

A competitive disinhibitory network for robust optic flow processing in *Drosophila*

Received: 10 September 2023

Accepted: 14 March 2025

Published online: 01 May 2025



Mert Erginkaya^{1,2}, Tomás Cruz^{1,3}, Margarida Brotas^{1,4}, André Marques¹, Kathrin Steck⁵, Aljoscha Nern⁶, Filipa Torrão¹, Nélia Varela¹, Davi D. Bock⁷, Michael Reiser⁶ & M. Eugenia Chiappe¹✉

Many animals navigate using optic flow, detecting rotational image velocity differences between their eyes to adjust direction. Forward locomotion produces strong symmetric translational optic flow that can mask these differences, yet the brain efficiently extracts these binocular asymmetries for course control. In *Drosophila melanogaster*, monocular horizontal system neurons facilitate detection of binocular asymmetries and contribute to steering. To understand these functions, we reconstructed horizontal system cells' central network using electron microscopy datasets, revealing convergent visual inputs, a recurrent inhibitory middle layer and a divergent output layer projecting to the ventral nerve cord and deeper brain regions. Two-photon imaging, GABA receptor manipulations and modeling, showed that lateral disinhibition reduces the output's translational sensitivity while enhancing its rotational selectivity. Unilateral manipulations confirmed the role of interneurons and descending outputs in steering. These findings establish competitive disinhibition as a key circuit mechanism for detecting rotational motion during translation, supporting navigation in dynamic environments.

Many animals, including humans, rely on vision to navigate, which depends on a fine control of their line of sight (gaze) during locomotion to minimize motion blur^{1,2}. Walking, for example, produces complex and periodic patterns of head translation and rotation^{2,3}, and without gaze stabilization mechanisms, visual processing would vary throughout the gait cycle⁴. Additionally, when exploring their environment, mammals and insects employ a fixate-saccade strategy, maintaining or quickly shifting gaze orientation through coordinated eye and head rotations, complemented by body steering (course control), allowing the patterns of retinal flow to remain less sensitive to the biomechanics of the gait cycle^{1,2,5–7}. Thus, by determining the body orientation relative to the gaze direction, the brain can extract critical information from

optic flow to support multilevel control of navigation, from planning where to place the next steps², to controlling body posture and balance², to deciding whether to maintain or change course direction⁸.

The motor programs underlying gaze and course stability are regulated by sensory feedback (Fig. 1a)^{9–12}, including visual feedback, which must extract relevant information, such as the rotation of eyes, head and body during locomotion. While visual feedback from distant landmarks can support slow (over several steps)¹³ course correction, finer moment-by-moment control is also needed to prevent trajectory drift amplification¹⁴. Optic flow (the coherent patterns of image velocity across the retina caused by the observer's motion relative to the environment¹⁵) provides reliable self-motion information over

¹Champalimaud Neuroscience Programme, Champalimaud Centre for the Unknown, Lisbon, Portugal. ²Neurobiology and Genetics, Theodor-Boveri-Institute, Biocenter, Julius-Maximilians-University of Würzburg, Würzburg, Germany. ³Friedrich Miescher Institute for Biomedical Research, and Biozentrum, Department of Cell Biology, University of Basel, Basel, Switzerland. ⁴CEDOC, iNOVA4Health, NOVA Medical School, Universidade Nova de Lisboa, Lisbon, Portugal. ⁵Faculty of Science and Medicine, Department of Neuro and Movement Sciences, Université de Fribourg, Fribourg, Switzerland. ⁶Janelia Research Campus, Howard Hughes Medical Institute, Ashburn, VA, USA. ⁷University of Vermont Larner College of Medicine, Burlington, VT, USA. ✉e-mail: eugenia.chiappe@neuro.fchampalimaud.org

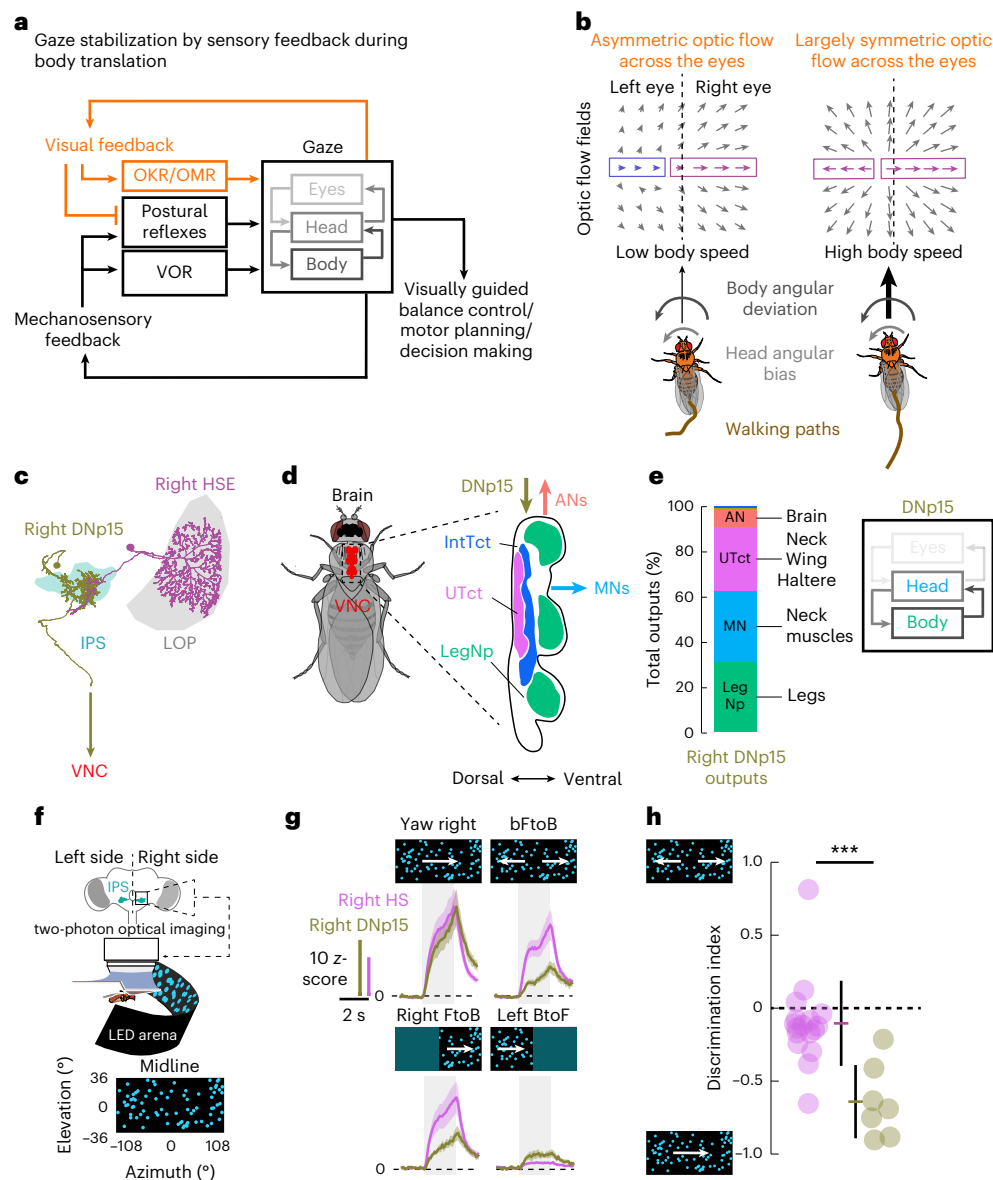


Fig. 1 | Examining optic flow processing for gaze stabilization during locomotion. **a**, Animals use sensory feedback, coordinating movements across the body to stabilize gaze. **b**, Simulated binocular optic flow in a fly walking at low (left) versus high (right) speed while drifting with the same angular velocity. Rectangles highlight back-to-front (BtoF, blue) or front-to-back (FtoB, magenta) motion detected by a horizontal motion-sensitive system. **c**, EM-reconstructed right HS (magenta) and right DNP15 (olive) skeletons, connected by chemical and electrical synapses in the IPS, a premotor region. HS dendrites reside in the lobula plate (LOP), whereas DNP15 axons project to the VNC. **d**, Schematic highlighting the fly's central nervous system (left). VNC regions targeted by DNP15 outputs (right). Ascending neurons (ANs; orange) project to the brain while motor neurons (MNs, light blue) project to neck and body muscles. **e**, DNP15 output distribution, grouped based on VNC regions (**d**) (left).

DNP15 targets premotor and motor circuits well suited to coordinate head and body movements for gaze control (right). **f**, Schematic of the fly brain and the IPS, where optical imaging was performed (top). Two-photon calcium imaging setup and the visual LED display (bottom). **g**, Calcium responses of HS (magenta) and DNP15 (olive) neurons to binocular (top, 'yaw' or 'bFtoB') and unilateral (bottom, 'left/right FtoB') horizontal visual motion. Traces represent grand mean z-scored responses $\pm$ s.e.m. (shaded area), with mean responses derived from 5–10 stimulus repetitions per fly. Gray rectangle shades indicate the stimulus window. HS ($N = 13$ flies, $n = 17$ regions of interest (ROIs)), DNP15 ($N = 6$, $n = 7$). **h**, Discrimination index (Methods) between 'bFtoB' and 'yaw' stimuli. Each circle represents an individual ROI. The colored horizontal line and black vertical line represent the mean and 95% CI, respectively. *** $P < 0.001$ (two-sided Wilcoxon rank-sum test). HS ($N = 13$ flies, $n = 17$ ROIs), DNP15 ($N = 6$, $n = 7$).

short timescales^{2,16}. Consistent with this function, optic flow-sensitive neurons^{10,17–20}, found across species, integrate visual and extra-retinal signals to distinguish self-generated from external motion^{21–24} while processing binocular visual motion asymmetries to detect path deviations^{25–27}. These asymmetries, however, become harder to detect visually at a high speed of locomotion due to stronger translational components in optic flow (Fig. 1b, colored rectangles). Ideally, optic flow-sensitive networks should reliably detect binocular asymmetries invariant to translational speed. The functional organization of these

visuomotor circuits supporting such robust processing of visual feedback and course (and gaze) control is poorly understood.

In *Drosophila*, rotational optic flow-sensitive neurons, such as the horizontal system (HS) and H2 cells are found in the lobula plate and project to premotor regions of the brain²⁰, and have been implicated in gaze and course stability control^{10,24}. These lobula plate tangential cells (LPTs) are sensitive to horizontal rotational optic flow and can drive body rotations²⁸, with HS cells contributing to body steering particularly during fast locomotion^{16,29}. Thus, HS cells and associated

central networks contribute to robust processing of rotational optic flow during body translation. One possibility is that feedforward excitation from HS cells, and other LPTs, is combined downstream with lateral inhibition to drive simple left–right categorization and behavioral choice^{30,31}; however, the identity, structure and function of central networks postsynaptic to HS and H2 cells remain to be elucidated.

This study identifies and characterizes a central network postsynaptic to HS and H2 cells to understand how it processes binocular optic flow. We found that DNp15, a descending neuron connected to HS cell^{27,32}, is less sensitive to translational optic flow compared to HS cells. Using electron microscopy (EM)-based reconstructions, we identified a multilayered network with inputs from HS and H2 cells. Our analysis revealed a middle layer consisting of several classes of GABAergic interneurons, and an output layer diverging into feedback to the lobula plate, descending paths to the ventral nerve cord (VNC; analogous to the vertebrate spinal cord), and an ascending path to deeper brain regions, such as the lateral accessory lobe (LAL). Combining two-photon calcium imaging, GABA receptor and synaptic activity manipulations and modeling, we found that feedforward excitation together with lateral disinhibition decrease translational sensitivity while enhancing rotational selectivity at the network's output layer. This fine-tuned optic flow processing transforms binocular asymmetries into categorical steering commands for course and gaze control, likely invariant to locomotion speed.

Results

DNp15 is less sensitive to translational motion than HS

To understand the role of HS cells in steering, we examined DNp15 neurons (also known as DNHS1; Fig. 1c); a pair of descending neurons (DNs) postsynaptic to HS cells that project to the VNC^{27,32} (Fig. 1d). Using a recent EM dataset of the male VNC³³, we mapped DNp15's primary outputs. About two-thirds project to regions associated with the neck, haltere and wing, aligning with DNp15's known projection field within the VNC³² (Fig. 1e). The remaining third targets leg neuropils (Fig. 1e), suggesting DNp15 may also influence steering during walking, a hypothesis that we test later. This analysis indicates that bilateral DNp15 activity may reflect asymmetries during body translation that could help stabilize both gaze and course direction.

To compare optic flow selectivity between HS and DNp15 cells, we conducted in vivo, two-photon calcium imaging in immobilized flies, focusing on the inferior posterior slope (IPS), where HS axons and DNp15 dendrites overlap (Fig. 1c,f and Methods). We presented a binocular asymmetric stimulus, mimicking the visual effect of horizontal, yaw rotations ('yaw') and a binocular symmetric front-to-back ('bFtoB') stimulus simulating forward translation projected onto a rotational-sensitive system (without vertical or local motion parallax components; Fig. 1f,g and Supplementary Video 1). Unilateral stimuli ('right FtoB' or 'left FtoB') were also used to assess binocular circuit interactions (Fig. 1g).

HS cells are sensitive to ipsilateral front-to-back motion, velocity tuned and have large receptive fields with a small binocular frontal region^{25,27,28}. Consistent with its chemical and electrical connections to HS cells^{27,32}, DNp15 exhibited similar velocity tuning (Extended Data Fig. 1a,b and Supplementary Video 2) and showed the highest sensitivity to 'yaw' stimulus (Extended Data Fig. 1c); however, unlike HS cells, DNp15 was strongly modulated by binocular interactions, displaying lower sensitivity to 'bFtoB' stimulus (Fig. 1g) but higher sensitivity to contralateral back-to-front motion compared to HS cells (Fig. 1g and Extended Data Fig. 1c, 'left BtoF'). These differences were also evident under contralateral-regressive translational optic flow (Extended Data Fig. 1d).

To quantify differences in selectivity for rotational versus translational stimuli, we defined a discrimination index comparing the neurons' responses to 'bFtoB' versus 'yaw' or to 'progressive' versus 'sideslip' stimuli (Fig. 1h, Extended Data Fig. 1e and Methods).

DNp15 showed enhanced selectivity for rotational over translational stimuli, indicating binocular integration of optic flow well suited to detect asymmetries during body translations.

Distinct central networks process rotational optic flow

To investigate potential circuit mechanisms underlying signal transformation between HS and DNp15 cells, we identified the main downstream synaptic partners of HS and two well-studied LPT cells: H2, which connect to HS cells via chemical and electrical synapses and responds to contralateral back-to-front horizontal motion^{20,26,27} and VS cells, which respond to vertical rotational optic flow¹⁰. Using the FAFB dataset³⁴ (Extended Data Fig. 2a), we mapped chemical synapses at HS, H2 and VS axon terminals. Manual tracing of pre- and postsynaptic partners showed that all three receive axonal input, indicating central modulation of their activity (Extended Data Fig. 2b–d; see Methods for tracing strategy). These cells form a mix of strong and weak synaptic connections (Supplementary Fig. 1), a finding validated using two additional EM datasets: FlyWire³⁵ and Hemibrain³⁶ (Extended Data Fig. 2a, Supplementary Fig. 1 and Supplementary Table 1). FlyWire semi-automatic reconstructions are based on FAFB, whereas Hemibrain is an independent, partial dataset from a female fly brain.

We then identified strong synaptic partners of HS, H2 and VS cells and analyzed their connectivity using a matrix derived from FAFB dataset (Fig. 2a and Extended Data Fig. 2d; see Methods for 'strong synaptic partners' definition). The matrix revealed mostly sparse connections, with some neuron pairs forming strong unidirectional links via hundreds of synapses. Hierarchical clustering analysis identified three primary clusters: (1) HS and VS cells with the interneuron IVLPT8; (2) a network with H2 and HS cells; and (3) a network with VS cells (Fig. 2a; see Supplementary Table 2 for neuron identities). This structure remained stable across different threshold settings (Supplementary Fig. 2).

The H2–HS network formed strong connections among central interneurons, H2 and HS cells and feedback CH cells²⁶ (Fig. 2b,c). Its outputs diverged into an ascending pathway via PS047 cells projecting to LAL, a region linked to orientation behaviors³⁷ (Fig. 2b,c), and descending pathways targeting VNC leg, neck and haltere neuropils³³, including DNp15 (ref. 32) and DNao2, a steering-related DN³⁸ (Fig. 2b,d). VS connections formed a small fraction of the H2–HS network (Fig. 2c), and instead had independent output pathways, including feedback to the ocelli (fly's secondary optical system) and central interneurons that partially overlapped with the H2–HS network (Extended Data Fig. 3a–c). This suggests that H2–HS and VS networks process optic flow through distinct circuits.

The physiological properties of DNp15 (ref. 32) (Fig. 1) and DNao2 (ref. 38), along with the LAL output pathway, support the H2–HS network's role in binocular optic flow processing and steering control. DNp15, for example, receives strong input from the contralateral H2 neuron (Fig. 2b), consistent with its sensitivity to contralateral back-to-front motion (Fig. 1g). This convergence of inputs from HS, H2 and central interneurons likely drives DNp15's selectivity to horizontal optic flow.

Recurrency within central, optic flow-sensitive networks

While the H2–HS and VS networks are largely distinct, they share recurrent connections through common LPT cell recurrent neurons (LPTCrns) in the middle layer. We identified five LPTCrn classes based on their anatomical features and connections to HS, H2 and VS cells (Fig. 2e,f and Extended Data Fig. 3c–e).

Crab LPTCrns (cLPTCrns), central to the VS network (Extended Data Fig. 3b), project bilaterally within the IPS and form reciprocal connections with most VS and HS cells (Fig. 2f). Bilateral LPTCrns (bLPTCrns) also project to both hemispheres of the dorsal IPS, reciprocally connecting with ipsilateral HS and VS cells (Fig. 2f). Unilateral LPTCrns (uLPTCrns), primarily associated with the H2–HS network

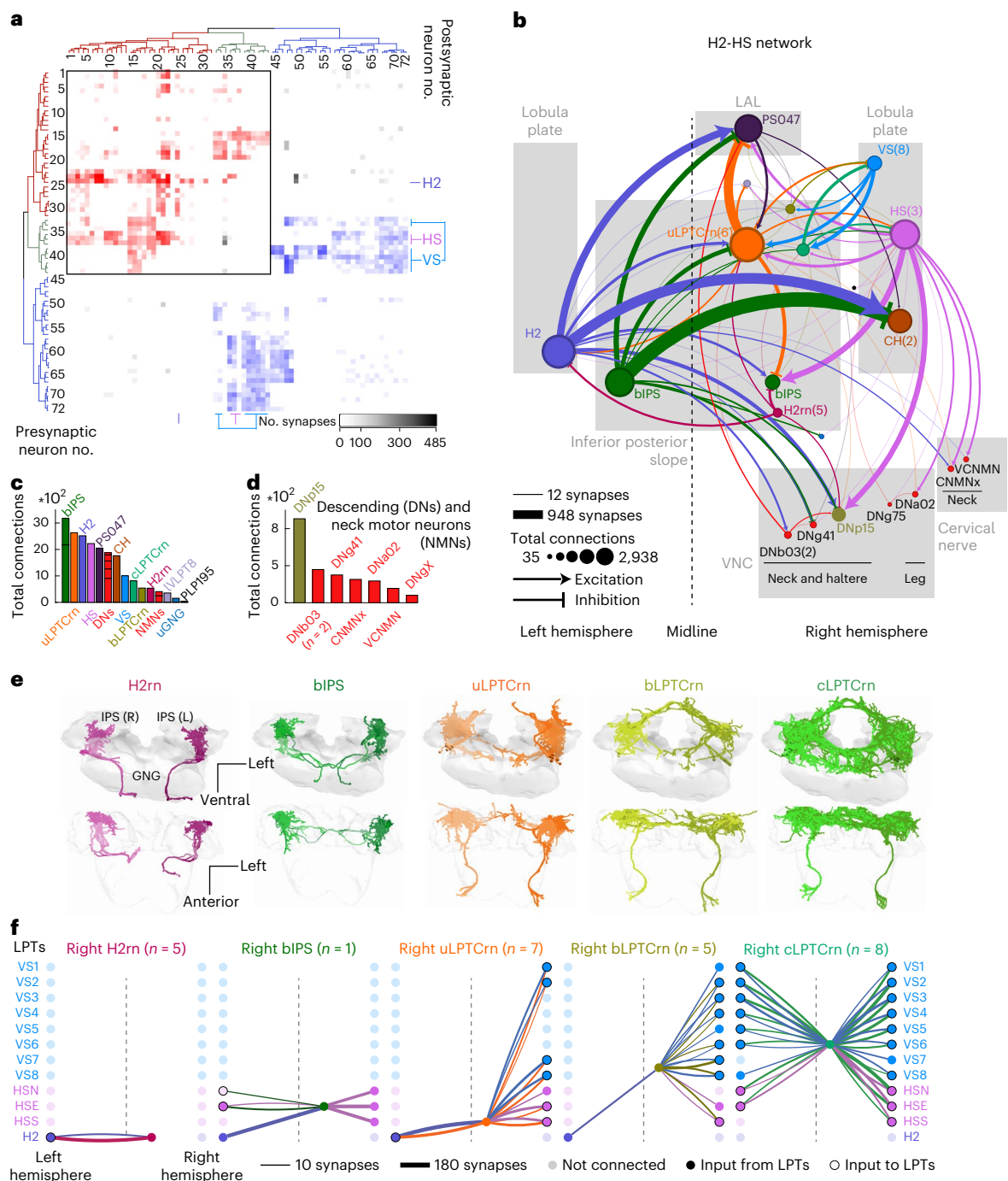


Fig. 2 | Identifying central networks processing optic flow-sensitive inputs.

a, Synaptic connectivity matrix of HS, H2 and VS cells with their strongest central synaptic partners. Grayscale indicates synapse counts; colored squares indicate H2-HS (red) and VS (blue) networks based on hierarchical clustering (dendrogram; Methods). Note that the HS and VS cells (green dendrogram cluster) take part in both networks. Supplementary Table 2 provides a full list of neurons and nomenclature. **b**, Connectivity graph of the H2-HS network (black rectangle in **a**). Nodes represent neurons, with edges depicting synaptic connections. Numbers in parentheses indicate cell counts within each node, grouped by anatomy. Node diameter reflects total connections (inputs and outputs). Except for LPTs, nodes are positioned within brain regions

(gray rectangles) where their axons reside. Edge color and thickness denote presynaptic neurons and synapse numbers. For clarity, edges with fewer than 50 synapses are shaded; those with fewer than 10 are not shown. Inhibitory edges are tee-shaped. **c**, Total synaptic connections per cell type within H2-HS network. **d**, Total synaptic connections per descending and neck motor neuron in the H2-HS network. **e**, Anatomical reconstructions of network interneurons within the IPS, shown in posterior (top) and dorsal (bottom) views. Each cell type forms a bilateral, mirror symmetric population. GNG, gnathal ganglion. **f**, Connectivity graph of each cell type in **e** with individual LPTs. Edge thickness indicates synapse count while edge color indicates the upstream (input) cell. LPTs that do not provide input are shaded; LPTs receiving inputs are circled.

(Fig. 2b,c), project ipsilaterally and form strong reciprocal connections with contralateral H2 and ipsilateral HS, VS1-2 and VS7-8 cells (Fig. 2f).

