flies compared to actual visually evoked behavior and robust optogenetic responses in blind flies (compare Figs. 3*Ai*, 4*Ai*, right; 3*Ai*, 4*Ai*, left, *Bi*) could be explained by unspecific prolonged perturbances of visual circuits by blue light pulses that potentially attenuate optogenetic effects in visually intact flies. Alternatively, stationary visual signals from the inactive arena that provide stable reference points might antagonize the effect of HS cell activation. It should also be noted that visual stimuli were presented to both eyes, leading to hyperpolarization of HS cells in one hemisphere and depolarization of HS cells in the other hemisphere. In contrast, optogenetic stimulation of HS cells involved the cells within only one hemisphere. The information provided by unilateral HS cell activation is partly ambiguous and might signal yaw rotation, but also sideway or forward translation. In fact, HS cells have been shown to encode translating optic flow provided by both eyes during intersaccadic flight intervals, which may serve to extract depth information (Boeddeker et al., 2005; Kern et al., 2005; Karmeier et al., 2006). Furthermore, HS cells might be functionally specialized, and processing the outputs in various combinations could be used to recover different aspects of self-motion-induced optic flow. For instance, HSN and HSE with dorsal and equatorial receptive fields, respectively, receive contralateral input tuned to back-to-front motion (Hausen, 1982; Schnell et al., 2010), rendering them sensitive to sideward translation in addition to yaw rotation, particularly so when activity is bilaterally subtracted. In contrast, HSS lacks contralateral input, and summating HSS activity from both hemispheres in higher processing centers might be used to recover parameters associated with forward translation (Karmeier et al., 2006). Although we cannot resolve such functional specializations, the fact that unilateral stimulation of all three HS cells produces clear turning responses toward the stimulated side supports the notion that the HSE and HSN cells, and potentially the HSS cell, are important for controlling compensatory yaw rotations of head and body. Nonetheless, it is well known that HS cells are part of a larger network of tangential cells in the lobula plate (Haag and Borst, 2001, 2002, 2003). There is no doubt that under natural conditions, more dynamic and finer-grained activity patterns in HS and perhaps other neurons than we were able to induce optogenetically are required to reduce ambiguities related to horizontal optic flow and to guide flies safely through their environment. It is also interesting to note that neither cessation of moving patterns nor ChR2-C128S channel closure by longer-wavelength light, both leading to repolarization of HS cells, immediately reestablishes baseline head angle and wing beat amplitudes. This might indicate that these behavioral parameters require active stimulation along the opposite direction and the concomitant depolarization of the contralateral HS cells to return to baseline.

Our results contribute to an emerging picture of how visually guided behavioral patterns are generated in flies. Certain visual cues contained in the raw image sequence such as direction of motion are extracted by intricate parallel circuits of the optic lobes and integrated and conveyed toward appropriate motor circuits via a cohort of wide-field projection neurons, each of which is endowed with a unique visual tuning profile. As an analogous example to the HS system, FOMA-1 cells of the lobula complex are tuned to looming stimuli and consequently elicit escape responses when activated (de Vries and Clandinin, 2012). In blow flies, part of the pathway downstream of the HS cells has been identified for the neck motor system via a motor neuron of the ventral cervical nerve, constituting a straight-forward link between unilateral HS cell activation and neck muscle contraction (Strausfeld et al., 1987; Huston and Krapp, 2008; Haag et al., 2010). In contrast, much less is known about the circuits underlying turning behavior during flight (Borst et al., 2010). Unilateral change of wing beat amplitude is controlled by small steering muscles, some of which have been shown to respond to horizontal wide-field motion in a direction-selective way (Egelhaaf, 1989; Heide and Götz, 1996). Such muscles are therefore probable downstream targets of the HS system, but the neural elements that convey the visual information to them remain to be functionally characterized (Gronenberg and Strausfeld, 1990).

The fact that HS cell responses are remarkably robust while optomotor behavior can be quite variable as explicitly shown, for instance, for *Calliphora* (Rosner and Warzecha, 2011) illustrates that visual reflexes are not unimodal all-or-nothing events, but rather are subject to multimodal modification as well as to central gating. This might reflect the ethological need to conditionally modify or disable optomotor compensation by conflicting visual cues (Reiser and Dickinson, 2010), other sensory modalities (Hengstenberg, 1991; Chow and Frye, 2008; Huston and Krapp, 2009), or during certain internally generated flight maneuvers like saccades (Bender and Dickinson, 2006). Identification of further constituents of the optomotor pathways would provide opportunities to elucidate the neural basis for such multimodal stimulus integration, modulation, and gating of the optomotor response. To this end, flies expressing bistable channelrhodopsin-2 in combination with specific driver lines can help to establish the participation of candidate neurons. Using channelrhodopsin-2 variants with longer time constants than ChR2-C128S (Yizhar et al., 2011), the consequence of activating certain neurons could perhaps even be tested in freely behaving, unrestrained animals.

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