The other LPTCrn classes connect specifically to HS and H2 cells. Bilateral IPS neurons (bIPS) receive convergent inputs from ipsilateral

HS and contralateral H2 cells, and project to the contralateral HSN and HSE cells (Fig. 2f). H2 recurrent neurons (H2rn) receive input from contralateral H2 cells and form feedback connections selectively with them (Fig. 2f).

Beyond the lobula plate, the H2–HS network's middle layer connects extensively with neurons within the IPS, gnathal ganglia, the LAL and other optic lobe regions (Extended Data Fig. 4 and Supplementary Fig. 3). Unlike H2, HS and VS cells, LPTCrns also receive direct ascending input from the VNC (Extended Data Fig. 4). These connections suggest that LPTCrns have distinct optic flow sensitivities, integrate extra-retinal signals with optic flow cues, and transmit additional signals from premotor centers, likely influencing the optic flow sensitivity of output neurons. In the following sections, we explore middle-layer responses to optic flow and compare them to their inputs from HS and H2 cells, as well as their main common output, DNp15 cells.

Optic flow responses within the network's middle layer

The connectivity of bIPS, uLPTCrns and H2rns suggest they play a key role in shaping optic flow responses between HS cells and DNp15 neurons (Fig. 3a). Using EM skeleton tracing and computational searches of GAL4 driver images³⁹, we generated Split-Gal4 lines for cell-type-specific genetic access to middle layer neurons (Supplementary Fig. 4). We then characterized LPTCrn optic flow responses through in vivo two-photon calcium imaging at their axon terminals under nonbehaving conditions to minimize extraretinal inputs^{23,24} (Fig. 3b, Supplementary Video 1 and Methods).

All three neuron types (bIPS, H2rns and uLPTCrns) showed strong, direction-selective responses to horizontal motion, like HS, H2 cells and DNp15 neurons (Fig. 3c and Extended Data Fig. 5a); however, their velocity-tuned responses differed from their upstream LPTs: uLPTCrns were more sensitive to slower speeds, bIPS to intermediate speeds and H2rns to faster speeds than HS and H2 cells (Extended Data Fig. 5b and Supplementary Video 2). Despite direct VS inputs (Fig. 2f), uLPTCrns showed minimal or negative responses to pitch and roll motion (Extended Data Fig. 5a), suggesting modulation by intrinsic properties or additional inputs, potentially through direct connections from other optic lobe neurons (Supplementary Fig. 3) or indirect inputs from the central brain (Extended Data Fig. 4c).

LPTCrn responses to unilateral stimuli revealed network-based modulation of visual inputs. Right bIPS and uLPTCrns, which receive inputs from right HS and left H2 neurons (Fig. 3a), responded as expected to ipsilateral FtoB motion. Right H2rns responded as expected to contralateral back-to-front, but also to ipsilateral FtoB motion despite lacking direct HS inputs (Fig. 3c and Extended Data Fig. 5c). Moreover, uLPTCrns and bIPS showed minimal responses to contralateral back-to-front motion, despite strong H2 inputs (Extended Data Fig. 5c), suggesting sensitivity to both rotational and translational optic flow.

To test this idea, we examined responses to symmetric binocular stimuli: progressive (or regressive) translation (Supplementary Video 1, 'progressive/regressive') or bFtoB (bBtoF) stimuli (Fig. 1g). Each neuron showed characteristic responses (Fig. 3c and Extended Data Fig. 5d). bIPS responded strongly to 'bFtoB' (Fig. 3d), while uLPTCrns showed minimal or negative responses to translational patterns, and H2rns weakly responded to both directions of translation (Fig. 3d and Extended Data Fig. 5d). The discrimination index (Methods and Fig. 3e) confirmed that HS and H2 cells preferred rotational motion, while H2rn, uLPTCrn and DNp15 were more selective for rotation than translation (Fig. 3e). bIPS, however, showed stronger sensitivity to translation, shifting its discrimination index toward positive values (Fig. 3e).

To determine whether these differences were due to symmetric versus asymmetric binocular stimuli sensitivity, we tested asymmetric translational motion (sideslip or combined sideslip and progressive/regressive motion; Extended Data Fig. 6a). All H2–HS network neurons responded to asymmetric translational stimuli (Extended Data Fig. 6b–f), with greater selectivity for asymmetric versus symmetric stimuli, except for bIPS. (Extended Data Fig. 6g). While HS, H2, uLPTCrns, and H2rns showed similar selectivity for sideslip versus

combined motion, bIPS displayed a slight, though not significant, preference for the compound stimulus (Extended Data Fig. 6h). This suggests bIPS may be sensitive to global perspective cues for forward motion, independent of local motion parallax cues. The key question remains: how does bIPS, embedded in a rotation-sensitive network, exhibit increased sensitivity to translation, and what functional role does this play in the broader network?

Recurrent, inhibitory interactions shape bIPS responses

We hypothesized that bIPS' lack of response to contralateral back-to-front motion and its enhanced sensitivity for translational stimuli are emergent properties of the H2–HS network. One possibility is that H2 excitation in bIPS is suppressed by indirect, H2-dependent inhibition. To examine this, we analyzed the predicted neurotransmitter profile⁴⁰ of bIPS inputs (Fig. 4a). The main inhibitory inputs (uLPTCrn and H2rn) are predicted to be GABAergic (Fig. 4a), whereas HS and H2 provide strong excitatory inputs (Figs. 2c and 4b). Immunostaining against GABA (Supplementary Fig. 5a and Table 1) and genetic targeting confirmed all LPTCrns are GABAergic (Supplementary Fig. 5b).

Next, we tested whether uLPTCrns and H2rns modulate H2–bIPS communication by analyzing the spatial organization of their inputs relative to bIPS' root point (the junction between soma tract and main neurite) (Extended Data Fig. 7a and Methods). H2rn inputs were closest to the root point (Extended Data Fig. 7b,c) and positioned nearer to H2 inputs than to HS inputs (Extended Data Fig. 7d). H2rn frequently formed microcircuit motifs with H2 cells (Extended Data Fig. 7e,g), but not with HS cells (Extended Data Fig. 7f,h), suggesting localized suppression of H2 excitation; however, this alone does not fully explain bIPS' lack of sensitivity to contralateral back-to-front motion (Extended Data Fig. 5c). It is possible that both H2rns and uLPTCrns contribute to this suppression.

To test this last idea, we disrupted fast-acting GABA signaling in bIPS using the FlpStop strategy (Methods and Table 1)⁴¹ to target the *resistance to dieldrin* (*rdl*) gene, essential for functional GABA-A receptors⁴² (Supplementary Fig. 4b). bIPS neurons in a *rdl* mutant background showed reduced optic flow responses compared to controls (Fig. 4c–e), indicating disrupted disinhibition. Responses to 'bFtoB' motion were significantly diminished (Fig. 4c,e; $P < 0.003$, Wilcoxon rank-sum test), shifting the discrimination index between 'bFtoB' and 'yaw' stimuli toward zero (Fig. 4f; $P < 0.0006$, Wilcoxon rank-sum test); however, the discrimination index between progressive and 'yaw' stimuli remained unchanged (Fig. 4f). Notably, the lack of response to contralateral back-to-front motion, which activates H2 cells, persisted in *rdl* mutants (Fig. 4c; 'left BtoF'), suggesting additional gating mechanisms. These findings demonstrate that GABAergic inhibition modulates bIPS' sensitivity to binocular symmetric optic flow and likely contributes to shaping horizontal optic flow responses during translation (Fig. 1).

Compound optic flow fine tuning by middle layer neurons

DNp15 responds strongly to rotational motion and weakly to translational motion (Fig. 1g). Within the H2–HS network, it receives strong connections from H2, HS and bIPS, followed by uLPTCrns and H2rns (Extended Data Fig. 4c). These GABAergic interneurons may modulate optic flow processing between HS and DNp15; however, during translational optic flow, only bIPS is active (Fig. 3c), making it the most likely modulator. To examine bIPS' influence on HS' and DNp15's responses to compound optic flow, we used stimuli combining fixed-velocity rotation, mimicking a walking fly's drift at typical angular velocities¹, with varying forward speeds ('low', 'intermediate' and 'fast'; see Methods for stimulus design). Given technical differences in stimuli design, we also tested responses to flicker, horizontal, and translational optic flow using a standardized design (Supplementary Video 3). We analyzed neural activity under control conditions and after manipulations, either disrupting GABA-A signaling in bIPS and DNp15 using the *Rdl*-FlpStop

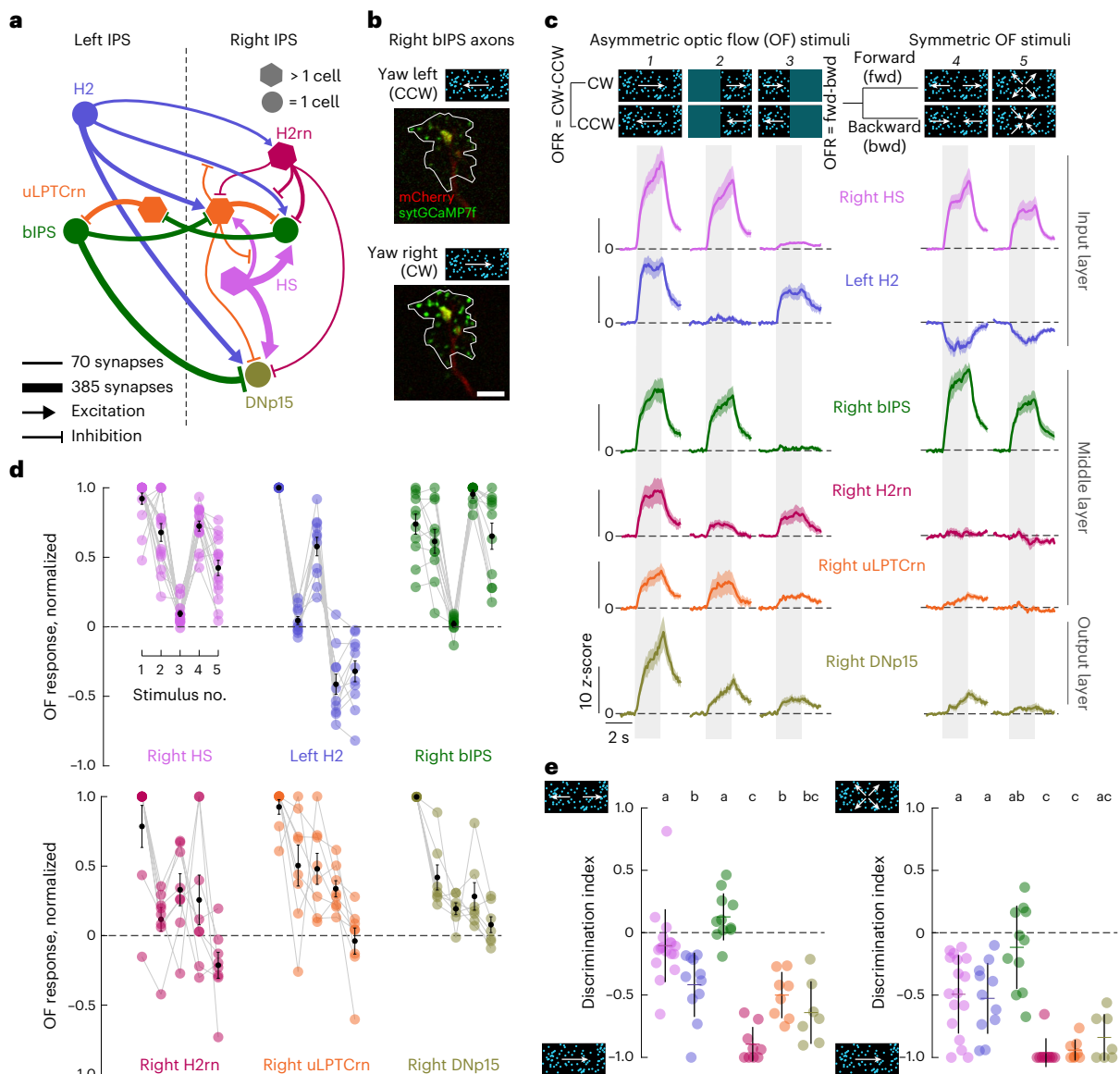


Fig. 3 | Characterizing optic flow sensitivity in the H2–HS network. **a**, Local connectivity between input, middle and output layers of the H2–HS network. **b**, Single-plane image of right bIPS axons from one example fly during left (CCW, top) and right (CW, bottom) horizontal ‘yaw’ motion. A single ROI, which covers the entire axonal region is outlined in white. The red signal represents mCherry fluorescence, and the green signal represents sytGCaMP7f fluorescence averaged over the full duration of stimulus motion from one example trial. Scale bar, 10 μ m. **c** Top: Visual stimuli, with arrows indicating motion direction. Optic flow calcium responses (OFRs), expressed as the difference in response amplitude between CW-CCW motion (rotational stimuli 1–3) or the difference in response amplitude between forward and backward motion (translational stimuli 4–5) (bottom). Traces represent grand mean

z-scored responses $\pm$ s.e.m. (shaded), with mean responses derived from 5–10 stimulus repetitions per fly. Gray rectangles indicate the stimulus window. For this and the remaining panels: HS ($N = 13$ flies $n = 17$ ROIs), H2: ($N = 8$, $n = 11$), bIPS ($N = 10$, $n = 11$), H2rn ($N = 6$, $n = 9$), uLPTCrns ($N = 6$, $n = 8$), DNp15 ($N = 6$, $n = 7$). **d**, Normalized OFRs relative to each neuron’s preferred stimulus, with error bars representing s.e.m. **e**, Discrimination index between binocular translation (stimuli 4 or 5) and rotational stimuli (stimulus 1; Methods). Circles represent individual ROIs. The horizontal and vertical lines represent mean and 95% CI, respectively. Letters indicate groups with significant difference ($P < 0.0033$, two-sided Wilcoxon rank-sum test with Bonferroni correction for multiple comparisons).

construct (Supplementary Fig. 4b,c) or blocking bIPS’ chemical synaptic output via TNT expression (Fig. 5a; see Methods for discussion on circuit manipulations).

While no cells responded to flicker (Extended Data Fig. 8a), circuit manipulations affected responses to pure rotations, translations and compound stimuli. Blocking bIPS synaptic output had no effect on HS responses to progressive motion but slightly reduced sensitivity to rotation (Fig. 5b,c,f; $P > 0.107$, Wilcoxon rank-sum test). HS responses to the compound stimuli were unaffected by bIPS synaptic activity but decreased sharply at intermediate and fast speeds (Fig. 5c,h).

Disrupting GABA-A signaling in bIPS had little effect on its ‘yaw’ or ‘progressive’ responses (Fig. 5b,d,f) but significantly reduced responses to ‘bFtoB’ stimuli (Fig. 4e). bIPS responses to compound optic flow were speed-dependent (Fig. 5d, left and right deviations). At low speeds, when rotational components dominated (Supplementary Video 3), bIPS showed similar directional selectivity to pure rotations (Figs. 4c and 5b). At higher speeds, bIPS’s selectivity to rotations decreased, a shift dependent on GABA-A signaling (Fig. 5d,g,h) and consistent with bIPS’ role in translational-sensitive modulation of its outputs.

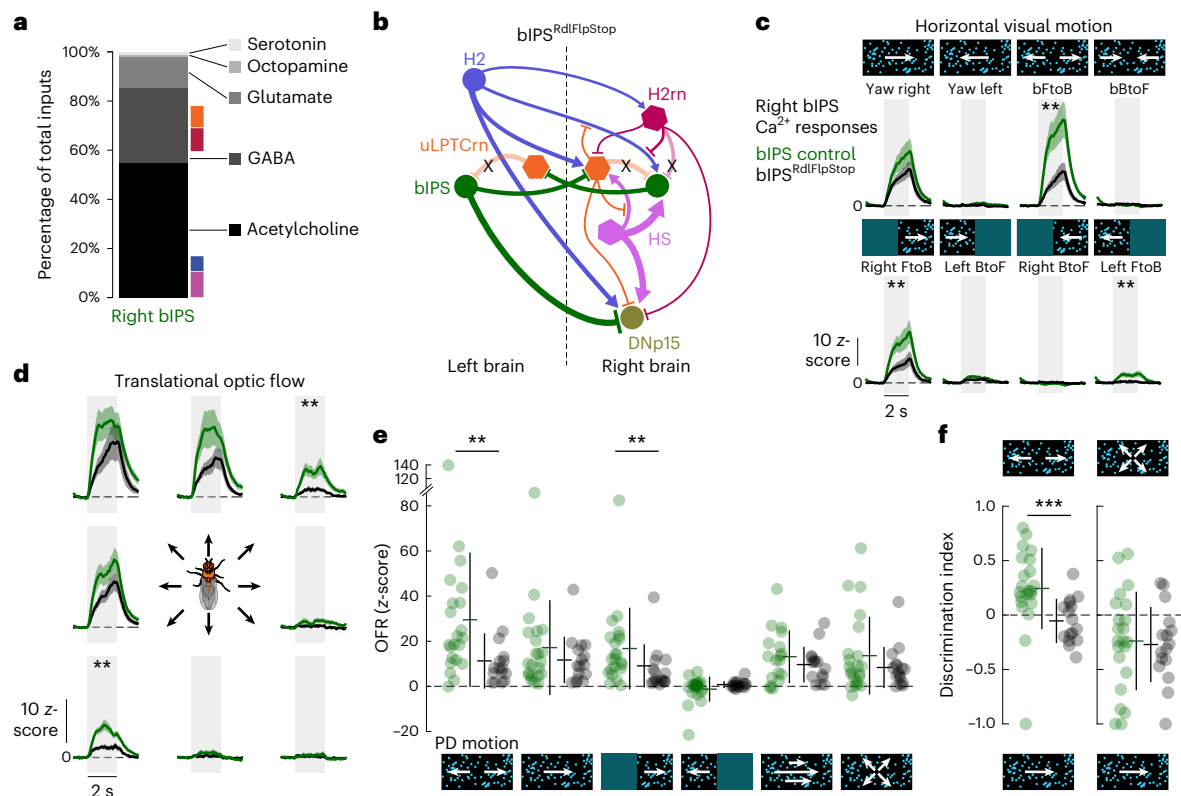


Fig. 4 | Inhibition fine-tunes optic flow responses in bIPS. **a**, Input profile of bIPS, grouped by their predicted primary neurotransmitter. Neurons in **b** are highlighted by colored lines. **b**, Local connectivity between input, middle and output layers of the H2–HS network. Inhibitory connections are shown as tee-shaped edges. Disrupting *rdl* gene with FlpStop blocks fast GABAergic signaling onto bIPS (crosses). **c**, Calcium responses of control (green) and disrupted (black) bIPS cells to unilateral and bilateral horizontal motion. Traces represent grand mean z-scored responses across flies $\pm$ s.e.m. (shaded), with mean responses derived from 5–10 stimulus repetitions per fly. Light-gray rectangles indicate the stimulus window. Stars indicate significant differences in

visual responses (area under the curve). bBtoF, bilateral back-to-front; right/left FtoB, unilateral front-to-back; right/left BtoF, unilateral back-to-front. **d**, Same as **c**, but for translational optic flow. **e**, Rotational and translational OFR (Fig. 3c). The preferred direction (PD) of motion is shown below each stimulus. **f**, bFtoB versus yaw discrimination index (Methods) for the neurons in **c** (left). Same as left, but for the progressive versus yaw discrimination index (right). Circles represent individual ROIs (**e**, **f**). Horizontal and vertical lines represent mean and 95% CI, respectively. bIPS ($N = 18$ flies, $n = 22$ ROIs), bIPS^{Rdl-FlpStop} ($N = 11$, $n = 16$). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (two-sided Wilcoxon rank-sum test).

Disrupting GABA-A signaling in DNp15 (Supplementary Fig. 4c) caused a noticeable, though not significant, increase in sensitivity to progressive motion (Fig. 5e,f; $P < 0.18$, low and intermediate speeds, Wilcoxon rank-sum test), and significantly increased rotation sensitivity (Fig. 5b; $P < 0.006$). This may be due to altered inhibitory and excitatory inputs from the H2–HS network (Fig. 2). Like bIPS, DNp15's responses to low-speed compound stimuli resembled its responses to rotation (Extended Data Fig. 8e, compare zero and low speed). With disrupted GABA-A signaling, DNp15's responses to compound stimuli at all speeds become more HS-like (Fig. 5e,f). At higher speeds, DNp15's responses decreased, likely due to reduced HS activity (Fig. 5e, arrows). These findings highlight that the H2–HS network balances excitation and inhibition to fine tune optic flow processing, with bIPS' speed-dependent activity modulation ensuring DNp15's robust selectivity for rotations.

Recurrent, lateral disinhibition for binocular asymmetry detection

bIPS' inhibitory inputs regulate its sensitivity to binocular symmetric stimuli (Figs. 4 and 5). As a GABAergic neuron (Supplementary Fig. 5), bIPS may help detect binocular asymmetries during translation. bIPS is also recurrently connected with uLPTCrns (Fig. 3a), complicating its role in a dynamic setting like locomotion. To explore this recurrent motif, we examined bIPS–uLPTCrn interactions during walking using a phenomenological model agent constrained by the H2–HS network's

connectivity (Methods). The agent mimicked an exploratory fly¹, using DNp15-like neurons to process horizontal visual inputs for steering.

The model included four input channels, each incorporating classical FtoB (HS cell-like) and back-to-front (H2 cell-like) visual motion detectors¹⁰, alongside H2–HS network's core lateral disinhibitory motif (Fig. 6a). The model was tuned to match real neural response amplitudes to horizontal motion at steady state (Fig. 6b, Extended Data Fig. 9a–c and Methods). Removing bIPS' inhibitory inputs by zeroing uLPTCrn synaptic weights reduced bIPS sensitivity to binocular symmetric stimuli (Fig. 6c) and impaired its ability to distinguish rotational from translational stimuli, consistent with experimental data (Fig. 6d). These findings suggest that feedforward excitation combined with lateral disinhibition replicates H2–HS network's responses to horizontal motion.

Next, we examined bIPS' role in high-speed course control when H2 cells are weakly active. The agent compared left versus right modeled DNp15 activity to steer through visuomotor noise and self-generated translational optic flow (Extended Data Fig. 9d–h). A larger activity difference between the modeled DNp15 neurons (quantified as higher 'asymmetry index' (AI; Methods) indicated improved binocular asymmetry detection during fast translation (Fig. 6e). The modeled DNp15 neurons showed strong asymmetry on average (Fig. 6f, 'control'); however, removing bIPS or both bIPS and uLPTCrns, reduced this asymmetry to HS cell levels (Fig. 6f, 'without B' and 'without B + U'). This supports the idea that bIPS activity enables DNp15 to extract rotational

Table 1 | Key resources

Reagent or resource	Source	ID
Antibodies		
Mouse anti-nc82 antibody	DSHB	RRID: AB_2314866
Chicken anti-GFP antibody	Abcam	RRID: AB_13970
Rabbit polyclonal anti-GFP antibody	Thermo Fisher Scientific	Cat. no. A6455 RRID: AB_221570
Rabbit anti-GABA antibody	Sigma	RRID: A2052
Rabbit anti-DsRed antibody	Abcam	AB356483
Goat Alexa-488 anti-chicken	Invitrogen	RRID: A11038
Goat Alexa-594 anti-rabbit	Invitrogen	RRID: AB_150088
Goat Alexa-633 anti-mouse	Invitrogen	RRID: AB_141431
Experimental Models: Organisms/Strains		
<i>Drosophila</i> UAS-2xEGFP	Bloomington <i>Drosophila</i> Stock Center (BDSC)	RRID: BDSC_6874
<i>Drosophila</i> UAS-mCD8::GFP	BDSC	RRID: BDSC_5137
<i>Drosophila</i> 40XUAS-mCD8::GFP	BDSC	RRID: BDSC_32195
<i>Drosophila</i> UAS-CD4::tdTomato	BDSC	RRID: BDSC_35837
<i>Drosophila</i> 20XUAS-6XmCherry in VK00018	BDSC	RRID: BDSC_52267
<i>Drosophila</i> 20XUAS-6XmCherry in attP2	BDSC	RRID: BDSC_52268
<i>Drosophila</i> 13XLexAop2-6XmCherry	BDSC	RRID: BDSC_52272
<i>Drosophila</i> MCFO-1	BDSC, Nern et al. ⁶¹	RRID: BDSC_64085
<i>Drosophila</i> 13XLexAop-jGCaMP7f	BDSC	RRID: BDSC_80910
<i>Drosophila</i> 20XUAS-jGCaMP7f	BDSC	RRID: BDSC_80906
<i>Drosophila</i> UAS-sytGCaMP7f	Gaby Maimon ⁶²	RRID: BDSC_94619
<i>Drosophila</i> 20XUAS-GCaMP6f, UAS-FLP	Molina-Obando et al. ⁶³	N/A
<i>Drosophila</i> {hsp70-FLP}1	BDSC	RRID: BDSC_23649
<i>Drosophila</i> {hsp70-FLP}3F	BDSC	RRID: BDSC_6420
<i>Drosophila</i> Otd-nls:FLPo	Asahina et al. ⁶⁴	N/A
<i>Drosophila</i> pJFRC56-10XUAS>Stop>eGFPKir2.1 in attP2	Gerry Rubin	N/A
<i>Drosophila</i> pJFRC34-5XUAS-DSCP-E86tetLC	Gerry Rubin	N/A
<i>Drosophila</i> UAS>Stop>Ricin	BDSC	RRID: BDSC_29001
<i>Drosophila</i> UAS-TrpA1(B).K	BDSC	RRID: BDSC_26263
<i>Drosophila</i> Rdl-FlpStopND	BDSC	RRID: BDSC_67682
<i>Drosophila</i> VT014724-Gal4.DBD,Rdl-FlpStopND	This study	N/A
<i>Drosophila</i> R77F05-Gal4.DBD,Rdl-FlpStopND	This study	N/A
<i>Drosophila</i> GAD1-p65.AD	BDSC	RRID: BDSC_60322
<i>Drosophila</i> VGlut-p65.AD	BDSC	RRID: BDSC_82986
<i>Drosophila</i> VGlut-Gal4.DBD	Diao et al. ⁶⁵	N/A
<i>Drosophila</i> ChaT-Gal4.DBD	BDSC	RRID: BDSC_60318
<i>Drosophila</i> R39E01-LexA	BDSC	RRID: BDSC_52776
<i>Drosophila</i> R39E01-LexA,13XLexAop2-6XmCherry	This study	N/A
<i>Drosophila</i> R39E01-LexA, 13XLexAop2-IVS-GCaMP6f	This study	N/A
<i>Drosophila</i> R77F05-LexA	BDSC	RRID: BDSC_54265
<i>Drosophila</i> R50G03-Gal4	This study	N/A
<i>Drosophila</i> VT058487-Gal4	Vienna <i>Drosophila</i> Resource Center	N/A
<i>Drosophila</i> w ¹¹¹⁸ ; R11E07-p65.AD; R77F05-Gal4.DBD	BDSC	RRID: BDSC_75953
<i>Drosophila</i> w ¹¹¹⁸ ; Empty-p65.AD; Empty-Gal4.DBD	BDSC	RRID: BDSC_79603
<i>Drosophila</i> R23C12-p65.AD	BDSC	RRID: BDSC_70130
<i>Drosophila</i> R25F07-p65.AD	BDSC	RRID: BDSC_70622
<i>Drosophila</i> R27B03-p65.AD	BDSC	RRID: BDSC_70164

Table 1 (continued) | Key resources

Reagent or resource	Source	ID
<i>Drosophila</i> R50G03-p65.AD	This study	N/A
<i>Drosophila</i> VT014724-p65.AD	BDSC	RRID: BDSC_72019
<i>Drosophila</i> VT039047-p65.AD	BDSC	RRID: BDSC_73268
<i>Drosophila</i> VT040346-p65.AD	BDSC	RRID: BDSC_74542
<i>Drosophila</i> VT045281-p65.AD	BDSC	RRID: BDSC_75164
<i>Drosophila</i> R32A11-Gal4.DBD	BDSC	RRID: BDSC_69228
<i>Drosophila</i> R39E01-Gal4.DBD	BDSC	RRID: BDSC_70217
<i>Drosophila</i> R50G03-Gal4.DBD	This study	N/A
<i>Drosophila</i> VT014724-p65.DBD	BDSC	RRID: BDSC_72146
<i>Drosophila</i> VT019845-Gal4.DBD	BDSC	RRID: BDSC_73315
<i>Drosophila</i> VT038829-Gal4.DBD	BDSC	RRID: BDSC_73679
<i>Drosophila</i> VT039047-Gal4.DBD	BDSC	RRID: BDSC_73724
<i>Drosophila</i> VT045281-Gal4.DBD	BDSC	RRID: BDSC_72221
<i>Drosophila</i> VT049923-Gal4.DBD	BDSC	RRID: BDSC_73921
Software and algorithms		
MATLAB	MathWorks	RRID:SCR_001622
R	Open-source	RRID:SCR_001905
natverse ⁶⁶ (R package)	Open-source	https://github.com/natverse
ScanImage	Vidrio Technologies	RRID:SCR_014307
Gephi	Open-source	RRID:SCR_004293
Fiji	Open-source	RRID:SCR_002285
CATMAID	Open-source	RRID:SCR_006278

components from compound optic flow (Fig. 6f). Removing bIPS also made uLPTCrn activity more symmetric (Fig. 6f), while removing uLPTCrn cells reduced bIPS symmetry (Fig. 6c,f), mirroring the effect of disrupting GABA-A signaling in bIPS (Fig. 6g).

Thus, the model suggests that recurrent and lateral disinhibitory interactions within the H2–HS network enhance binocular asymmetry sensitivity during high-speed translation. Asymmetry in modeled DNp15 and uLPTCrn activity remained high across forward velocities typical of exploratory flies¹ (Fig. 6h). We propose that bIPS-mediated lateral disinhibition maintains these asymmetries at high speed. Symmetric bIPS activity directly suppresses DNp15s and indirectly disinhibits DNp15 via uLPTCrns ipsilateral to the drifting side, promoting asymmetric DNp15 activity for steering over a broad range of forward velocities.

DNp15’s and bIPS’ asymmetric activity drive steering

A prediction of the model is that asymmetric activity of bIPS and DNp15 should lead to steering during translation and DNp15’s connectivity within the VNC includes interneurons projecting to leg neuropiles, suggesting these cells may contribute to steering during walking (Fig. 1). Then, to test DNp15’s role in steering, we examined flies exploring a mildly aversive, novel environment while perturbing activity of these neurons symmetrically or asymmetrically. Under these conditions, flies primarily walk in straight ‘forward runs’ (of about ten body lengths per second), interspersed with abrupt course direction changes (‘saccades’)¹ (Fig. 7a, red circles). We manipulated DNp15 activity using stochastic Kir2.1 potassium channel expression, generating flies with DNp15 intact, bilaterally silenced or unilaterally silenced (Supplementary Fig. 6a and Methods). To assess course control, we aligned forward runs and calculated mean path deviation per unit of forward locomotion (Fig. 7b,c). Consistent with the agent model predictions (Fig. 7d left), flies with both or neither DNp15 silenced showed no systematic path deviations or saccades biases (Fig. 7d right and Extended

Data Fig. 10d,f, ‘none’ or ‘both’ silenced DNp15), whereas unilateral DNp15 silencing caused contralateral path deviations without affecting saccades (Fig. 7d and Extended Data Fig. 10d, ‘left’ or ‘right’ silenced DNp15). To confirm these effects were DNp15-specific, we repeated these experiments using ricin toxin A subunit expression⁴³ to ablate DNp15 stochastically (Supplementary Fig. 6b). This manipulation reproduced the previous results (Extended Data Fig. 10a–c), confirming that DNp15’s asymmetric activity influences course control during walking.

Agent-based modeling predicted a similar effect in bIPS (Fig. 7e, left), which experiments confirmed. Like DNp15, unilateral bIPS silencing (Supplementary Fig. 6c) caused drifting forward runs (Fig. 7e, ‘left’ or ‘right’ silenced bIPS), whereas bilateral silencing or intact bIPS produced no bias (Fig. 7e, ‘none’ or ‘both’ silenced bIPS). Silencing bIPS activity had no impact on saccade biases (Extended Data Fig. 10e), but bilateral bIPS activation—unlike DNp15—reduced saccades (Extended Data Fig. 10f,g and Supplementary Fig. 6d). These results show that H2–HS network’s role in behavior is context-specific: it stabilizes course direction to support stable gaze, consistent with HS cells’ proposed role in walking behavior¹⁶.

Discussion

As animals navigate, they must stabilize their gaze to control their trajectories, gather high-fidelity environmental information and detect moving objects. Gaze stabilization relies on accurately estimating self-motion. While previous studies in vertebrates and insects have explored how visual systems encode translation independent of rotations^{10,21}, few have examined the reverse, how rotational motion is processed in the context of translation^{44–46}. This study investigated how visuomotor circuits in *Drosophila* extract horizontal optic flow during translation to aid gaze and course control (Figs. 1a and 7g).

Using full-brain EM reconstruction, optical imaging, modeling and behavioral experiments, we identified compact, multilayer networks that estimate rotational optic flow. The first layer comprises optic

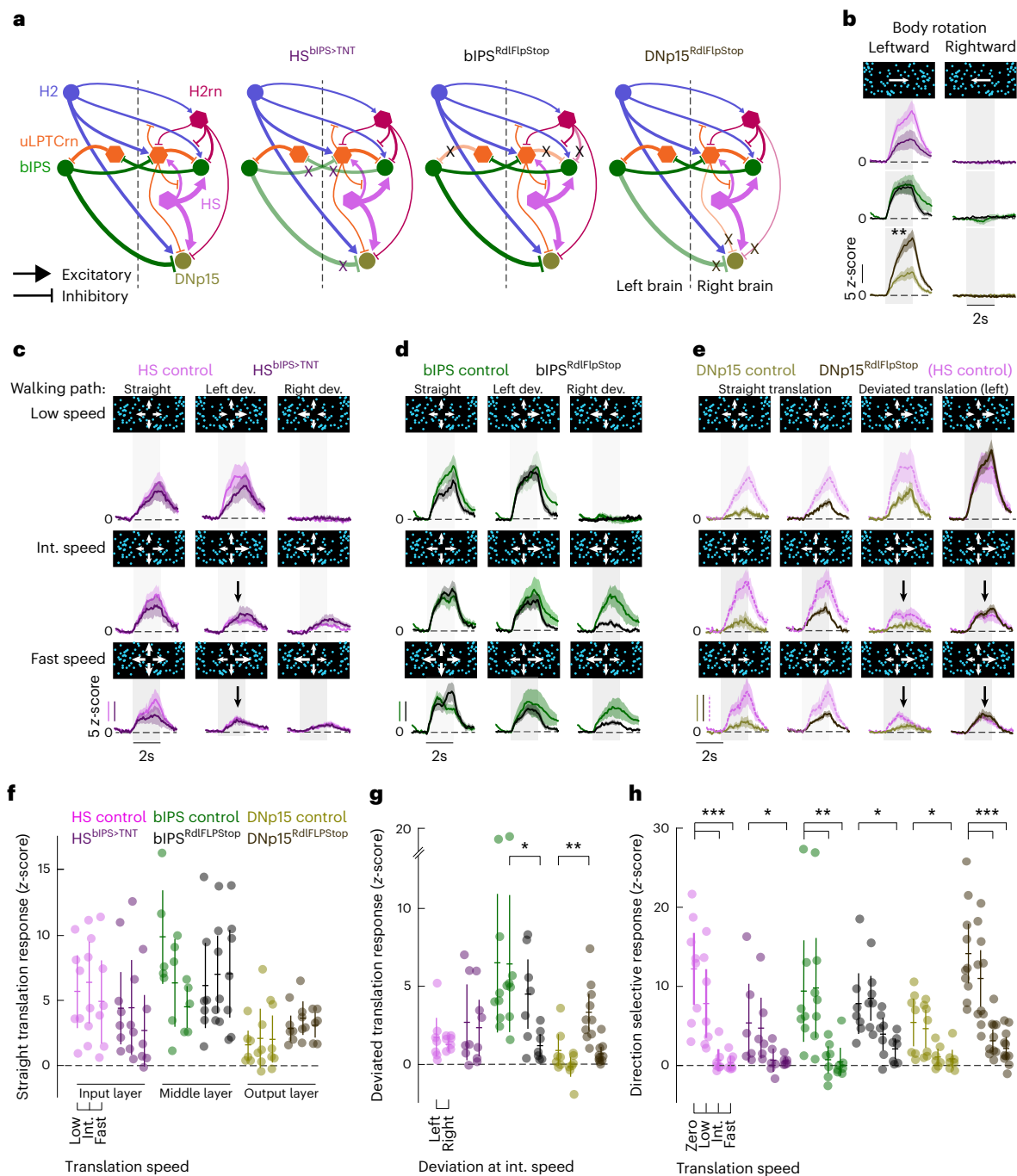


Fig. 5 | Inhibition modulates compound optic flow processing. **a**, Schematic of the H2–HS network illustrating the circuit manipulations (top). These include (1) disruption of chemical synaptic activity in bIPS ($HS^{bIPS>TNT}$, dark magenta) and (2) GABA-A receptor knockdown in bIPS ($bIPS^{RdlFlpStop}$, dark green) or in DNp15 ($DNp15^{RdlFlpStop}$, maroon). **b**, z-scored calcium responses of wild-type HS (magenta), bIPS (green), DNp15 (olive) and their manipulated counterparts (darker colors) to rotational motion. For panels **b–e**, traces represent grand mean z-scored responses $\pm$ s.e.m. (shaded), with mean responses derived from 5–10 stimulus repetitions per fly. Gray rectangles indicate the stimulus window. **c**, HS responses to translational and compound (translational + rotational) optic flow at varying translational speeds (‘low’, ‘intermediate’ and ‘fast’) under control (magenta) versus manipulated (dark magenta) conditions. Arrows indicate reduced HS response amplitude during compound optic flow with preferred direction of body deviation (left). **d**, Same as **c**, but for bIPS responses under control (green) and GABA-A receptor knockdown (dark green) conditions. **e**, Same as **c**, but for DNp15 responses under control (olive) and GABA-A receptor knockdown (maroon) conditions, with HS responses (magenta) replotted for

comparison. Arrows indicate reduced HS response amplitude during compound optic flow. Note that disrupting inhibition in DNp15 increases compound optic flow responses to HS levels. **f**, Neuronal responses to translational optic flow, shown as the average z-scored response during the last 500 ms of visual stimulation. Circles represent individual ROIs; horizontal and vertical lines represent mean and 95% CI, respectively. **g**, Same as **f**, but for compound optic flow at intermediate speed. **h**, Direction-selective responses (the difference between preferred (left deviation) versus nonpreferred (right deviation) responses), to compound optic flow at a given forward speed. For progressive stimuli, HS control ($N = 4$ flies, $n = 6$ ROIs), $HS^{bIPS>TNT}$ ($N = 5$, $n = 6$), bIPS control ($N = 5$, $n = 5$), $bIPS^{RdlFlpStop}$ ($N = 5$, $n = 7$), DNp15 control ($N = 6$, $n = 6$), $DNp15^{RdlFlpStop}$ ($N = 6$, $n = 6$). For compound stimuli, HS control ($N = 6$, $n = 8$), $HS^{bIPS>TNT}$ ($N = 5$, $n = 6$), bIPS control ($N = 6$, $n = 7$), $bIPS^{RdlFlpStop}$ ($N = 5$, $n = 7$), DNp15 control ($N = 7$, $n = 7$), $DNp15^{RdlFlpStop}$ ($N = 8$, $n = 10$). Statistics of the figure: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (two-sided Wilcoxon rank-sum test with Bonferroni’s correction for multiple comparisons). Supplementary Video 3 shows the visual stimuli and Supplementary Table 3 provides the full list of genotypes.

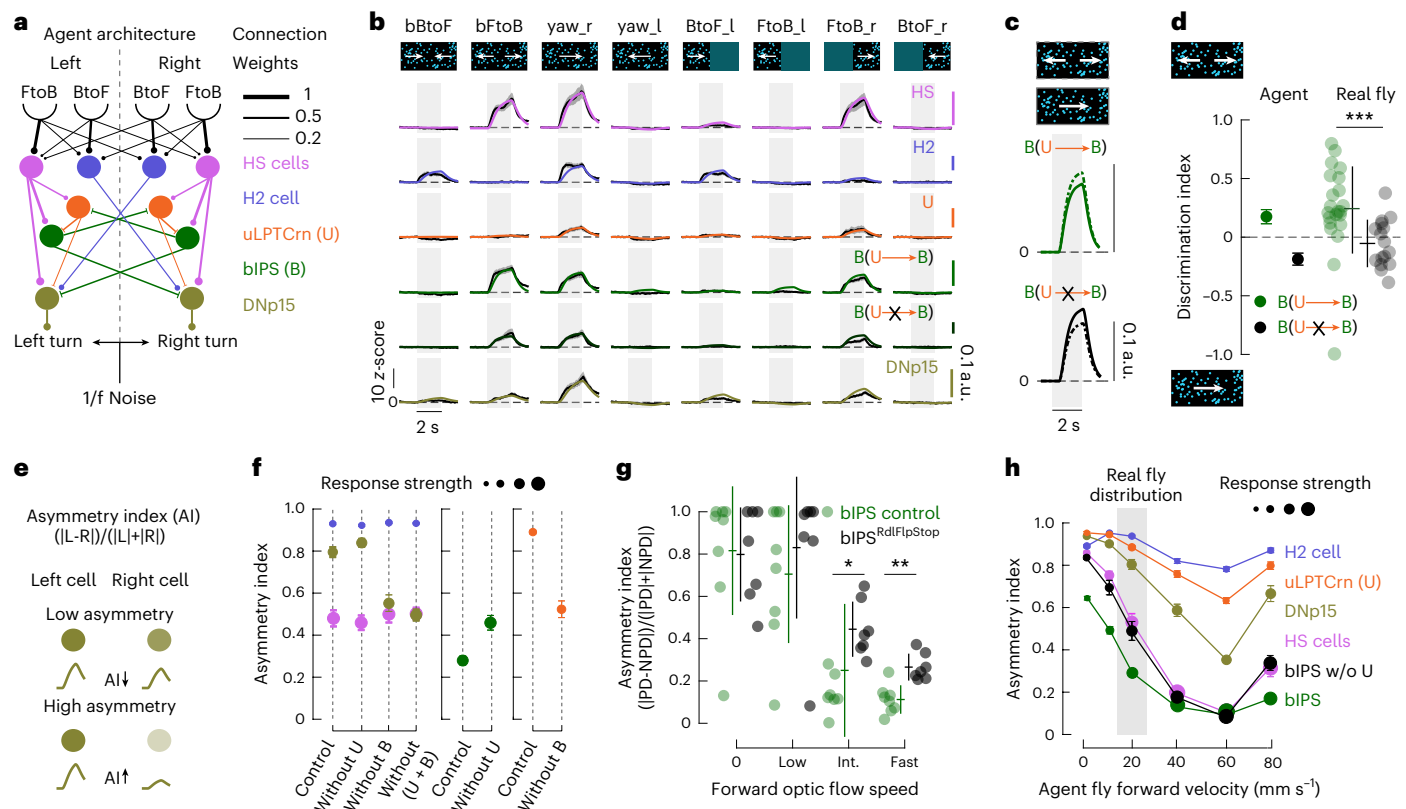


Fig. 6 | A simple model tests mechanisms for rotational optic flow estimation.

a, Schematic of the model architecture: two sets of Reichardt detectors, tuned to FtoB or BtoF motion detect visual motion in a simulated walking fly (Methods and Extended Data Fig. 9). This visual input is further processed by a network mimicking the core circuit motif of H2–HS subnetwork (Fig. 3a), with connection weights tuned to measured visual responses (Extended Data Fig. 9a–c and Methods). **b**, Simulated (colored) and real neuron (black) responses to unilateral and bilateral motion. Simulated responses are scaled to match the maximum response of each recorded cell, which are replotted from Extended Data Fig. 5 and Fig. 4c. Real traces represent grand mean z-scored responses $\pm$ s.e.m. (shaded). 'yaw_l/r', yaw left/right; 'BtoF_l/r', BtoF left/right; 'FtoB_l/r', FtoB left/right. **c**, Simulated bIPS responses to FtoB (dashed line) and yaw motion (solid line), with (green) or without (dark green) input from uLPTCrns. **d**, Simulated bIPS FtoB-Yaw discrimination index with or without input from uLPTCrns (left). $n = 5$ simulations per condition, error bars indicate s.d. Real bIPS discrimination index with or without functional GABA-A receptors

(replotted from Fig. 4f) (right). Colored horizontal lines and black vertical lines represent mean and 95% CI, respectively. bIPS ($N = 18$ flies, $n = 22$ ROIs), bIPS^{Rdl-FlpStop} ($N = 11$, $n = 16$). **e**, Calculation of the asymmetry index (AI) with examples of low and high AI values. **f**, AI of simulated neurons (colored) in the full model (control), under bIPS silencing (without B), uLPTCrn silencing (without U) or both bIPS and uLPTCrn silencing (without U + B). Dot size corresponds to the neuronal response amplitude; $n = 20$ simulations per condition, error bars indicate s.d. **g**, AI of real bIPS neurons to compound optic flow at varying forward velocities, with (green) or without (dark green) functional GABA-A receptors. Each circle represents an individual ROI. Colored horizontal lines and black vertical lines represent mean and 95% CI, respectively. bIPS: $N = 7$ flies $n = 8$ ROIs, bIPS^{Rdl-FlpStop}: $N = 6$ flies $n = 7$ ROIs. **h**, AI of simulated neurons (colored) in the full model at varying forward velocities. $n = 20$ simulations per condition, error bars indicate s.d. Shaded area corresponds to the range of velocities observed for forward runs in real flies¹. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (two-sided Wilcoxon rank-sum test).

flow-sensitive neurons (HS, H2 and VS cells), which provide input to and receive feedback from two partially overlapping networks with distinct interneurons and outputs. Both networks feature reciprocal connections between GABAergic interneurons and LPTs (Fig. 2), shaping the output's sensitivity to both translational and rotational optic flow (Figs. 1 and 3–5). Notably, we identified a competitive disinhibition motif³¹ within the H2–HS network that likely amplifies binocular asymmetries in optic flow during body translation (Figs. 6 and 7). Given its projection to higher-order premotor areas and the VNC, we propose that the H2–HS network enables robust horizontal optic flow processing for gaze stabilization⁹, continuous course adjustments¹⁶ and internal representations of the fly's angular movements⁴⁷.

Traditionally, estimating gaze direction relative to body orientation has been considered dependent on extra-retinal information. In vertebrates, optic flow-sensitive neurons typically receive extra-retinal signals related to eye or head movement²¹, often integrated congruently⁴⁴. Similarly, during walking, HS cells receive extraretinal input related to angular velocity²³. The H2–HS network architecture suggests these signals originate from brain premotor areas and ascending

pathways from the VNC, converging in the network's middle layer (Extended Data Fig. 4). These multimodal inputs likely enhance self-motion estimation under ambiguous visual conditions, such as in open spaces or low light levels, or when distinguishing between self-generated versus externally generated optic flow based on expected retinal flow statistics²⁴. Indeed, decoding optic flow depends on locomotion, influencing visual neurons' speed sensitivity^{48–50}, perception⁸ and internal representations⁴⁷.

Here, we examined how visual signals alone are transformed within the H2–HS network, recording neural activity under nonbehaving conditions, which limited LPTs' speed sensitivity to naturalistic optic flow (Fig. 5 and Extended Data Fig. 1b). HS cells' weak responses to fast compound optic flow could be explained by their flicker-rate sensitivity, potentially interfering with motion detection, particularly in the presence of local parallax cues known to excite bona fide translational systems but not HS cells⁵¹. These constraints likely limited the network's output for fast, naturalistic optic flow stimuli. Future studies will investigate H2–HS network activity during locomotion to clarify its context-dependent properties. Nevertheless, our findings

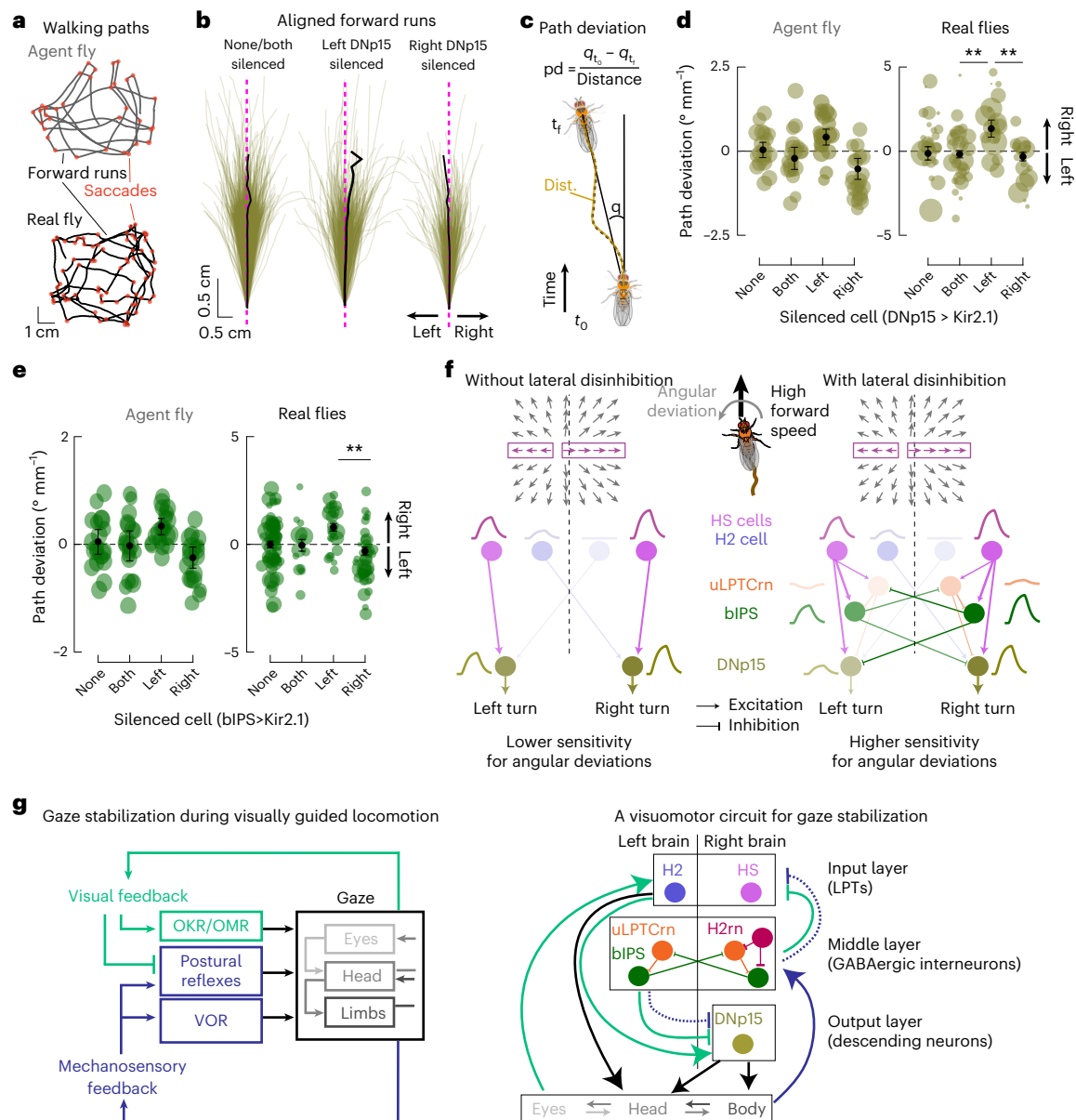


Fig. 7 | Examining model predictions in fly behavior. **a**, Example walking paths of the agent (top) and the real fly (bottom) under visual feedback. Rapid body turns (saccades) in between forward runs are highlighted in red. **b**, Forward walking paths covering at least four body lengths, aligned at their origin, in flies with both or none DNP15 expressing Kir2.1 (left) or with the left DNP15 (middle), or right DNP15 expressing Kir2.1 (right). Black lines show average left-right position as a function of forward displacement across all trajectories. **c**, Definition of path deviation. **d**, Simulated (left) and real fly (right) path deviations during forward runs with bilateral or unilateral expression of Kir2.1 in DNP15. Colored circles show mean bias, with circle size indicating number of walking bouts per fly. Black dots represent grand means weighted by walking bout number (dot size). Error bars show s.e.m. Number of flies: none $N = 25$; both $N = 31$; left $N = 22$; right $N = 22$. **e**, Same as **d**, but for bIPS silencing. Number of flies: none

$N = 65$; left $N = 26$; right $N = 38$; both $N = 13$. $**P < 0.01$ (two-sided Wilcoxon rank-sum test with Bonferroni's correction). Supplementary Table 3 provides a full list of genotypes. **f**, The middle layer of the H2-HS network supports steering by enhancing the difference in the activity levels of the output layer across hemispheres (higher activity illustrated by larger signal amplitude and higher node and edge opacity). This function depends on a competitive disinhibitory motif that is well suited for high-speed locomotion. We propose that this competitive disinhibition at the middle layer dynamically modulates the output layer, yielding a more robust estimate of deviations from a straight course. **g**, Core visuomotor circuit identified in this study extracting rotational components of naturalistic compound optic flow for eyes, head and body movement coordination in the context of horizontal (yaw) gaze stabilization.

suggest that the middle layer modulates DNP15's sensitivity to both translational and rotational components, emphasizing inhibition's key role for processing naturalistic optic flow and estimating self-rotation.

Fine tuning of inhibition for rotational optic flow estimation

In several brain regions, including the vertebrate optic tectum⁴⁵ and higher cortical areas in mammals^{22,52}, distinct optic-flow-sensitive neurons selectively encode either rotational or translational motion,

or both components jointly⁴⁴. During translation, when its binocular interactions with H2 cells tend to be silent (Extended Data Fig. 6 and Fig. 6), HS cells' activity can be ambiguous due to their response to rotational and translational motion (Fig. 1)⁴⁶. We show that the H2-HS network also integrates binocular information at the middle layer to extract rotational components during translation.

This study identified three classes of inhibitory neurons (bIPS, uLPTCrn and H2rn cells) (Fig. 2) with optic flow sensitivities

not fully explained by their synaptic input from LPTs (Fig. 3). Recurrent connections among these neurons play a key role in transforming optic flow signals within the middle layer (Figs. 4 and 5). Experiments and modeling suggest that GABAergic inputs to bIPS from uLPTCrns and H2rns, enhance selectivity to binocular symmetric horizontal optic flow (Fig. 6). We propose two primary roles for the inhibitory motifs in the H2–HS network. First, the competitive cross-disinhibition via recurrent, lateral interactions between bIPS and uLPTCrns biases binary steering choices (left or right) and stabilizes gaze (Fig. 7f,g, right). This cross-disinhibition also provides feedback to optic lobes (Fig. 2), likely modulating visual inputs to LPTs. In fact, a similar motif is found at earlier stages of optic flow processing, highlighting the fine tuning of optic flow by inhibition⁵³.

Second, reciprocal inhibition between LPTCrns and LPTs regulate the gain of the visual inputs and outputs, allowing flexible responses to internal and external conditions. With its selectivity and motor-related inputs, the middle layer of the H2–HS network is well suited for monitoring the motor state, influencing DNs, feedback pathways and central premotor circuits during walking. These findings emphasize the middle layer's crucial role in extracting rotational optic flow for gaze control in dynamic environments.

Optic flow networks: divergence and convergence in gaze control

Like vertebrates¹⁹, the *Drosophila* brain contains multiple optic flow-sensitive pathways, with about 60 classes of LPTs converging onto distinct central brain regions²⁰. Many LPTs, including VS cells, form lateral connections with other LPTs. A notable exception are the HS cells, which connect laterally only with contralateral H2 (refs. 26,27) (Fig. 2); however, VS, H2 and HS cells partially converge onto interneurons within the H2–HS and VS networks' middle layer. These LPTs encode three rotational axes (yaw, pitch and roll) aligning with their specialized role in gaze control^{16,24,29}.

Among middle layer interneurons, cLPTCrns connect to HS and VS cells, with stronger connections between yaw- and roll-sensitive LPTs, whereas bLPTCrns and uLPTCrns integrate yaw- and pitch-sensitive inputs (Fig. 2). This connectivity structure suggests a transformation, from encoding three independent axes of rotation at the LPT level to representing combined axes within LPTCrns in a reduced, abstract representation. Meanwhile, these central GABAergic interneurons receive distinct inputs from other visual, premotor and motor areas (Extended Data Fig. 4 and Supplementary Fig. 3). We propose that this recurrent, yet partially divergent network enables accurate processing of naturalistic optic flow while transforming visual input into a reference frame suited for head and body control, facilitating the integration of multisensory information in a common coordinate system for accurate feedback in gaze control. Such abstract transformations have been found in other gaze-related visuomotor systems^{54,55}.

Outlook

Visual feedback, including optic flow, is essential for multiple functions of locomotion^{9,19}. Optic flow pathways contribute to online, error-based movement adjustments, heading perception, smooth eye pursuit, object motion detection, calibration of internal models and gaze and course control^{21,56–58}; however, how these pathways, each encoding distinct features of optic flow, interact with the motor hierarchy underlying locomotion control remains unclear⁵⁹.

Our detailed characterization of the H2–HS network provides a foundation for exploring how visuomotor activity in these networks relates to locomotion across the motor hierarchy. The H2–HS network's output is highly divergent, with motor neurons, such as VCNMN⁶⁰ and DNs, such as DNp15 (ref. 32) and DNα02 (ref. 38), likely contributing to continuous control of head and body rotations. However, their specific roles may depend on behavioral contexts. While silencing these DNs bilaterally does not affect walking⁹, unilateral perturbations that

create bilateral asymmetries in their activity lead to biases in walking direction. This suggests that angular movement control depends on the bilateral activity of DN: even in coordinated configurations, slight asymmetries in a single DN class can produce subtle but measurable walking effects (Fig. 7b,d). Understanding when and how these gaze-control pathways function will provide insight into the complex interplay between VNC and brain circuits.

An important output of the H2–HS network, PS047, does not project to VNC but instead strongly targets the PS and LAL (premotor areas involved in steering and saccade control³⁷). These regions connect to higher-order centers for learning and navigation, such as the mushroom body and central complex, suggesting that PS047 may serve a different role than VNC projecting outputs. Given the conserved nature of optic flow processing across species^{10,21} and universal locomotor constraints, our findings offer valuable insights into how similar systems in other animals are functionally organized to support navigation.

Online content

Any methods, additional references, Nature Portfolio reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at <https://doi.org/10.1038/s41593-025-01948-9>.

References

- Cruz, T. L., Pérez, S. M. & Chiappe, M. E. Fast tuning of posture control by visual feedback underlies gaze stabilization in walking *Drosophila*. *Curr. Biol.* **31**, 4596–4607 (2021).
- Matthis, J. S., Yates, J. L. & Hayhoe, M. M. Gaze and the control of foot placement when walking in natural terrain. *Curr. Biol.* **28**, 1224–1233.e5 (2018).
- Kress, D. & Egelhaaf, M. Gaze characteristics of freely walking blowflies in a goal-directed task. *J. Exp. Biol.* **217**, 3209–3220 (2014). jeb.097436-.
- Davidson, M. J., Verstraten, F. A. J. & Alais, D. Walking modulates visual detection performance according to stride cycle phase. *Nat. Commun.* **15**, 1–12 (2024).
- Imai, T., Moore, S. T., Raphan, T. & Cohen, B. Interaction of the body, head, and eyes during walking and turning. *Exp. Brain Res.* **136**, 1–18 (2001).
- Michaël, A. M., Abe, E. T. T. & Niell, C. M. Dynamics of gaze control during prey capture in freely moving mice. *eLife* **9**, 1–27 (2020).
- Blaj, G. & Van Hateren, J. H. Saccadic head and thorax movements in freely walking blowflies. *J. Comp. Physiol. A Neuroethol. Sensory Neural Behav. Physiol.* **190**, 861–868 (2004).
- Horrocks, E. A. B., Mareschal, I. & Saleem, A. B. Walking humans and running mice: perception and neural encoding of optic flow during self-motion. *Philos. Trans. R. Soc. B Biol. Sci.* **378**, 20210450 (2023).
- Cruz, T. L. & Chiappe, M. E. Multilevel visuomotor control of locomotion in *Drosophila*. *Curr. Opin. Neurobiol.* **82**, 102774 (2023).
- Mauss, A. S. & Borst, A. Optic flow-based course control in insects. *Curr. Opin. Neurobiol.* **60**, 21–27 (2020).
- Simpson, J. I. The accessory optic system. *Ann. Rev. Neurosci.* **7**, 13–41 (1984).
- Robinson, D. A. The use of control systems analysis in the neurophysiology of eye movements. *Ann. Rev. Neurosci.* **4**, 463–503 (1981).
- Souman, J. L., Frissen, I., Sreenivasa, M. N. & Ernst, M. O. Walking straight into circles. *Curr. Biol.* **19**, 1538–1542 (2009).
- Berstein, N. *The Coordination and Regulation of Movements* (Pergamon, 1967).
- Koenderink, J. J. Optic flow. *Vision Res.* **26**, 161–179 (1986).

16. Fujiwara, T., Brotas, M. & Chiappe, M. E. Walking strides direct rapid and flexible recruitment of visual circuits for course control in *Drosophila*. *Neuron* **110**, 2124–2138.e8 (2022).
17. Wurtz, R. H. Optic flow: a brain region devoted to optic flow analysis? *Curr. Biol.* **8**, 554–556 (1998).
18. Barnes, W. J. P., Horseman, B. G. & Macauley, M. W. S. in: *The Crustacean Nervous System* (ed. Wiese, K.) 468–485 (Springer, 2002).
19. Matsuda, K. & Kubo, F. Circuit Organization underlying optic flow processing in zebrafish. *Front. Neural Circuits* **15**, 73 (2021).
20. Zhao, A. et al. A comprehensive neuroanatomical survey of the *Drosophila* lobula plate tangential neurons with predictions for their optic flow sensitivity. *eLife* <https://doi.org/10.7554/eLife.93659.1> (2024).
21. Britten, K. H. Mechanisms of self-motion perception. *Annu. Rev. Neurosci.* **31**, 389–410 (2008).
22. Duffy, C. J. MST neurons respond to optic flow and translational movement. *J. Neurophysiol.* **80**, 1816–1827 (1998).
23. Fujiwara, T., Cruz, T. L., Bohnslav, J. P. & Chiappe, M. E. A faithful internal representation of walking movements in the *Drosophila* visual system. *Nat. Neurosci.* **20**, 72–81 (2017).
24. Kim, A. J., Fenk, L. M., Lyu, C. & Maimon, G. Quantitative predictions orchestrate visual signaling in *Drosophila*. *Cell* **168**, 280–294.e12 (2017).
25. Krapp, H. G., Hengstenberg, R. & Egelhaaf, M. Binocular contributions to optic flow processing in the fly visual system. *J. Neurophysiol.* **85**, 724–734 (2001).
26. Farrow, K., Haag, J. & Borst, A. Nonlinear, binocular interactions underlying flow field selectivity of a motion-sensitive neuron. *Nat. Neurosci.* **9**, 1312–1320 (2006).
27. Pokusaeva, V. O., Satapathy, R., Symonova, O. & Jösch, M. Bilateral interactions of optic-flow sensitive neurons coordinate course control in flies. *Nat. Commun.* **15**, 8830 (2024).
28. Schnell, B. et al. Processing of horizontal optic flow in three visual interneurons of the *Drosophila* brain. *J. Neurophysiol.* **103**, 1646–1657 (2010).
29. Busch, C., Borst, A. & Mauss, A. S. Bi-directional control of walking behavior by horizontal optic flow sensors. *Curr. Biol.* **28**, 4037–4045.e5 (2018).
30. Koyama, M. & Pujala, A. Mutual inhibition of lateral inhibition: a network motif for an elementary computation in the brain. *Curr. Opin. Neurobiol.* **49**, 69–74 (2018).
31. Jovanic, T. et al. Competitive disinhibition mediates behavioral choice and sequences in *Drosophila*. *Cell* **167**, 858–870 (2016).
32. Suver, M. P., Huda, A., Iwasaki, N., Safarik, S. & Dickinson, M. H. An array of descending visual interneurons encoding self-motion in *Drosophila*. *J. Neurosci.* **36**, 11768–11780 (2016).
33. Takemura, S. et al. A connectome of the male *Drosophila* ventral nerve cord. *eLife* <https://doi.org/10.7554/eLife.97769.1> (2024).
34. Zheng, Z. et al. A complete electron microscopy volume of the brain of adult *Drosophila melanogaster*. *Cell* **174**, 730–743.e22 (2018).
35. Dorkenwald, S. et al. Neuronal wiring diagram of an adult brain. *Nature* **634**, 124–138 (2024).
36. Scheffer, L. K. et al. A connectome and analysis of the adult *Drosophila* central brain. *eLife* **9**, 1–74 (2020).
37. Namiki, S. & Kanzaki, R. in: *Insect Sex Pheromone Research and Beyond*, 243–264 (Springer, 2020).
38. Rayshubskiy, A. et al. Neural circuit mechanisms for steering control in walking *Drosophila*. *eLife* **13**, RP102230 (2024).
39. Clements, J. et al. NeuronBridge: an intuitive web application for neuronal morphology search across large data sets. *BMC Bioinform.* **25**, 1–25 (2024).
40. Eckstein, N. et al. Neurotransmitter classification from electron microscopy images at synaptic sites in *Drosophila melanogaster*. *Cell* **187**, 2574–2594.e23 (2024).
41. Fisher, Y. E. et al. FLPStop, a tool for conditional gene control in *Drosophila*. *eLife* **6**, e22279 (2017).
42. Stilwell, G. E. & Ffrench-Constant, R. H. Transcriptional analysis of the *Drosophila* GABA receptor gene resistance to dieldrin. *J. Neurobiol.* **36**, 468–484 (1998).
43. Moffat, K. G., Gould, J. H., Smith, H. K. & O’Kane, C. J. Inducible cell ablation in *Drosophila* by cold-sensitive ricin A chain. *Development* **114**, 681–687 (1992).
44. Sunkara, A., DeAngelis, G. C. & Angelaki, D. E. Joint representation of translational and rotational components of optic flow in parietal cortex. *Proc. Natl Acad. Sci. USA* **113**, 5077–5082 (2016).
45. Zhang, Y., Huang, R., Nöthenberg, W. & Arrenberg, A. B. A robust receptive field code for optic flow detection and decomposition during self-motion. *Curr. Biol.* **32**, 2505–2516.e8 (2022).
46. Kern, R., Petereit, C. & Egelhaaf, M. Neural processing of naturalistic optic flow. *J. Neurosci.* **21**, 1–5 (2001).
47. Hulse, B. K., Stanoev, A., Turner-Evans, D. B., Seelig, J. D. & Jayaraman, V. A rotational velocity estimate constructed through visuomotor competition updates the fly’s neural compass. Preprint at *bioRxiv* <https://doi.org/10.1101/2023.09.25.559373> (2023).
48. Jung, S. N., Borst, A. & Haag, J. Flight activity alters velocity tuning of fly motion-sensitive neurons. *J. Neurosci.* **31**, 9231–9237 (2011).
49. Chiappe, M. E., Seelig, J. D., Reiser, M. B. & Jayaraman, V. Walking modulates speed sensitivity in *Drosophila* motion vision. *Curr. Biol.* **20**, 1470–1475 (2010).
50. Andermann, M. L., Kerlin, A. M., Roumis, D. K., Glickfeld, L. L. & Reid, C. Functional specialization of mouse higher visual cortical areas. *Neuron* **72**, 1025–1039 (2011).
51. Isaacson, M. D., Eliason, J. L. M., Nern, A., Rogers, E. M. & Gus, K. Small-field visual projection neurons detect translational optic flow and support walking control. Preprint at *bioRxiv* <https://doi.org/10.1101/2023.06.21.546024> (2023).
52. Rasmussen, R. N., Matsumoto, A., Arvin, S. & Yonehara, K. Binocular integration of retinal motion information underlies optic flow processing by the cortex. *Curr. Biol.* **31**, 1165–1174.e6 (2021).
53. Ammer, G. et al. Multilevel visual motion opponency in *Drosophila*. *Nat. Neurosci.* **26**, 1894–1905 (2023).
54. Masino, T. & Knudsen, E. I. Horizontal and vertical components of head movement are controlled by distinct neural circuits in the barn owl. *Nature* **345**, 434–437 (1990).
55. Soechting, J. F. & Flanders, M. Moving in three-dimensional space: frames of reference, vectors, and coordinate systems. *Annu. Rev. Neurosci.* **215**, 167–191 (1992).
56. Warren, J., Kay, B. A., Zosh, W. D., Duchon, A. P. & Sahuc, S. Optic flow is used to control human walking. *Nat. Neurosci.* **4**, 213–216 (2001). 2001 42.
57. Madhav, M. S. et al. Control and recalibration of path integration in place cells using optic flow. *Nat. Neurosci.* **27**, 1599–1608 (2024).
58. Krause, T., Spindler, L., Poeck, B. & Strauss, R. *Drosophila* acquires a long-lasting body-size memory from visual feedback. *Curr. Biol.* **29**, 1833–1841.e3 (2019).
59. Chiappe, M. E. Circuits for self-motion estimation and walking control in *Drosophila*. *Curr. Opin. Neurobiol.* **81**, 102748 (2023).
60. Gorko, B. et al. Motor neurons generate pose-targeted movements via proprioceptive sculpting. *Nature* **628**, 596–603 (2024).
61. Nern, A., Pfeiffer, B. D. & Rubin, G. M. Optimized tools for multicolor stochastic labeling reveal diverse stereotyped cell arrangements in the fly visual system. *Proc. Natl Acad. Sci. USA* **112**, E2967–E2976 (2015).

62. Lyu, C., Abbott, L. F., & Maimon, G. Building an allocentric travelling direction signal via vector computation. *Nature* **601**, 92–97 (2021).
63. Molina-Obando, S. et al. On selectivity in the *Drosophila* visual system is a multisynaptic process involving both glutamatergic and GABAergic inhibition. *eLife* **8**, e49373 (2019).
64. Asahina, K. et al. Tachykinin-expressing neurons control male-specific aggressive arousal in *Drosophila*. *Cell* **156**, 221–235 (2014).
65. Diao, F. et al. Plug-and-play genetic access to *Drosophila* cell types using exchangeable exon cassettes. *Cell Rep.* **10**, 1410–1421 (2015).
66. Bates, A. S. et al. The natverse, a versatile toolbox for combining and analysing neuroanatomical data. *eLife* **9**, e53350 (2020).

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

Open Access This article is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International License, which permits any non-commercial use, sharing, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if you modified the licensed material. You do not have permission under this licence to share adapted material derived from this article or parts of it. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by-nc-nd/4.0/>.

© The Author(s) 2025

Methods

Key resources

The key resources used are listed in Table 1.

Animal research organism

This study used adult *Drosophila melanogaster*. The European Council Directive 86/609/EEC, updated and replaced by Directive 2010/63/EU of the European Parliament and of the Council, on the protection of animals used for scientific purposes, does not apply to insects, including *D. melanogaster*.

Drosophila husbandry

D. melanogaster flies were reared on standard fly medium and kept on a 12-h light–dark cycle at 25 °C. All experiments were performed with 3–4-day-old flies. Calcium imaging experiments were carried out with females due to their larger brain size, while behavioral experiments were performed with males, which exhibit more continuous walking in our experimental conditions. The complete list of transgenic flies and their specific sources is provided in Table 1, with full genotypes used in each experiment detailed in Supplementary Table 3.

Generation of transgenic lines and mutant flies

We regenerated the R50G03-Gal4 transgenic line by reinjecting the original plasmid. R50G03-AD and R50G03-DBD stocks were generated using standard procedures⁶⁷. For the R39E01–LexA, 13xLexAop–6xmCherry and R39E01–LexA, 13xLexAop2–IVS–GCaMP6f recombinants, transgenic lines carrying R39E01–LexA were crossed with mCherry or GCaMP6f lines. Recombinant progeny were identified by eye color and confirmed via immunostaining.

To generate cell type-specific mutants for the GABA-A receptor (*rdl*), we used the Rdl-FlpStopND cassette⁴¹. Instead of combining it with a *rdl* null mutant, we used two copies of the FlpStop cassette. Given that many neurons in the H2–HS and VS networks are sensitive to GABA-A signaling and that these networks likely balance excitation and inhibition, we aimed to avoid unintended disruptions to network function, particularly due to existing reciprocal connections (Fig. 2).

For the VT014724-p65.DB, Rdl-FlpStopND and R77F05-p65.DB, Rdl-FlpStopND recombinants, eye color selection was not possible, as the FlpStop cassette lacks the *mini-white* gene. Instead, we established stable lines from single progeny and confirmed recombinants by PCR, using previously designed primers targeting the FlpStop cassette in the ND orientation⁴¹:

Mil-F: GCGTAAGCTACCTTAATCTCAAGAAGAG
FRTspacer_5p_rev: AAATGGTGCAAGAGAAGTTC

We also attempted to specifically silence b1PS while recording DNp15 activity (DNp15^{b1PS>TNT}), to replicate experiments with HS cells (Fig. 5c); however, the DNp15–LexA line exhibited a trans-repression effect⁶⁸. While DNp15 expression and calcium signals were detectable with DNp15–LexA alone at the attP40 landing site, introducing Empty-AD or b1PS-AD constructs suppressed DNp15 expression. We generated other DNp15–LexA lines, but these gave no stable expression. To circumvent these challenges, we adopted the Rdl-FLPStop silencing strategy for DNp15. Although this method disrupts all GABA-A-dependent inputs and is less specific than targeting b1PS, it avoids the trans-repression problem by eliminating dependence on LexA lines.

Electron microscopy datasets

We used three different EM datasets: a full female adult *Drosophila* brain imaged using serial section transmission EM (FAFB³⁴), a dense reconstruction of a partial adult female brain imaged using focused ion-beam scanning EM, referred to as Hemibrain³⁶ (<https://neuprint.janelia.org/>, Hemibrain v.1.2.1) and a male adult VNC, referred to as MANC³³.

In FAFB, neuron skeletons were either manually traced using CATMAID (FAFB v.14)^{69,70}, following the procedure described previously³⁴, or automatically segmented within the FlyWire³⁵ environment (<https://flywire.ai/>, data snapshot v.630) along with cell typing⁷¹ and chemical synapse predictions⁷². None of the EM datasets contained information about electrical synapses.

A limitation of our EM connectomics approach is the inability to detect gap junctions. Dye injection into HS cells has revealed potential electrical coupling with b1PS^{27,28}, DNp15 (refs. 27,32) and VCNMN²⁷ cells, suggesting that the H2–HS network uses both chemical and electrical synapses to operate at different timescales, fulfilling both reflexive and modulatory functions.

Neuronal EM reconstructions and synaptic tagging

Manual neuron tracing in FAFB was performed either for ‘identification’ or ‘completion’⁷³. Completion involved reconstructing the full arbor of a neuron, while identification involved tracing the soma and major axonal and dendritic branches containing microtubules. The identification approach reduced manual tracing time considerably by omitting small microtubule-free twigs while allowing for unique cell type identification.

All HS, H2 and VS cells were traced to completion. Strong LPT partners were traced for identification first and later completed. Extended Data Fig. 2c shows the number of identified inputs and outputs of HS, H2 and VS cells relative to their total synaptic connections. For nearly all manually traced FAFB neurons, we obtained complete reconstructions in FlyWire with only minor differences in their twigs. Semi-automatic segmentation in Hemibrain and FlyWire proved challenging for a small subset of neurons with darker cytosol and heavily striated dendritic branching. One such cell (DNp15) was manually traced for completion, and its input profile was analyzed in the FAFB dataset (Extended Data Fig. 4c).

A recent connectome analysis found that the FAFB dataset is left–right inverted⁷¹. This means that right hemisphere neurons in FAFB/FlyWire correspond to the left hemisphere neurons in real-world coordinates. As inter-hemisphere connectivity is highly stereotyped within and across EM datasets⁷¹, we retain the inverted neuron naming in FAFB. This allows for a more intuitive comparison with the Hemibrain dataset, which primarily covers the right side of the brain. Notably, this does not affect our results.

We tagged the incoming (postsynaptic) and outgoing (presynaptic) chemical synapses of HS, VS and H2 cells in their axon terminals, as well as those of strong LPT partners in FAFB. As a rule, we tagged all synaptic connections between any pair of traced neurons. Therefore, even if a neuron does not have complete tagged synapses, we still obtained the total number of synaptic connections among all fully or nearly fully traced neurons in FAFB. Additionally, we used FlyWire to plot the input and output profiles of LPTCrns (Extended Data Fig. 4) and MANC to visualize the output profile of DNp15 in the VNC (Fig. 1e).

Selection of LPT partners to reconstruct

We traced all HS, all H2, along with over 90% of VS axo-axonic inputs for identification (Extended Data Fig. 2c). As LPT axo-dendritic outputs are approximately ten times more numerous than axo-axonic inputs, we used a pseudo-random sampling approach for output tracing. In FAFB, strongly connected partners often form multiple axo-dendritic connections around the same presynaptic bouton. To identify ‘putative strong’ partners, we visually prioritized neurons that sent multiple dendrites to LPTs within a small area. Overall, we identified about 35% of HS outputs, and 20% of H2 and VS outputs, focusing on the most strongly connected neurons shared across datasets (Supplementary Fig. 1). We also cross-checked the strongest LPT partners in the Hemibrain and FlyWire datasets, reconstructing any missing one in FAFB. This strategy enabled us to capture all major LPT partners with minimal manual tracing.

EM to light microscopy matching

We used the natverse R libraries⁶⁶, particularly templatebrains and rcattmaid, to register FAFB neurons onto the JFRC2010 template, widely used in Janelia FlyLight transgenic line libraries. Aligned FAFB skeletons were transformed onto two-dimensional (2D) ColorMIP images⁷⁴ and computationally match these images with Gal4 lines from FlyLight. These shortlisted lines were then combined to generate Split-Gal4 lines with sparse expression⁶⁷. Stability and sparseness of each split combination were assessed by examining GFP expression in several brains using confocal microscopy.

Immunohistochemistry

Brains from 2–4-day-old flies were dissected PBS. Immunostaining followed a slightly modified protocol²³. After dissection, brains were fixed with 4% paraformaldehyde (PFA) for 20 min at room temperature or 1 h on ice. Before primary antibodies application, samples were incubated in 10% normal goat serum for 15 min (or in 5% serum for 1 h) with 0.1% Triton X-100 in PBS.

Green fluorescent protein (GFP) and GCaMP expression were labeled using chicken anti-GFP (Abcam, ab13970, 1:1,000 dilution). Brain neuropile was labeled with mouse anti-nc82 (Developmental Studies Hybridoma Bank, 1:10 dilution). For flies expressing tdTomato or mCherry, rabbit anti-DsRed (Abcam, ab356483, 1:500 dilution) was included. GABA staining used chicken anti-GFP with rabbit anti-GABA (1:100 dilution; Sigma-Aldrich, A2052). Secondary antibodies included AlexaFluor594 goat anti-rabbit (Abcam, ab150088, 1:500 dilution), AlexaFluor488 goat anti-chicken (Abcam, ab150173, 1:500 dilution) and AlexaFluor633 goat anti-mouse (Life Technologies, A-21050, 1:500 dilution). Confocal sections were acquired with Zeiss LSM710 confocal microscope (using Zeiss ZEN software) at 1 or 20- μ m intervals, and maximum intensity projections were generated using Fiji/ImageJ (v.1.54f) (<https://imagej.net/software/fiji/>).

Fly preparation and calcium imaging

To ensure consistent body size, six flies were anesthetized with CO₂ after eclosion and housed together. On imaging day (3–4-day-old flies), flies were cold-anesthetized and their wings and legs were removed to reduce sensory re-afference. Leg openings were sealed with beeswax, and the proboscis was gently extended and waxed from below to minimize brain movement. This preparation has limited wing and abdomen movement and preserved haltere and antenna mobility. Flies were mounted on a custom-made holder by pinning the thorax to a tungsten wire (A-M Systems, 716100) using UV-cured glue (Bondic). A micromanipulator and triple camera system were used to ensure consistent head and body orientation for mounting with wax and removing the pin. The cuticle at the back of the head was removed with sharp forceps (Dumont 55F). Fat body tissue was gently suctioned out and trachea and muscles 1 and 16 were removed with fine forceps. High-resolution images of head orientation (pitch, yaw and roll) were taken post-experiment²⁴.

Flies were mounted under an upright microscope (Movable Objective Microscope, Sutter) equipped with a $\times 40$ water-immersion objective lens (CFI Apo 40XW NIR, Nikon). Calcium imaging was performed using a custom-built MIMMs 2.1 two-photon laser scanning system (<https://www.janelia.org/open-science/mimms-22-2024>), with a Chameleon Ultra II Ti:Sapphire femtosecond laser (Coherent) tuned to 930 nm (for GCaMP and tdTomato) and 780 nm (for mCherry) controlled by ScanImage (v.3.8, MATLAB 2013b). Maximum power under the objective lens was 6 mW. Emission was collected on GaAsP PMT detectors (Hamamatsu H10770PA-40) through 535/50 nm (green) and 605/70 nm (red) bandpass filters (Chroma). A 128 $\times$ 128-pixel image slice was acquired at the dorsal IPS, where all imaged neurons overlap, with a frame rate of 12.2–15.0 Hz. When mCherry or tdTomato was coexpressed with GCaMP, high-resolution 3D z-stacks were taken pre- or post-experiment. The preparation was continuously perfused

with an external solution (270–275 mOsm) containing 103 mM NaCl, 3 mM KCl, 5 mM TES, 8 mM D-trehalose, 10 mM D-glucose, 26 mM NaHCO₃, 1 mM NaH₂PO₄, 4 mM MgCl₂ and 2 mM CaCl₂ in MilliQ water, bubbled with 95% O₂ and 5% CO₂ (–pH 7.3).

Visual display and visual stimuli

The visual display consists of a 32 $\times$ 96 array of blue LEDs (465 nm, Bright LED Electronics) based on the Generation 3 (G3) modular display⁷⁵ design. To minimize light leakage into the two-photon detection system, four layers of blue filter (Rosco R385) were added. For a centrally positioned fly, the display covered 216° azimuth and 72° elevation, with a pixel size of 2.25°. The display was then tilted 74° to match the head pitch angle of imaged flies.

Each trial began with a stationary pattern, followed by 2-s rotation (clockwise/counter-clockwise for pitch, yaw and roll) or translational (forward/backward thrust and left/right sideslip) motion sequences interspersed with still periods. Trials lasted 13 s (2, 2, 5, 2 and 2 s per sequence), with a 5-s dark intertrial interval. Stimuli were repeated 3–12 times per fly in a randomized order. Speed-tuning trials used a 6-s still-motion-still sequence (2 s per phase), with randomized order per fly (Supplementary Videos 1 and 2 show motion and speed-tuning stimuli).

Visual stimuli design

Starfield stimuli⁷⁶ were generated using MATLAB (MathWorks) using modified scripts for G3 display compatibility⁷⁷ (https://github.com/misaacson01/Motion_Maker_G4). A virtual 2D spherical volume space was populated with 500 uniformly distributed dots (5° solid angle diameter) and only overlapping pixels between the virtual sphere and the LED were illuminated. To enhance motion perception, edges were blurred using 16 grayscale intensity levels, reducing motion-independent flicker.

Compound stimuli, simulating naturalistic optic flow, were generated based on the average angular velocity during forward runs of freely walking flies in our arena (111° s^{–1}) and added varying forward velocities:

- 0 mm s^{–1} pivoting (pure rotation)
- 5.5 mm s^{–1} slow walking
- 11 mm s^{–1} intermediate walking speed
- 16.5 mm s^{–1} fast walking

Using an 8-mm spherical treadmill, we converted angular velocity (1.937 rad s^{–1}) and the corresponding forward velocities to 0, 1.375 ('low'), 2.751 ('intermediate') and 4.146 ('fast') rad s^{–1}. Note that these conversions facilitate combining rotational and translational velocities in a principled manner and do not directly correspond to velocities on a flat surface.

Here, the translational component has no relative distance information (no looming cues) while rotations are simplified to laminar flow and no perspective cues were considered^{78,79}.

For each LED pixel, horizontal and vertical motion vectors were computed as follows:

For uniform horizontal rotation, the vectors are:

$$\mathbf{U}_{\text{rot}} = \alpha \times \omega_{\text{rot}}$$

$$\mathbf{V}_{\text{rot}} = 0 \times \omega_{\text{rot}},$$

with $\alpha = 1$ or $\alpha = -1$ for the two opposite directions of rotation.

For pure forward translation with a weight of ω_{trans} , the vectors are:

$$\mathbf{U}_{\text{trans}} = \left(X / \left((X^2 + Y^2)^{0.5} \right) \right) \times \omega_{\text{trans}}$$

$$\mathbf{V}_{\text{trans}} = \left(Y / \left((X^2 + Y^2)^{0.5} \right) \right) \times \omega_{\text{trans}}$$

The compound vectors are the sum of the rotational and translational vectors:

$$\mathbf{U} = \mathbf{U}_{\text{rot}} + \mathbf{U}_{\text{trans}}$$

$$\mathbf{V} = \mathbf{V}_{\text{rot}} + \mathbf{V}_{\text{trans}}$$

We set ω_{rot} at 1 and varied ω_{trans} at 0, 0.71, 1.42 and 2.14 to simulate different walking speeds. We also included a translation-only control ($\omega_{\text{rot}} = 0$), and a flicker control ($\omega_{\text{trans}} = 0$ and $\omega_{\text{rot}} = 0$).

Preliminary experiments indicated rapid response attenuation at high velocities, likely exceeding the cells' speed-tuning sensitivity^{48,49}. Therefore, we decreased the overall velocity of all compound stimuli by half (Supplementary Video 3 shows compound optic flow stimuli).

Choice of indicators for neural activity

H2 and HS cells hyperpolarize in response to nonpreferred motion²⁸, suggesting that their downstream partners exhibit similar responses. Ideally, voltage changes in these neurons would be recorded directly, but their small somas and locations restricted visual stimulation during whole-cell patch recordings. Additionally, genetically encoded voltage sensors (ASAP4)⁸⁰ proved unstable for long recordings (>1 h).

To record neural activity through the H2–HS network over time, we opted for calcium imaging. While this method primarily detects depolarization due to rectification effects, it offered a stable and informative compromise.

We tested several GCaMP variants and found that sytGCaMP7f⁶² provided the best signal-to-noise ratio and enabled axon terminals imaging, allowing differentiation of right and left bIPS axons. Thus, sytGCaMP7f was used in most of our recordings. For DNp15 neurons, which lack brain axon terminals, we used GCaMP7f⁶¹ instead. In FlpStop experiments with bIPS and DNp15, we used cytoplasmic GCaMP6f⁶², which was previously recombined with UAS–FLP⁶³. Anatomical landmarks provided by tdTomato expression (or mCherry expression in controls) and the direction selectivity of recorded ROIs helped distinguish dendritic from axonal signals in bIPS.

Data acquisition and alignment for imaging

Post-experiment, imaging frames were motion-corrected in MATLAB (2018a) using subpixel image registration⁸³ to align each frame with a time-averaged template. Multiple recordings from the same fly were registered to a common template if positional shifts were minimal. In a few cases where motion correction caused abnormal frame shifts despite negligible brain movement, raw image traces were analyzed instead. Trials where neurons moved out of the imaging plane were discarded.

Exploratory walking arena

The virtual reality setup for exploratory walking was previously described¹. In brief, 2–4-day-old males with clipped wings walked freely in a 90-mm circular arena with heated walls, while a random dot array projected from below provided prominent visual feedback. The stimulus moved with the fly using real-time tracking, so only body rotations provided optic flow.

To label single bIPS or DNp15 cells, flies received a 30 °C heat shock for up to 15 min after wing removal post-eclosion. To prevent interference with genetic expression from wall heating, brains were dissected and fixed immediately after each experiment to confirm cell labeling.

Agent simulations

A simulated rigid-body agent moved forward or performed a saccade (Extended Data Fig. 9d), with bout lengths matching real flies¹. The agent explored a virtual 90-mm circular arena with heated walls, where

saccades were modeled as stereotyped rapid changes in angular velocity influenced by wall distance. Time intervals between saccades were forward runs at a constant translational speed (20 mm s^{−1}), except for Fig. 6h, where the speed varied. Slow rotations were introduced using 1/f noise (η), scaled by the 'noise level' (w_{noise}) parameter to match real fly movements in darkness (Extended Data Fig. 9f).

Agent rotations were controlled by a visual system based on optic flow, detected using a 'two-quadrant' type Hassenstein–Reichardt (HR) correlator in four channels⁸⁴. Visual motion cues were processed via high pass filtering ($\tau = 50$ ms), followed by a half-wave rectification, low-pass filtering (first order, $\tau = 15$ ms) and spatial multiplication. The final opponent direction-selective output signal was scaled by the parameter 'visual weight' (w_{visual}) to match real fly path straightness under visual feedback¹ (Extended Data Fig. 9g,h). Each eye independently detected FtoB and BtoF visual rotations, simulating HS and H2 cell responses.

$$HS_{\text{right}} = w_{\text{HSPD}} \times FtoB_{\text{right}} - w_{\text{HSNDP}} \times BtoF_{\text{right}} + w_{\text{HSContra}} \times BtoF_{\text{left}}$$

$$H2_{\text{right}} = w_{\text{H2PD}} \times BtoF_{\text{right}} - w_{\text{H2NDP}} \times FtoB_{\text{right}} + w_{\text{H2Contra}} \times FtoB_{\text{left}}$$

Weights were manually selected across six simulations to best match experimental data, evenly distributed between 0 and 1, and optimized sequentially for input, middle and output layers. Specifically, we first adjusted and fixed the weights for HS and H2, then for bIPS and uLPTCrns, and finally for DNp15. At the simulated walking speed¹, H2 is only minimally excited, and the model simulations confirms this expectation (Fig. 6h). H2rns were excluded to avoid overfitting as their activation depended on H2 activity. Weight tuning was validated against additional visual stimuli beyond those used for fitting (Fig. 6b and Extended Data Fig. 9a,b). This approach allowed us to ensure that the model's responses generalized beyond the stimuli used for fitting.

Modeled responses were compared to calcium imaging data by convolving outputs with GCaMP rise and decay times (Fig. 6b). It is important to note that this is intended for visualization purposes only. Our model focuses on steady-state responses matched to the steady-state GCaMP response amplitudes without temporal dynamics. Following HS and H2, the middle layer inhibitory neurons, bIPS (B) and uLPTCrns (U), were simulated according to the architecture outlined in Fig. 6a.

$$B_{\text{right}} = w_{\text{HSB}} \times HS_{\text{right}} + w_{\text{H2B}} \times H2_{\text{left}} - w_{\text{UB}} \times U_{\text{right}}$$

$$U_{\text{right}} = w_{\text{HSU}} \times HS_{\text{right}} + w_{\text{H2U}} \times H2_{\text{left}} - w_{\text{BU}} \times B_{\text{left}}$$

All neurons converge onto DNp15, modeled after the H2–HS network architecture.

$$DNp15_{\text{right}} = w_{\text{HSp15}} \times HS_{\text{right}} + w_{\text{H2p15}} \times H2_{\text{left}} - w_{\text{Up15}} \times U_{\text{right}} - w_{\text{Bp15}} \times B_{\text{left}}$$

Final DNp15 weights were tuned iteratively to match horizontal visual motion responses in Extended Data Fig. 1c. The final table of weights is presented in Extended Data Fig. 9c. The agent's course control depended on DNp15's left versus right activity difference

$$V_{\text{angular}} \left(^\circ / s \right) = w_{\text{visual}} \times (DNp15_{\text{right}} - DNp15_{\text{left}}) + w_{\text{noise}} \times \eta$$

Where w_{visual} , w_{noise} and η correspond to the visual weight, noise level and injected 1/f noise, respectively. Neuronal silencing was simulated by setting activity to zero, while activation was set to 0.1. The AI quantified differences between left and right neural activity:

$$AI_{\text{neuron}} = \frac{|Neuron_{\text{left}} - Neuron_{\text{right}}|}{|Neuron_{\text{left}}| + |Neuron_{\text{right}}|}$$

Data analysis

EM connectivity matrices and synaptic clustering. Using natverse⁶⁶ libraries (v.0.2.3) in R Studio (v.1.1.453), we loaded HS, VS and H2 cells from FAFB, pruning optic lobe dendrites to retain only central brain projections. Next, we retrieved their synaptic partners and generated an all-by-all connectivity matrix, where each entry represents the number of synapses from a row neuron to a column neuron (Supplementary Fig. 2).

To simplify and focus our connectivity analysis on the strongest synaptic partners, we removed weak partners that did not meet our connectivity threshold. We analyzed the distributions of synaptic connections to LPTs and selected thresholds where these distributions flattened (Extended Data Fig. 2d), thereby emphasizing the strongest synaptic partners for both inputs and outputs. This method ensures that we capture the most influential input and output partners without including many weakly connected cells.

To identify neuron clusters, we summed input and output synapses per neuron pair, creating a symmetric connectivity table. We then binarized it (setting all nonzero connections to 1) to prevent strong pairwise connections from skewing results while amplifying the role of weaker ones. Weak connections (fewer than five synapses) were set to zero before binarization. A similarity distance matrix was generated from this table, followed by hierarchical clustering using Ward's method, producing the connectivity dendrogram (Fig. 2a). The dendrogram reordered the connectivity matrix, grouping clustered neurons together.

We also repeated the clustering without binarization or partner removal (Supplementary Fig. 2) to confirm the network structure remained consistent, ensuring our analysis was not biased by parameter choices.

Network connectivity graphs. After clustering, neurons in each group were loaded into CATMAID (FAFB v.14)⁷⁰ and classified into cell types based on anatomy, forming a graph where nodes represent cell types and directed edges indicate synapse counts. The adjacency matrix was then imported into Gephi, where edge and node properties (such as color and size) were adjusted for visualization. Edge end points were shaped based on predicted primary neurotransmitters⁴⁰.

To assess the prominence of each neural class, we plotted the weighted degree, representing the total incoming and outgoing synapses per node (Fig. 2c,d and Extended Data Fig. 3b).

Anatomical clustering of LPT inputs. To quantify anatomical similarities among LPTCrn cell types (Extended Data Fig. 3e), we performed NBLAST⁸⁵ on their primary neurite tracts within dendrites, excluding distal twigs to enhance sensitivity to stereotyped features. We then applied hierarchical clustering using Ward's method on Euclidean distance matrices derived from the NBLAST scores.

Synaptic input and output profiles. We plotted the input profile of HS, H2 and VS cells (Extended Data Fig. 3c), grouping them by LPTCrn cell type. To map LPTCrn neuropil input (Extended Data Fig. 4c) and output (Extended Data Fig. 4b), we retrieved upstream partners from FlyWire using CAVE⁸⁶ and natverse⁶⁶, analyzing synaptic sites⁷² with a cleft score⁸⁷ greater than 50 to minimize false positives. The input neuropil was assigned based on the brain region with the most post-synaptic (dendritic) sites. Similarly, the output neuropil was defined by the region with the most presynaptic (axonal) sites. Fragments without a soma or with <200 synaptic sites were visually inspected. Fragments that project to the neck connective were classified as VNC/neck neurons, while the remaining fragments were excluded from the analysis.

For DNp15 outputs (Fig. 1e), we performed the same analysis using MANC³³, classifying soma-bearing, neck-projecting partners as ascending neurons (ANs). For DNp15 inputs (Extended Data Fig. 4c) we fully reconstructed the right DNp15 in FAFB, manually tagging all synapses.

Approximately 89% of partners were traced for identification, and their primary input neuropil was determined. DNp15 lacks axon terminals in the brain.

For the neurotransmitter input profile of bIPS (Fig. 4a) we regrouped right bIPS upstream partners from FlyWire based on their predicted neurotransmitter⁴⁰.

Distance-to-root and inter-synaptic distance analyses on bIPS. We retrieved right bIPS input locations from FAFB using natverse R⁶⁶. Synapse locations from right HS, H2rn, uLPTCrn and left H2 cells were mapped onto a bIPS graph via nabor library. The root point of bIPS was defined as the soma-axon-dendrite junction, where dendritic arbors converge before crossing the midline (Extended Data Fig. 7a). Using igraph library, we computed geodesic distances from each input synapse to the root point (Extended Data Fig. 7b,c), following an approach similar to Hulse et al.⁸⁸. Geodesic distances between H2rn inputs and the nearest H2 or HS input were calculated similarly (Extended Data Fig. 7d).

The nonrandom input locations of bIPS suggest a structured microcircuit organization. We assumed for each presynaptic cell, only one synapse contributes to a microcircuit and set 1.865 μm as the maximum inter-synapse distance (Extended Data Fig. 7d, black line), based on the distances between H2rn and H2 synapses. We then counted how often a cell type (cell X) formed a microcircuit with H2 (Extended Data Fig. 7e,g) or HS (Extended Data Fig. 7f,h) upstream of bIPS (Extended Data Fig. 7e,f) or DNp15 (Extended Data Fig. 7g,h), normalizing by total occurrences per motif.

Calcium responses to visual motion. ROIs were manually defined post-imaging. For single neuron lines (for example, H2 and bIPS), we used a single ROI. For multi-neuron lines, we initially selected multiple ROIs. After confirming qualitatively similar responses among all ROIs, we chose the one with the highest signal-to-noise ratio. No more than two ROIs were recorded per fly, one per hemisphere.

Fluorescence (F) was measured per trial, with baseline fluorescence (F_0) taken from the last pre-stimulus second. Calcium response change was calculated as:

$$\Delta F = F - F_0$$

To avoid artifacts from $\Delta F/F$ calculations⁸⁹, we instead z-normalized the ΔF data for comparison across neurons. Imaging data were down-sampled to 12.2 Hz and left-side recordings were flipped to match right-side responses. Stimuli (for example, roll clockwise, yaw left) were converted to their midline-symmetric versions (for example, roll counter-clockwise, yaw right) as described previously⁷⁶. All stimulus repetitions were averaged to obtain a mean response per ROI.

Optic flow response and discrimination indices. Optic flow (OF) responses were calculated using the area under the curve over 2 s of visual stimulation or by taking the 'peak' response averaged over the final 500 ms. Preferred (PD) and nonpreferred (NPD) motion directions were defined using right HS responses. OF responses (Fig. 3c) were plotted as:

$$\text{OF}_{\text{yaw}} = \text{Peak}_{\text{PD}} - \text{Peak}_{\text{NPD}}$$

In Fig. 3d, OF responses were normalized to peak OF per ROI. The discrimination index (DI) quantified neuronal selectivity between optic flow patterns (for example, yaw versus progressive) and is calculated as the difference between OF values divided by the sum of the absolute OF values:

$$\text{DI} = \frac{\text{OF}_{\text{Prog}} - \text{OF}_{\text{Yaw}}}{|\text{OF}_{\text{Prog}}| + |\text{OF}_{\text{Yaw}}|}$$

Classification of walking paths, straightness, angular deviations, and biases. Walking paths were classified as saccadic events or forward runs¹ (Fig. 7a), where a forward run is defined by movement that lasted for at least 333 ms with translational speed greater than 0.5 mm s⁻¹, or angular speed greater than 20° s⁻¹. Path straightness was calculated by measuring local deviations from an ideal path within a running window of 333 ms for each point of the fly trajectory, defined as:

$$\text{Path straightness} = \frac{\text{total distance traveled}}{\text{the sum of local deviations}}$$

Angular path deviation per forward segment was measured as the cumulative angular deviation divided by the total distance traveled. Finally, the angular bias was the binarized version of angular path deviation (Extended Data Fig. 9e). Saccade bias measured the proportion of left versus right saccades per fly. A value of -1 indicated that all saccades were to the left and +1 indicated that all saccades were to the right. A value of zero indicated an equal number of left and right saccades. Flies that walked for less than 50% of the time (<6 min) were excluded.

Quantification and statistical analysis

Statistics and reproducibility. No statistical methods were used to predetermine sample sizes. Sample sizes were chosen according to standard sample sizes in the field^{62,90}. For most experiments, data collection and analysis were not performed blind to the conditions of the experiments; however, data acquisition and analysis were performed identically for all genotypes. In stochastic silencing behavioral experiments (Fig. 7 and Extended Data Fig. 10), experimenters were blind to the genotype of flies while running the experiments. Each fly's genotype was determined post hoc with immunostaining. To ensure significance testing among comparable distributions, we discarded flies with injured legs and flies that walked for less than 50% of the time (<6 min) during the experiment. In calcium imaging experiments, recordings with large z-movement were discarded.

Statistics. We performed a two-sided Wilcoxon rank-sum test for comparisons between two independent groups, with Bonferroni correction for multiple comparisons where applicable. In all figure panels, unless specified by an asterisk, the comparisons were not significant.

Inclusion and ethics statement

While citing references scientifically relevant for this study, we worked to promote gender balance in our reference list as much as possible.

Reporting summary

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

Data availability

Data generated in this paper are publicly available at Zenodo at <https://doi.org/10.5281/zenodo.14967806> (ref. 91). Manually reconstructed FAFB neurons are available at Virtual Fly Brain (<https://fafb.catmaid.virtualflybrain.org/>). Source data are provided with this paper.

Code availability

All code and preprocessed data used for the analysis is publicly available at https://github.com/ChiappeLab/Erginkaya_et_al_2025.

References

67. Dionne, H., Hibbard, K. L., Cavallaro, A., Kao, J. C. & Rubin, G. M. Genetic reagents for making split-GAL4 lines in *Drosophila*. *Genetics* **209**, 31–35 (2018).
68. Mellert, D. J. & Truman, J. W. Transvection is common throughout the *Drosophila* genome. *Genetics* **191**, 1129–1141 (2012).
69. Schneider-Mizell, C. M. et al. Quantitative neuroanatomy for connectomics in *Drosophila*. *eLife* **5**, 1–36 (2016).
70. Saalfeld, S., Cardona, A., Hartenstein, V. & Tomančák, P. CATMAID: Collaborative annotation toolkit for massive amounts of image data. *Bioinformatics* **25**, 1984–1986 (2009).
71. Schlegel, P. M. et al. Whole-brain annotation and multi-connectome cell typing of *Drosophila*. *Nature* **634**, 139–152 (2024).
72. Buhmann, J. et al. Automatic detection of synaptic partners in a whole-brain *Drosophila* electron microscopy data set. *Nat. Methods* **18**, 771–774 (2021).
73. Bates, A. S. et al. Complete connectomic reconstruction of olfactory projection neurons in the fly brain. *Curr. Biol.* **30**, 3183–3199.e6 (2020).
74. Otsuna, H., Ito, M. & Kawase, T. Color depth MIP mask search: a new tool to expedite Split-GAL4 creation. Preprint at *bioRxiv* <https://doi.org/10.1101/318006> (2018).
75. Reiser, M. B. & Dickinson, M. H. A modular display system for insect behavioral neuroscience. *J. Neurosci. Methods* **167**, 127–139 (2008).
76. Weir, P. T. & Dickinson, M. H. Functional divisions for visual processing in the central brain of flying *Drosophila*. *Proc. Natl Acad. Sci. USA* **112**, E5523–E5532 (2015).
77. Isaacson, M. et al. A high-speed, modular display system for diverse neuroscience applications. Preprint at *bioRxiv* <https://doi.org/10.1101/2022.08.02.502550> (2022).
78. Sunkara, A., DeAngelis, G. C. & Angelaki, D. E. Role of visual and non-visual cues in constructing a rotation-invariant representation of heading in parietal cortex. *Elife* **2015**, 1–22 (2015).
79. Zhao, A. et al. Eye structure shapes neuron function in *Drosophila* motion vision. Preprint at *bioRxiv* <https://doi.org/10.1101/2022.12.14.520178> (2022).
80. Evans, S. W. et al. A positively tuned voltage indicator for extended electrical recordings in the brain. *Nat. Methods* **20**, 1104–1113 (2023).
81. Dana, H. et al. High-performance calcium sensors for imaging activity in neuronal populations and microcompartments. *Nat. Methods* **16**, 649–657 (2019).
82. Chen, T. W. et al. Ultrasensitive fluorescent proteins for imaging neuronal activity. *Nature* **499**, 295–300 (2013). 2013 4997458.
83. Thurman, S. T., Guizar-Sicairos, M. & Fienup, J. R. Efficient subpixel image registration algorithms. *Opt. Lett.* **33**, 156–158 (2008).
84. Eichner, H., Joesch, M., Schnell, B., Reiff, D. F. & Borst, A. Internal structure of the fly elementary motion detector. *Neuron* **70**, 1155–1164 (2011).
85. Costa, M., Manton, J. D., Ostrovsky, A. D., Prohaska, S. & Jefferis, G. S. X. E. NBLAST: rapid, sensitive comparison of neuronal structure and construction of neuron family databases. *Neuron* **91**, 293–311 (2016).
86. Dorkenwald, S. et al. CAVE: connectome annotation versioning engine. *Nat. Methods* <https://doi.org/10.1038/s41592-024-02426-z> (2025).
87. Heinrich, L., Funke, J., Pape, C., Nunez-Iglesias, J. & Saalfeld, S. Synaptic cleft segmentation in non-isotropic volume electron microscopy of the complete *Drosophila* brain. *Lect. Notes Comput. Sci.* **11071 LNCS**, 317–325 (2018).
88. Hulse, B. K. et al. A connectome of the *Drosophila* central complex reveals network motifs suitable for flexible navigation and context-dependent action selection. *eLife* **10**, e66039 (2021).
89. Vanwalleghe, G., Constantin, L. & Scott, E. K. Calcium imaging and the curse of negativity. *Front. Neural Circuits* **14**, 607391 (2021).
90. Seelig, J. D. & Jayaraman, V. Neural dynamics for landmark orientation and angular path integration. *Nature* **521**, 186–191 (2015).
91. Erginkaya, M. et al. A competitive disinhibitory network for robust optic flow processing in *Drosophila*. *Zenodo* <https://doi.org/10.5281/zenodo.14967806> (2025).

Acknowledgements

We thank R. Parekh and Janelia CAT for introducing us to FAFB and training us in EM tracing. K. Coates and F. Li for reviewing some of our manually traced neurons in FAFB. S. Huston for tracing of CNMNs, VCNMN and some cLPTCrn cells together with S. Imtiaz and B. Gorko, and for helping identify neck motor neurons. A. Li and R. Wilson for their help in reconstructing DNa02. S. Namiki and G. Card for their help in identifying DNns. J. Goldammer for identifying potential split-Gal4 candidates for H2rn cells and helping identify S-Neurons. H. Otsuna, G. Jefferis and P. Schlegel for introducing us to their tools for computational neuroanatomy and for helping with EM data analysis. A. Zhao for his input on LPT naming, response predictions and feedback on the manuscript. N. Eckstein and J. Funke for sharing neurotransmitter predictions for LPT partners before publication. We thank the Princeton FlyWire team and members of the Murthy and Seung laboratories, as well as members of the Allen Institute for Brain Science, for development and maintenance of FlyWire (supported by BRAIN Initiative grants MH117815 and NS126935 to Murthy and Seung). We also acknowledge members of the Princeton FlyWire team and the FlyWire consortium for neuron proofreading and annotation (Supplementary Table 2 lists all contributors). We thank the Murphy and Seung laboratories and the Jefferis laboratory for their contribution with 31.83% and 46.27% of the total editions in FlyWire neurons, respectively. In addition, we acknowledge the contribution of the Seeds and Hampel laboratory, the Wilson laboratory, the Bidaye laboratory, the Borst laboratory, the Kim laboratory and the Selcho laboratory for additional contributions. We thank members of the Cambridge Connectomics Group (G. Jefferis and M. Costa) including L. Serratos, A. Javier, S. Fang, K. Eichler and P. Schlegel for contributing to the proofreading of FlyWire Neurons. Proofreading in Cambridge was supported by the Wellcome Trust (Collaborative Award 203261/Z/16/Z) and National Institutes of Health (NIH) (BRAIN Initiative 1RF1MH120679-01). Development and administration of the FAFB tracing environment and analysis tools were funded in part by NIH BRAIN Initiative grant 1RF1MH120679-01 to D. Bock and G. Jefferis, with software development effort and administrative support provided by T. Kazimiers (Kazmos) and E. Perlman (Yikes). We also thank G. Maimon and C. Lyu for sharing sytGCaMP7f flies before publication. G. Rubin and H. Dionne (Rubin laboratory) for transgene constructs and reinjections. M. Silies for sharing the UAS-GCaMP6f, UAS-FLP recombinant line. E. Sönmez for help with testing Split-Gal4 combinations and help with validating FLPStop recombinants. The CR Fly Platform for assisting fly stock generation, maintenance and GABA staining. L. Venkatasubramanian for her help with the initial bIPS>TrpA1 behavior experiments. T. Fujiwara for help with finding and testing

potential Split-Gal4 combinations for H2rn and uLPTCrns and help with building the two-photon calcium imaging setup. W. Stagnaro for sharing unpublished work about multisensory processing in LPTCrns and bIPS cells. Past and present Chiappe Laboratory members for useful discussions and feedback on experiments and the manuscript. This work was supported by the Champalimaud Foundation and the research infrastructure Congento, LISBOA-01-0145-FEDER-022170. M.E.C. is supported by European Research Council Starting Grant ERC-2017-STG-759782 537 and European Research Council Consolidator Grant ERC-2022-CoC-101088936. The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript.

Author contributions

M.E. and M.E.C. conceived the present study. M.E. and M.E.C. designed the experiments. D.D.B. Curated and provided access to FAFB dataset pre-publication. M.E., M.B. and K.S. performed the EM work, with the help of F.T. and M.B.R. M.E., A.N. and N.V. generated transgenic lines. N.V. performed immunostainings. M.E. performed calcium imaging experiments. M.E., T.C. and N.V. performed behavioral experiments. T.C. designed the agent fly and performed model simulations. M.E. analyzed the data. A.M. provided input and code for the model and analysis. M.E. and M.E.C. wrote the manuscript with input from all authors. M.E.C. acquired funding and supervised the study.

Competing interests

The authors declare no competing interests.

Additional information

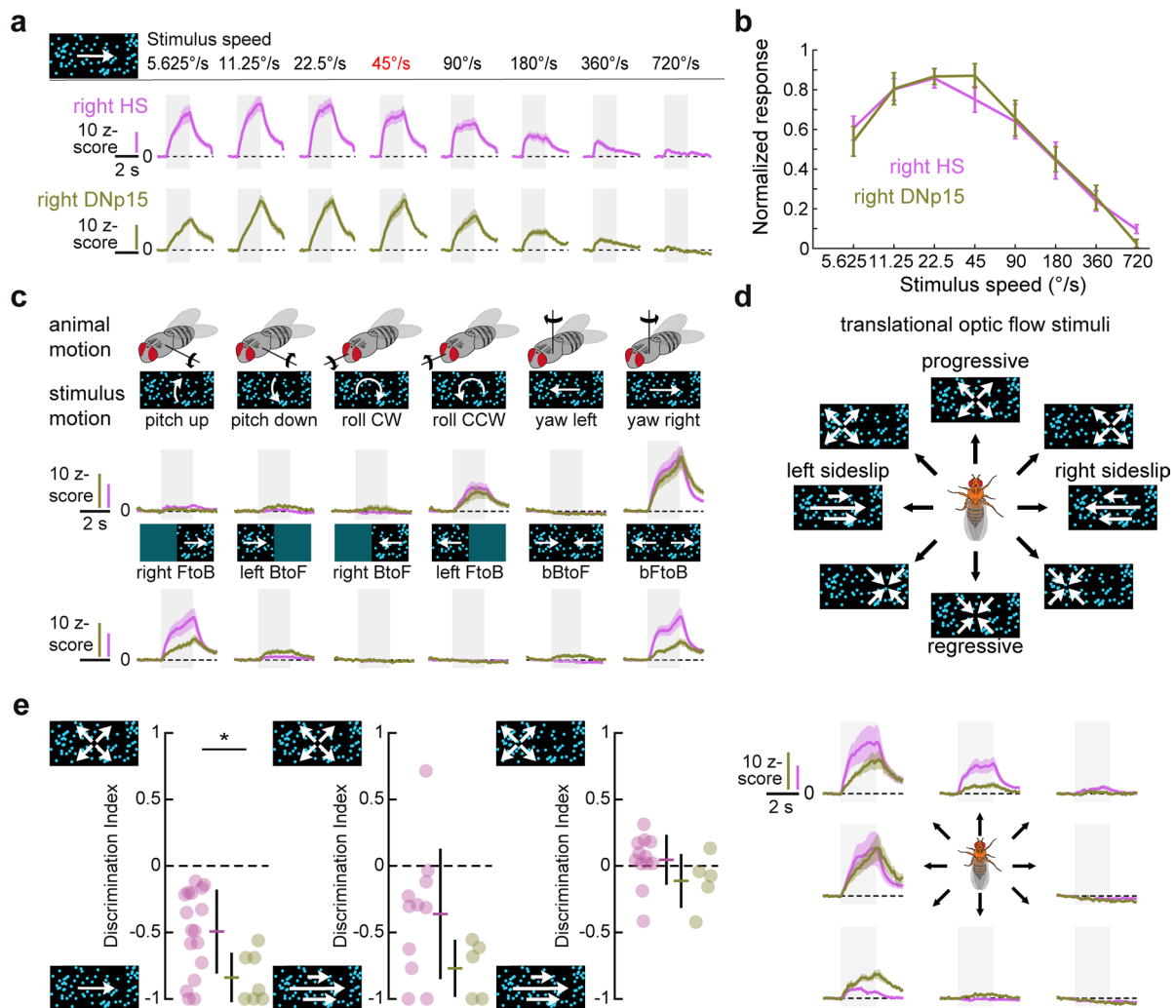
Extended data is available for this paper at <https://doi.org/10.1038/s41593-025-01948-9>.

Supplementary information The online version contains supplementary material available at <https://doi.org/10.1038/s41593-025-01948-9>.

Correspondence and requests for materials should be addressed to M. Eugenia Chiappe.

Peer review information *Nature Neuroscience* thanks Aman Saleem and the other, anonymous, reviewer(s) for their contribution to the peer review of this work.

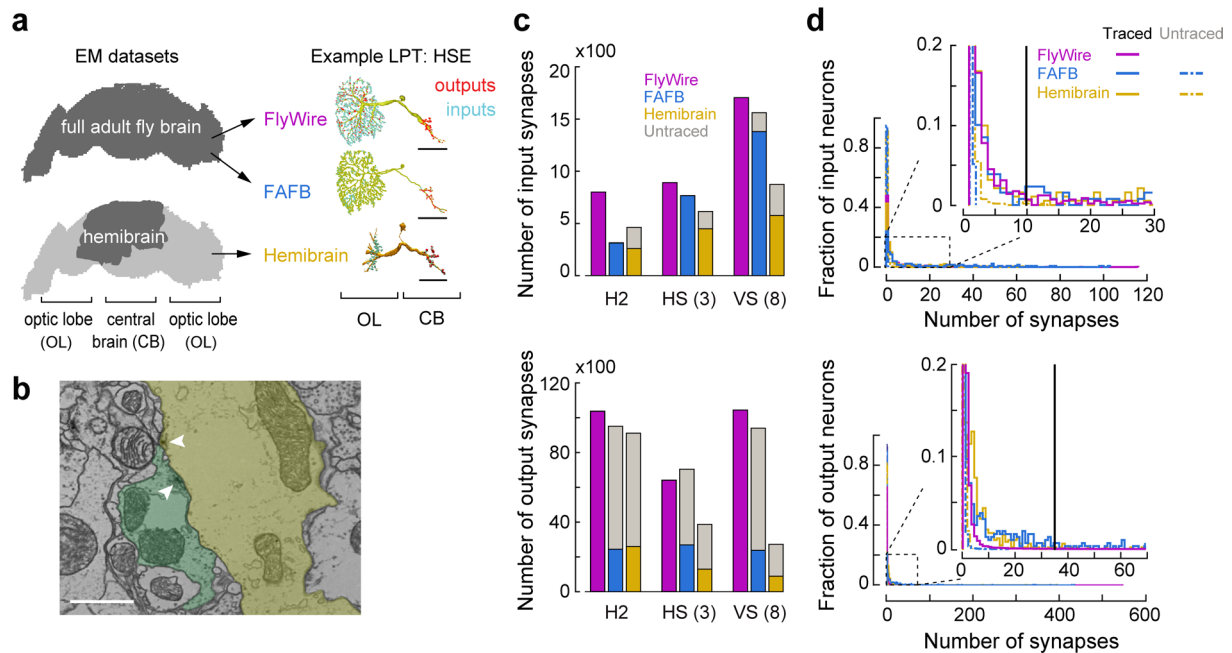
Reprints and permissions information is available at www.nature.com/reprints.



Extended Data Fig. 1 | Comparison of HS and DNP15 responses to optic flow.

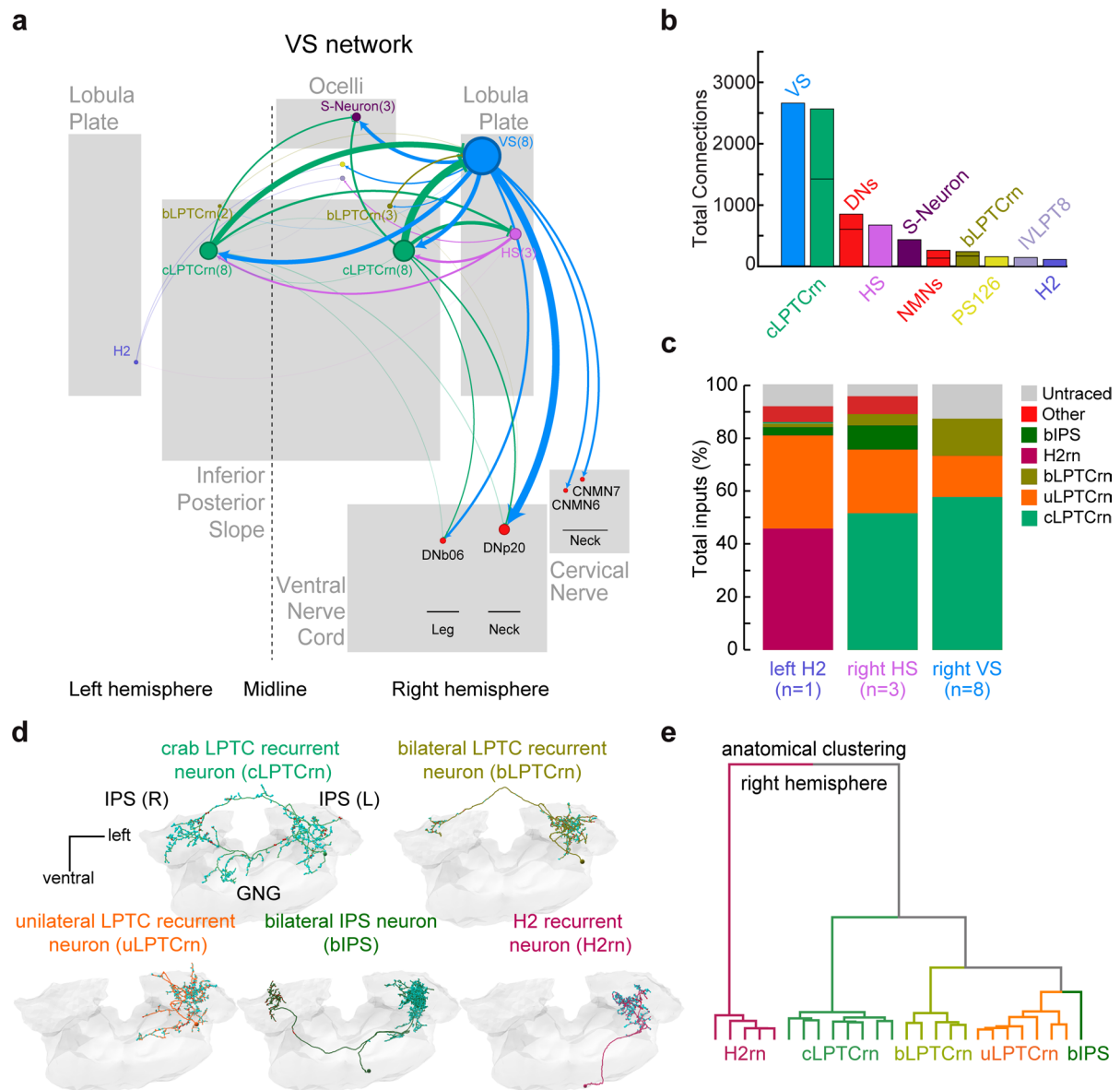
a Average responses of right HS (magenta) and right DNP15 (olive) neurons to 'yaw' stimuli moving at different speeds. The stimulus speed used in subsequent panels is highlighted in red. Traces represent grand mean z-scored responses $\pm$ SEM (shaded), with mean responses derived from 5–10 stimulus repetitions per fly. Gray rectangles indicate the stimulus window. HS (N = 8 flies, n = 12 ROIs), DNP15 (N = 5, n = 6). **b** Speed tuning curves calculated as the area under the curve during stimulus motion in (a), normalized by the maximum response per ROI. Error bars represent SEM. **c** Responses of HS and DNP15 cells to different binocular and unilateral visual stimuli. CW, clockwise; CCW, counter-clockwise; left/right FtoB, unilateral front-to-back visual motion; left/right BtoF, unilateral back-to-front visual motion; 'bFtoB' or 'bBtoF', bilateral front-to-back or

back-to-front visual motion, respectively. Traces represent grand mean z-scored responses $\pm$ SEM (shaded). HS (N = 13 flies, n = 17 ROIs), DNP15 (N = 6, n = 7). **d** Top, schematic illustrating directions of body motion and the resulting optic flow. Bottom: Responses of right HS (magenta) and DNP15 (olive) to the corresponding translational optic flow. Traces represent grand mean z-scored responses $\pm$ SEM (shaded). For progressive and regressive motion, HS (N = 13 flies, n = 17 ROIs), DNP15 (N = 6, n = 7). For the rest, HS (N = 8, n = 11), DNP15 (N = 4, n = 5). **e** Discrimination index for HS and DNP15 between different patterns of optic flow. Each circle represents an individual ROI. Colored horizontal line and black vertical line represents mean and 95% CI. *P < 0.05 (two-sided Wilcoxon's rank-sum test).



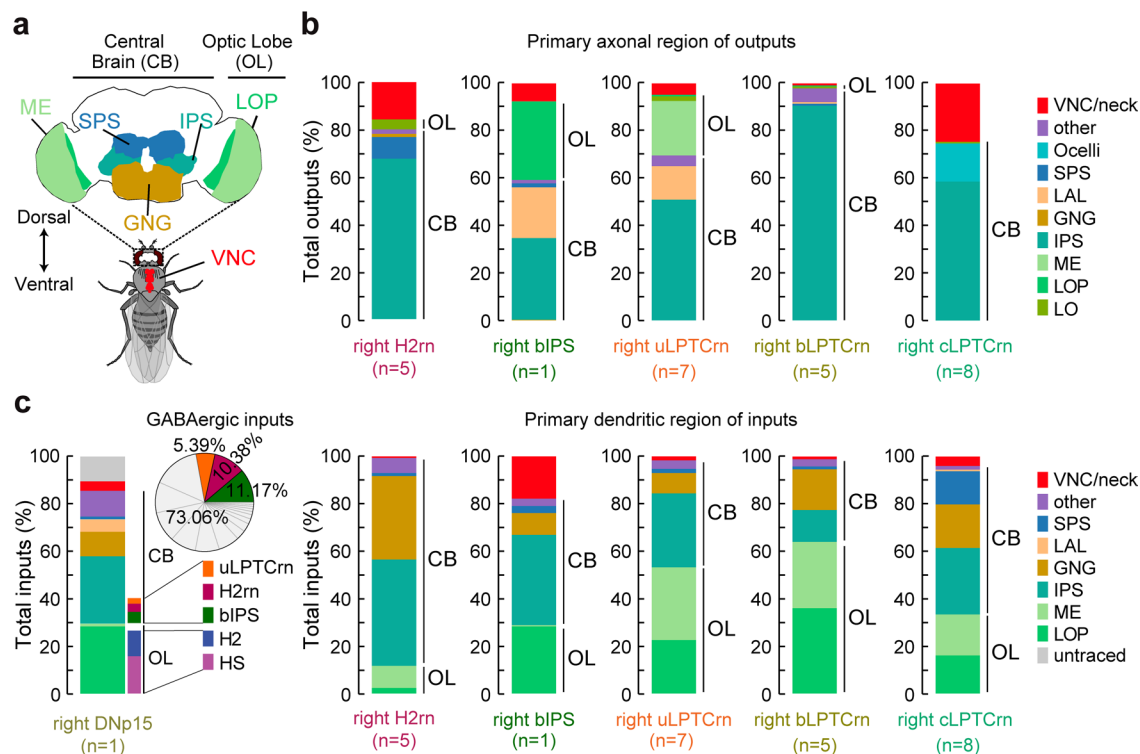
Extended Data Fig. 2 | LPTs show pre- and postsynaptic sites at their axon terminals. **a**) Left: Schematic showing the data coverage of the fly brain (dark gray) available from two large-scale electron microscopy (EM) datasets – FAFB and Flywire (top), and Hemibrain (bottom). Right: Reconstructions of the right HSE neuron across the three EM datasets. FAFB contains skeletonized neurons, while FlyWire and Hemibrain provide volumetric reconstructions. Postsynaptic (input) and presynaptic (output) sites are marked with cyan and red dots, respectively. **b**) Example EM section at the axon terminals of HSE (yellow) and a synaptic partner (green). Arrowheads indicate the location of presynaptic active zones. Scale bar, 1 μm . **c**) Top: Number of inputs (that is upstream partners) for HS, H2, and VS neurons in the fly central brain. Numbers in parentheses

indicate the number of cells present in each cell type. Presynaptic neurons traced for identification are colored based on the EM dataset. Synapses from untraced neurons are shown in gray. Bottom: same as Top, but for outputs (that is downstream partners). **d**) Top: Distribution of the number of synaptic connections between LPTs and their inputs. Color code indicates EM dataset and tracing status. Each distribution is normalized by the total number of synapses. Inset shows a zoomed-in view of the region outlined by the dashed rectangle. Neurons with more than 10 synapses (black line) are considered strong inputs. Bottom: Same as Top, but for LPT outputs. Neurons with more than 35 synapses (black line) are considered strong outputs (see Methods).



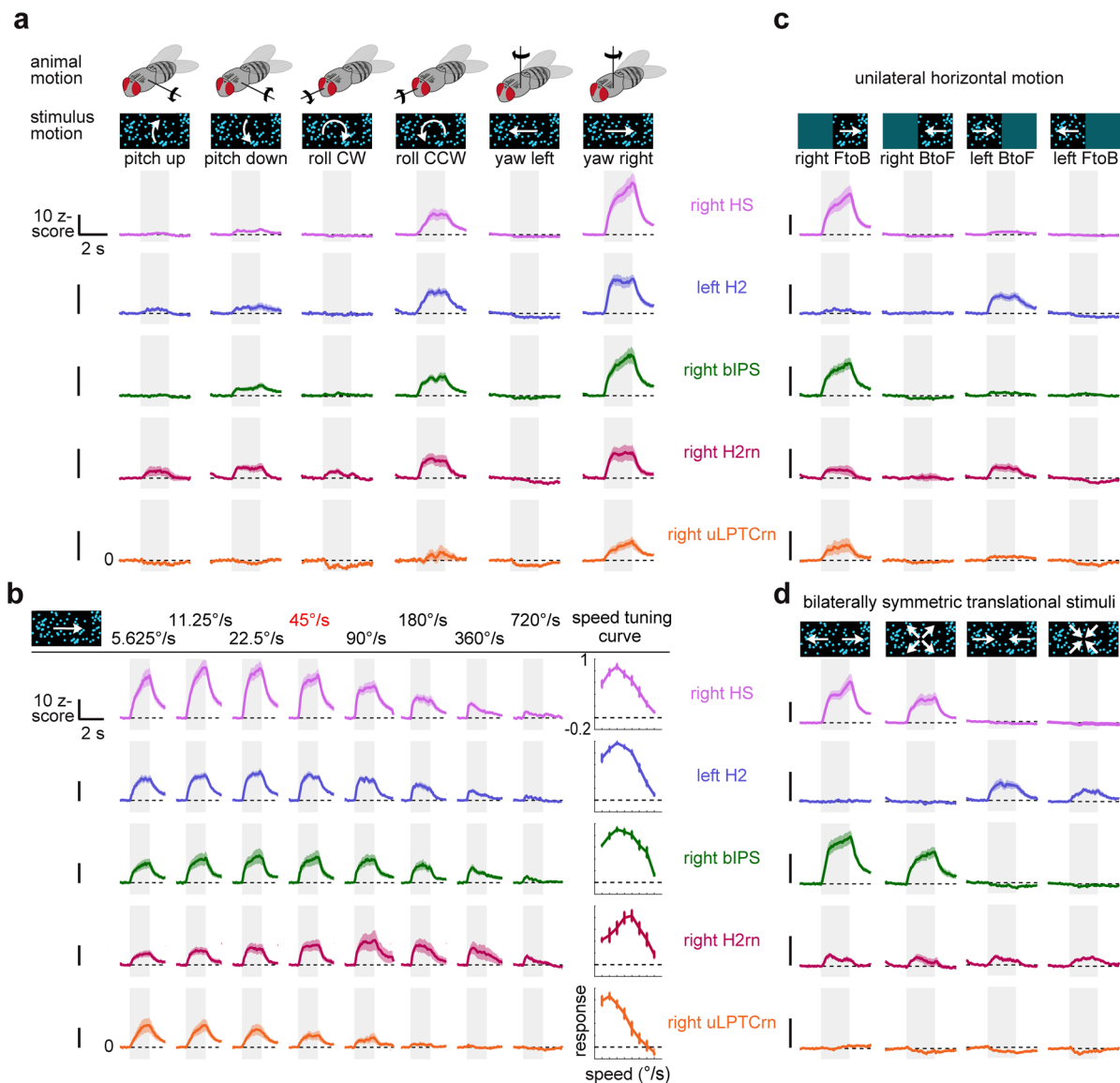
Extended Data Fig. 3 | Divergence and convergence in the central optic flow networks. **a)** Connectivity graph of the VS network, plotted as in Fig. 2b. **b)** Total synaptic connections per cell type within the VS network. **c)** Percentage of total inputs to left H2, right HS, and right VS cells, grouped by cell type (color-coded). Inputs outside the five major interneuron types are grouped as 'other'.

d) Posterior view of single-cell morphologies from each LPTCrn type, with pre- and postsynaptic sites marked in red and cyan, respectively. Regions of innervation are outlined in gray. IPS - inferior posterior slope; GNG - Gnathal Ganglion. **e)** NBLAST-based anatomical clustering of LPTCrn main backbones.



Extended Data Fig. 4 | Central neurons receive and send projections to other brain regions and the VNC. a Schematic of the fly brain with key neuropils highlighted. **b** Percentage of LPTCrn outputs, grouped by the main axonal output region of their downstream partners. **c** Percentage of inputs, grouped by the main dendritic input region of their upstream partners. For DNP15, additional colored bars represent H2-HS subnetwork inputs identified in Fig. 2. Inset next to

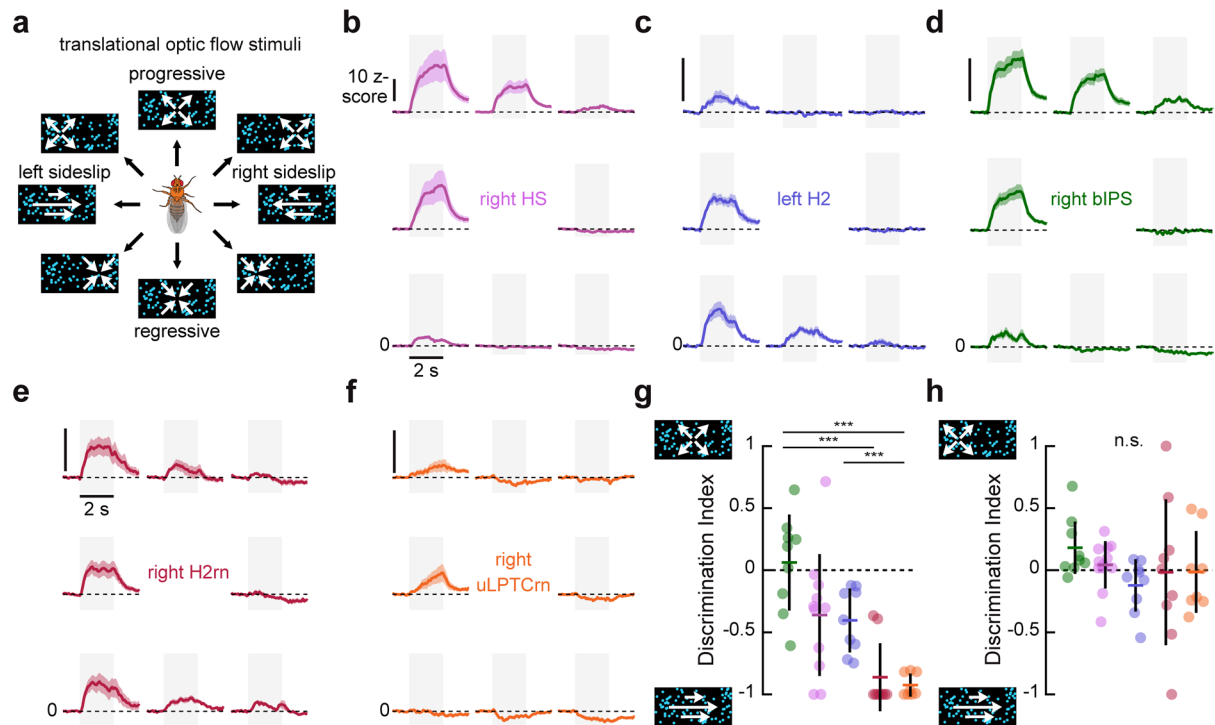
DNP15 shows proportion of GABAergic inputs provided by bIPS (green), H2rn (red) and uLPTCrn (orange) onto DNP15. Other cell types are colored in light gray. DNP15 input profile from FAFB, DNP15 GABAergic input profile and interneuron data from FlyWire. ME - medulla; LO - lobula; LOP - lobula plate; IPS - inferior posterior slope; SPS - superior posterior slope; GNG - Gnathal Ganglion; LAL - lateral accessory lobe; VNC - Ventral Nerve Cord.



Extended Data Fig. 5 | Responses of H2-HS network neurons to optic flow.

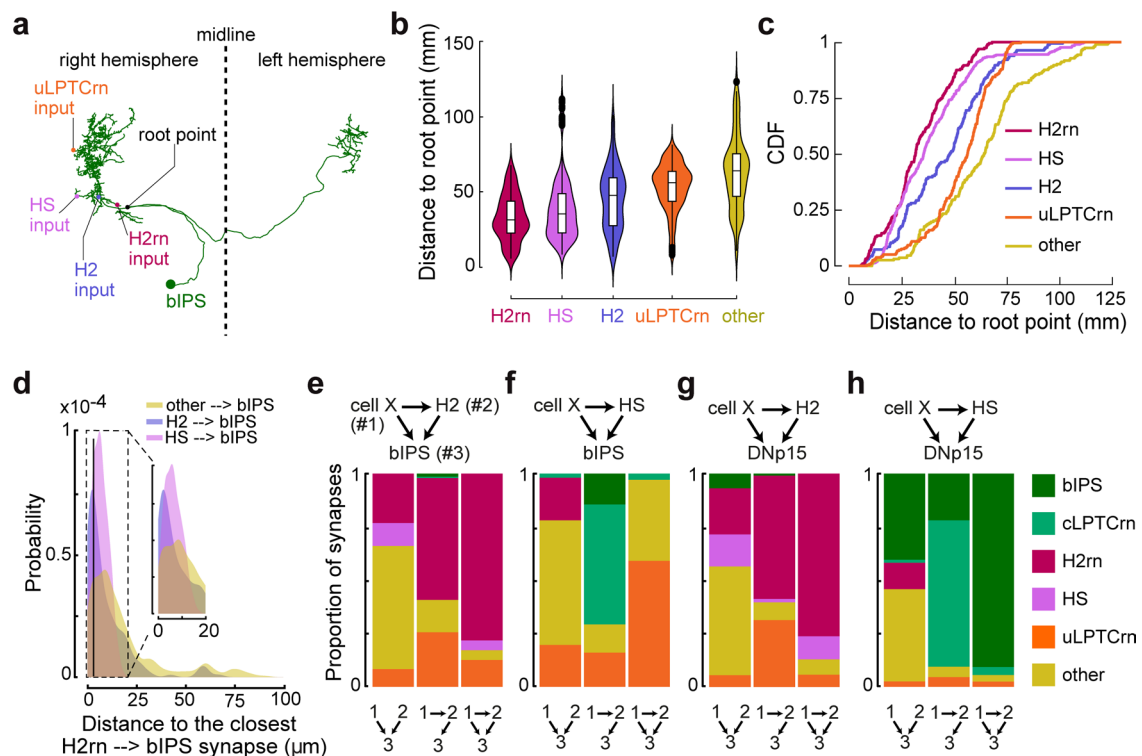
a) Calcium responses in H2-HS network neurons to rotational optic flow. Top: Schematics illustrate body rotations along six directions and the resulting optic flow. Bottom: Z-scored calcium responses of right HS (magenta) left H2 (blue), right bIPS (green), right H2rn (red) and right uLPTCrn (orange) neurons. For all panels, traces represent grand mean z-scored responses $\pm$ SEM (shaded), with mean responses derived from 5–10 stimulus repetitions per fly. Gray rectangles

indicate the stimulus window. **b**) Left: Responses to yaw optic flow at varying speeds. The speed used in other figures is highlighted in red. Right: Speed tuning curves for each cell type. Error bars represent SEM. **c**) Same as **a**), but for unilateral horizontal motion. FtoB: front-to-back, BtoF: back-to-front visual motion. **d**) Same as **a**), but for bilaterally symmetric visual motion. For all panels: HS ($N = 12$ flies, $n = 16$ ROIs), H2 ($N = 8$, $n = 11$), bIPS ($N = 15$, $n = 16$), H2rn ($N = 5$, $n = 8$), uLPTCrn ($N = 5$, $n = 5$).



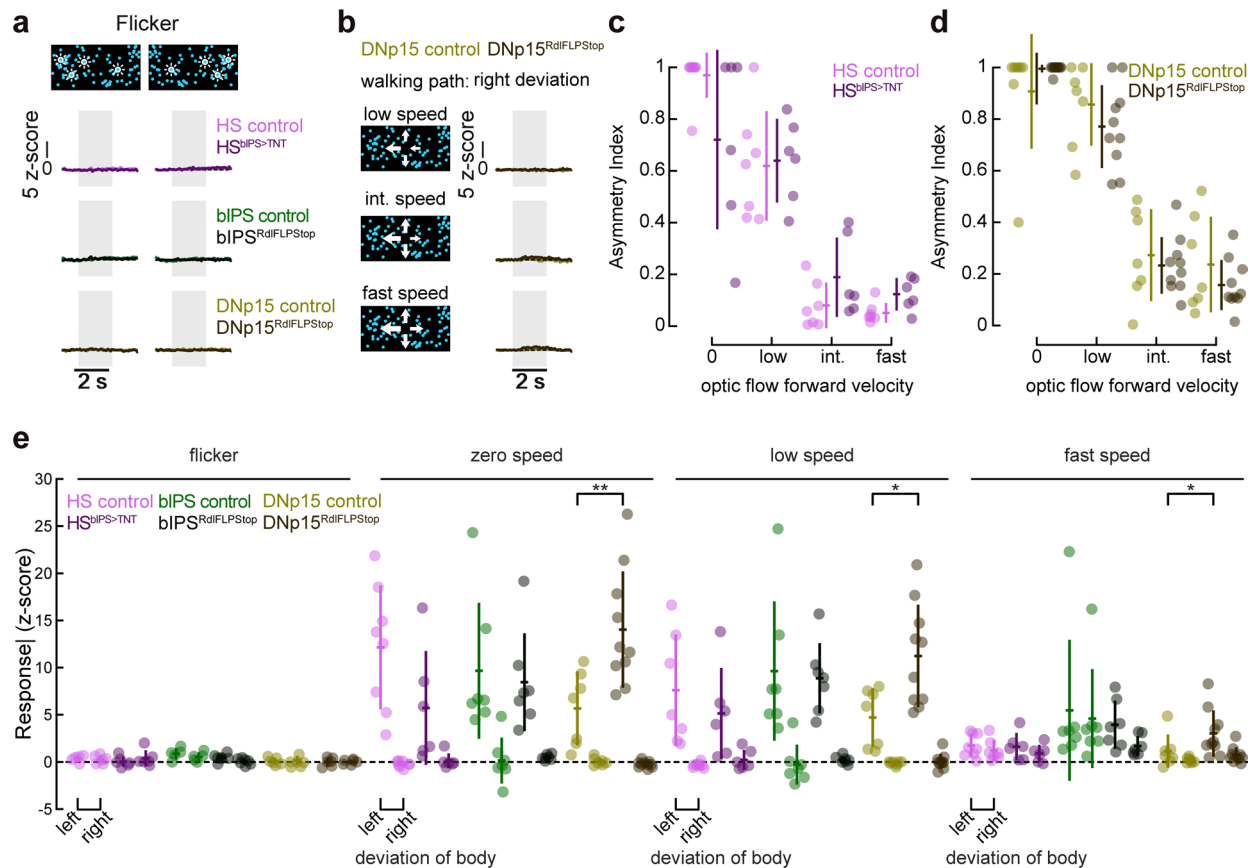
Extended Data Fig. 6 | Responses of H2-HS network to translational optic flow. **a**) Schematic illustrating directions of body translations and the resulting optic flow. **b–f**) Calcium responses of right HS **b**), left H2 **c**), right bIPS **d**), right H2rn **e**) and right uLPTCrn **f**) to translational optic flow patterns arranged in the same order as in **a**). Traces represent grand mean z-scored responses $\pm$ SEM (shaded), with mean responses derived from 5–10 stimulus repetitions per fly. Gray rectangles indicate the stimulus window. **g**) Progressive-sideslip discrimination

index (see Methods) of neurons shown in panels **b–f**). Each circle represents an individual ROI. Colored horizontal line and black vertical line represent mean and 95% CI, respectively. **h**) Same as **g**), but for diagonal translation vs. sideslip discrimination index. For all panels: HS ($N = 10$ flies, $n = 12$ ROIs), H2 ($N = 8$, $n = 9$), bIPS ($N = 9$, $n = 9$), H2rn ($N = 6$, $n = 9$), uLPTCrn ($N = 8$, $n = 8$). *** $p < 0.001$ (Two-sided Wilcoxon's rank-sum test with Bonferroni correction for multiple comparisons).



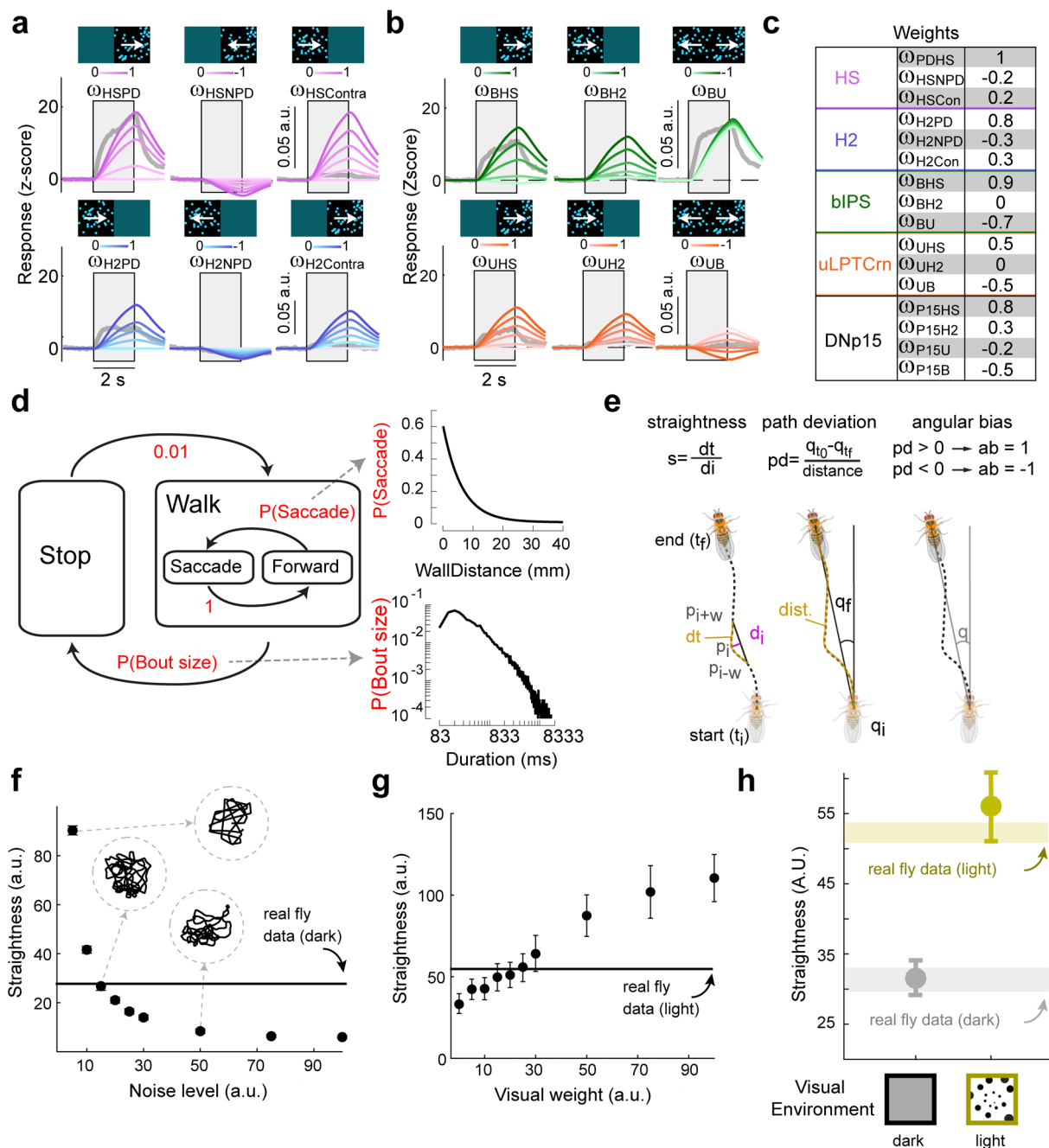
Extended Data Fig. 7 | The synaptic inputs of bIPs are organized non-randomly. **a**) Example of inputs to bIPs and the root point of the neuron. **b**) Geodesic distance between bIPs root point and synaptic inputs from H2rn ($n = 192$), HS ($n = 380$), H2 ($n = 164$), uLPTCrn ($n = 238$), or a random subsample of the remaining inputs ($n = 192$, see Methods). The horizontal line and the box depict the median with the 1st and 3rd quartiles. Vertical lines depict the minimum and maximum, with black dots showing outliers. **c**) Cumulative

distributions from **(b)**. **d**) Distance between an HS, H2 or other input synapse to bIPs and the closest H2rn input to bIPs. The vertical black line indicates the maximum inter-synapse distance used to define local connectivity. **e-h**) Proportion of local connectivity motifs that involve H2 (**e,g**) or HS (**f,h**) that converge onto bIPs (**e-f**) or DNp15 (**g-h**) with an additional neuron (cell X) color-coded by cell type. Schematics at the top of each panel show the three cells involved. Numbered schematics show the connectivity motif plotted per graph.



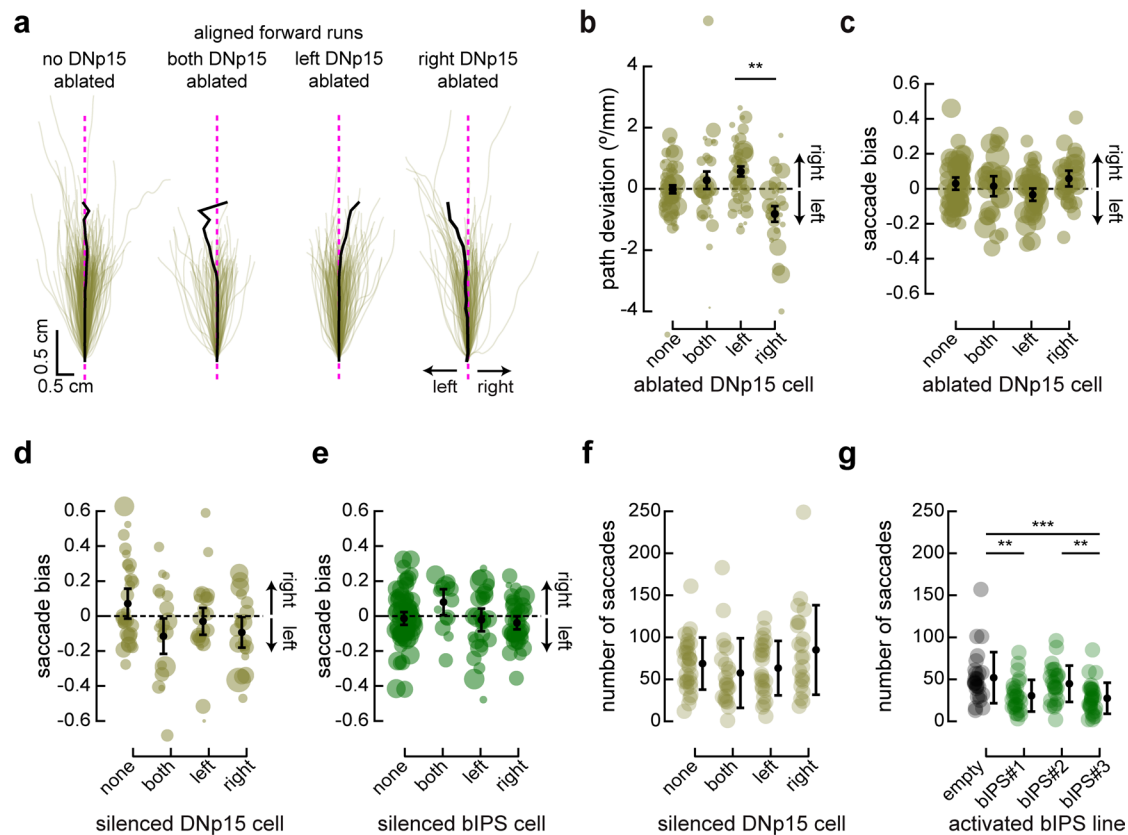
Extended Data Fig. 8 | Additional quantification of compound optic flow responses. **a**) Z-scored calcium responses of wild-type HS (magenta), bIPs (green), DNP15 (olive), and their manipulated counterparts (darker colors, see Fig. 5a) to visual flicker. For **(a–b)**, traces represent grand mean z-scored responses $\pm$ SEM (shaded), with mean responses derived from 5–10 stimulus repetitions per fly. Gray rectangles indicate the stimulus window. **b**) Z-scored calcium responses of DNP15 cells with or without functional GABA-A receptors to compound optic flow with rightward body deviation at varying translational speeds. **c**) Asymmetry index (see Methods) of HS under control (magenta) and bIPs silenced (dark magenta) conditions to compound optic flow at different translational velocities. **d**) Same as **(c)**, but for DNP15 responses under control

(olive) or GABA-A receptor knockdown (maroon) conditions. **e**) Neuronal responses to flicker, rotational (zero speed) and compound optic flow at low and fast translational speeds, shown as the average z-scored response during the last 500 ms of visual motion for each genotype in **(a)**. For **(c–e)**, each circle represents an individual ROI; horizontal and vertical lines represent mean and 95% CI. For flicker stimuli, HS control (N = 4 flies, n = 6 ROIs), HS^{bIPs>TNT} (N = 5, n = 6), bIPs control (N = 5, n = 5), bIPs^{Rdl-FlpStop} (N = 5, n = 7), DNP15 control (N = 6, n = 6), DNP15^{Rdl-FlpStop} (N = 6, n = 6). For the rest, HS control (N = 6, n = 8), HS^{bIPs>TNT} (N = 5, n = 6), bIPs control (N = 6, n = 7), bIPs^{RdlFlpStop} (N = 5, n = 7), DNP15 control (N = 7, n = 7), DNP15^{Rdl-FlpStop} (N = 8, n = 10). *P < 0.05; **P < 0.01 (Two-sided Wilcoxon's rank-sum test).



Extended Data Fig. 9 | Calibration and parameters of the visually guided agent. **a**) Visual responses of simulated HS (magenta) and H2 (blue) cells for different preferred (PD), non-preferred (NPD) and contralateral (Con) weights. Gray traces show mean neuronal responses recorded from real cells. **b**) Visual responses of simulated bIPS (green) and uLPTCrn (orange) cells for different HS, H2 and cross-connection weights. Gray traces show mean neuronal responses recorded from real cells. **c**) Final table with manually adjusted weights based on visual responses from **(a-b)**. **d**) Schematic of the state flow of the modeled agent, and the associated distributions for saccades (inset, top) and activity bout size (inset, bottom) estimated from the real data. **e**) Definitions of quality parameters:

straightness, path deviation and angular bias. **f**) Simulated straightness under dark conditions, as a function of $1/f$ noise level. Black line represents the mean straightness observed in real flies. **g**) Simulated straightness under light conditions with visual feedback, as a function of visual weight. Black line represents the mean straightness observed in real flies. **h**) Simulated straightness under dark (black) and light (gold) conditions for the chosen noise and visual weights. Shaded areas represent the standard error of straightness observed in real flies under light and dark conditions¹. For **(f-h)**, the plotted values are the mean of bootstrapped simulations, and the error bars represent SEM (see Methods).



Extended Data Fig. 10 | Effect of bIPS and DNp15 on body saccades. **a**) Walking paths of forward runs covering at least four body lengths, aligned at their origin, for flies with none, both, left or right DNp15 ablated neurons using stochastic expression of the Ricin toxin. Black lines show average left-right position as a function of forward displacement across all trajectories. **b**) Path deviation during forward runs (see Methods) for flies in **a**). Colored circles show mean bias, with circle size indicating number of walking bouts per fly. Black dots represent grand means weighted by walking bout number (dot size). Error bars show SEM. **c-e**) Same as **b**), but for left-right bias during body saccades (see Methods) for stochastic DNp15 ablation (**c**), DNp15 silencing (**d**) and bIPS silencing (**e**). Black dots represent the grand mean per genotype, weighted by the number of

saccades (dot size). **f**) Number of saccades for flies with none, both, left, or right DNp15 cells silenced. Black dots represent the grand mean per genotype, with error bars representing the 95% CI. **g**) Number of saccades for control flies (black) and for transgenic lines with both bIPS activated using TrpA1 (green). Number of flies in **a-c**): None, $N = 52$; Both, $N = 29$; Left, $N = 40$; Right, $N = 28$. For **d,f**): None, $N = 25$; Both, $N = 31$; Left, $N = 22$; Right, $N = 22$. For **e**): None, $N = 65$; Left, $N = 26$; Right, $N = 38$; Both, $N = 13$. For **g**): Empty, $N = 27$; bIPS#1, $N = 28$; bIPS#2, $N = 26$; bIPS#3, $N = 30$. ** $p < 0.01$, *** $p < 0.001$ (Two-sided Wilcoxon's rank-sum test with Bonferroni's correction for multiple comparisons). See Supplementary Table 3 for the full list of genotypes.

Reporting Summary

Nature Portfolio wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Portfolio policies, see our [Editorial Policies](#) and the [Editorial Policy Checklist](#).

Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

n/a Confirmed

- ☐ ☒ The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
- ☐ ☒ A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- ☐ ☒ The statistical test(s) used AND whether they are one- or two-sided
Only common tests should be described solely by name; describe more complex techniques in the Methods section.
- ☒ ☐ A description of all covariates tested
- ☐ ☒ A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
- ☐ ☒ A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
- ☐ ☒ For null hypothesis testing, the test statistic (e.g. F , t , r) with confidence intervals, effect sizes, degrees of freedom and P value noted
Give P values as exact values whenever suitable.
- ☒ ☐ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- ☒ ☐ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- ☒ ☐ Estimates of effect sizes (e.g. Cohen's d , Pearson's r), indicating how they were calculated

Our web collection on [statistics for biologists](#) contains articles on many of the points above.

Software and code

Policy information about [availability of computer code](#)

Data collection

Electron microscopy data were obtained from CATMAID-FAFB (v14), Flywire (v630) and Hemibrain (v1.2.1). Calcium imaging data were collected using Matlab 2013b with ScanImage (v3.8). Confocal images were acquired using the Zeiss ZEN LSM710 microscopy software. Behavioral data acquisition is performed in using previously published, non-commercially available custom-made arenas described in the Methods.

Data analysis

Electron microscopy data were analyzed with custom-written codes using publicly available natverse packages (v0.2.3) in R Studio (v1.1.453). and in Gephi (v0.9.2). Calcium imaging and behavioral data were analyzed using custom-written codes in Matlab R2018a. Confocal images were processed in Fiji/Image J (1.54f). All code and preprocessed data used for the analysis will be publicly available at GitHub: https://github.com/ChiappeLab/Erginkaya_et_al_2025

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Portfolio [guidelines for submitting code & software](#) for further information.

Data

Policy information about [availability of data](#)

All manuscripts must include a [data availability statement](#). This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A description of any restrictions on data availability
- For clinical datasets or third party data, please ensure that the statement adheres to our [policy](#)

Data generated in this paper are publicly available at Zenodo (DOI: 10.5281/zenodo.14967806). Manually reconstructed FAFB neurons are available at Virtual Fly Brain (<https://fafb.catmaid.virtualflybrain.org/>). Source Data are provided with this paper.

Research involving human participants, their data, or biological material

Policy information about studies with [human participants or human data](#). See also policy information about [sex, gender \(identity/presentation\), and sexual orientation](#) and [race, ethnicity and racism](#).

Reporting on sex and gender

Reporting on race, ethnicity, or other socially relevant groupings

Population characteristics

Recruitment

Ethics oversight

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☒ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see [nature.com/documents/nr-reporting-summary-flat.pdf](https://www.nature.com/documents/nr-reporting-summary-flat.pdf)

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	No sample size calculations were performed before experiments. Sample sizes were chosen according to standard sample sizes in the field (e.g., Seelig & Jayaraman, Nature, 2015; Lyu et al., Nature, 2021).
Data exclusions	LPTs respond to rotational stimuli along one of the three rotational axes of the head and body. A lack of responses indicates poor preparation quality. For other neurons in the H2-HS network, which we were characterizing for the first time, the physiological preparation involved immobilized flies, preventing behavioral assessments to verify preparation quality. Therefore, we excluded data from flies that showed no detectable calcium responses to any visual stimuli. Additionally, data from freely walking flies with injured limbs were excluded, as such impairments confounded neural manipulations and compromised the proper interpretation of behavioral experiments
Replication	For all physiological experiments, each experiment included 5-10 replications. For most cell-types and experiments we include at least 8 independent flies, and more frequently 10-15. For the behavioral experiments, given the random-based strategy to manipulate neural activity, each fly was an independent experiments, and we repeated these experiments until we reached at least 20 flies for each condition
Randomization	Female flies from the same genotype were chosen randomly from vials where they were group-housed for all experiments. There was no covariate analysis since these flies can be considered similar from the perspective of their genotype and environmental and social experience (we maintained equal density of flies in vials, and we used 3 to 4 day-old flies consistently). In addition, due to the challenges of these experiments, the sample size is limited to allow for an accurate cross-variate analysis. Order of all visual stimuli in calcium imaging experiments were randomized.
Blinding	Experimenters were not blind to the genotype of individual flies. However, data collection and analysis were automated and equivalent across genotypes.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☐	☒ Antibodies
☒	☐ Eukaryotic cell lines
☒	☐ Palaeontology and archaeology
☐	☒ Animals and other organisms
☒	☐ Clinical data
☒	☐ Dual use research of concern
☒	☐ Plants

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Antibodies

Antibodies used	Mouse anti-nc82 antibody, DSHB, RRID: AB_2314866. Chicken anti-GFP antibody, Abcam, RRID: AB_300798. Rabbit polyclonal anti-GFP antibody, Thermo Fisher Scientific, RRID: AB_221570. Rabbit anti-GABA antibody, Sigma, RRID: AB_477652. Rabbit anti-DsRed antibody, Abcam, AB356483. Goat AlexaFluor-488 anti-chicken, Invitrogen, RRID: AB_2534096. Goat Alexa-594 anti-rabbit, Invitrogen, RRID: AB_150088. Goat AlexaFluor-633 anti-mouse, Invitrogen, RRID: AB_141431.
Validation	All antibodies used in this study are commercially available and have been applied to the Drosophila nervous system in previous studies. Validation information can be found at manufacturers' websites: mouse nc82 (https://dshb.biology.uiowa.edu/nc82) chicken anti-GFP (https://www.abcam.com/en-us/products/primary-antibodies/gfp-antibody-ab13970) rabbit anti-GFP (https://www.thermofisher.com/antibody/product/GFP-Antibody-Polyclonal/A-11122) rabbit anti-GABA (https://www.sigmaaldrich.com/US/en/product/sigma/a2052) rabbit anti-DsRed (https://www.sigmaaldrich.com/US/en/product/mm/ab356483) Goat Alexa-488 anti-chicken (https://www.thermofisher.com/antibody/product/Goat-anti-Chicken-IgY-H-L-Secondary-Antibody-Polyclonal/A-11039) Goat Alexa-594 anti-rabbit (https://www.thermofisher.com/antibody/product/Goat-anti-Rabbit-IgG-H-L-Cross-Adsorbed-Secondary-Antibody-Polyclonal/A-11012) Goat Alexa-633 anti-mouse (https://www.thermofisher.com/antibody/product/Goat-anti-Mouse-IgG-H-L-Cross-Adsorbed-Secondary-Antibody-Polyclonal/A-21050)

Animals and other research organisms

Policy information about [studies involving animals](#); [ARRIVE guidelines](#) recommended for reporting animal research, and [Sex and Gender in Research](#)

Laboratory animals	W1118 Adult Drosophila melanogaster flies were used in all experiments. All experiments were performed with 3-to-4-days old flies.
Wild animals	No wild animals were used in the study.
Reporting on sex	Electron microscopy data were obtained from two individual adult female fly brains and one adult male ventral nerve cord. Female flies were used in all calcium imaging experiments due to their larger body size, providing easier preparation for physiology. Male flies were used in all behavior experiments since they exhibit more continuous locomotion in our experimental conditions.
Field-collected samples	No field collected samples were used in the study.
Ethics oversight	In Europe, no ethical approval is required for conducting research using Drosophila melanogaster

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Seed stocks	Report on the source of all seed stocks or other plant material used. If applicable, state the seed stock centre and catalogue number. If plant specimens were collected from the field, describe the collection location, date and sampling procedures.
Novel plant genotypes	Describe the methods by which all novel plant genotypes were produced. This includes those generated by transgenic approaches, gene editing, chemical/radiation-based mutagenesis and hybridization. For transgenic lines, describe the transformation method, the number of independent lines analyzed and the generation upon which experiments were performed. For gene-edited lines, describe the editor used, the endogenous sequence targeted for editing, the targeting guide RNA sequence (if applicable) and how the editor was applied.
Authentication	Describe any authentication procedures for each seed stock used or novel genotype generated. Describe any experiments used to assess the effect of a mutation and, where applicable, how potential secondary effects (e.g. second site T-DNA insertions, mosaicism, off-target gene editing) were examined.