Feature detection and orientation tuning in the Drosophila central complex

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Many animals, including insects, are known to use visual landmarks to orient in their environment. In Drosophila melanogaster, behavioural genetics studies have identified a higher brain structure called the central complex as being required for the fly’s innate responses to vertical visual features1 and its short- and long-term memory for visual patterns2–4. But whether and how neurons of the fly central complex represent visual features are unknown. Here we use two-photon calcium imaging in head-fixed walking and flying flies to probe visuomotor responses of ring neurons—a class of central complex neurons that have been implicated in landmark-driven spatial memory in walking flies5–7 and memory for visual patterns in tethered flying flies8. We show that dendrites of ring neurons are visually responsive and arranged retinotopically. Ring neuron receptive fields comprise both excitatory and inhibitory subfields, resembling those of simple cells in the mammalian primary visual cortex. Ring neurons show strong and, in some cases, direction-selective orientation tuning, with a notable preference for vertically oriented features similar to those that evoke innate responses in flies1–2. Visual responses were diminished during flight, but, in contrast with the hypothesized role of the central complex in the control of locomotion9, not modulated during walking. Taken together, these results indicate that ring neurons represent behaviourally relevant visual features in the fly’s environment, enabling downstream central complex circuits to produce appropriate motor commands10. More broadly, this study opens the door to mechanistic investigations of circuit computations underlying visually guided action selection in the Drosophila central complex.

Flies display a variety of visual pattern- and position-dependent behaviours, including stripe fixation7, short-term orientation memory5, pattern learning7 and place learning1. Common to these behaviours is a need to detect and respond to specific features in the insect’s visual surroundings. In addition, all these behaviours require the central complex11–13, a deep brain region that has also been implicated in motor control14. We used two-photon calcium imaging in genetically targeted populations of central complex input neurons in behaving flies to investigate their potential visuomotor role. We focused on the dendritic responses of ring neurons—neurons that connect the lateral triangle (LTR) to the ellipsoid body15–16 (Fig. 1a) and have been specifically implicated in visuomotor memory14,17.

Electron microscopy in the locust has shown that dendrites of ring neuron analogues arborize in specialized structures in the LTR called microglomeruli, where they are contacted by axonal projections from visual areas18. Confocal images of the Drosophila LTR labelled with green fluorescent protein (GFP) under the control of a pan-neuronal driver line, R57C10 (ref. 11), revealed a similar dense microglomerular substructure in the region (Fig. 1b, Supplementary Videos 1–4).

We asked if LTR microglomeruli respond to visual input. We used two-photon imaging with the calcium indicator GCaMP expressed pan-neurally to record neural activity in the LTR of head-fixed Drosophila placed at the centre of a visual arena (Fig. 1c, d). Flies were presented with small bright vertical bars moving horizontally at different elevations and LTR calcium transients were recorded from several planes of focus on one or both sides of the brain in a single experiment (see Methods). Calcium transients showed strong temporal correlations at the spatial scale expected of LTR microglomeruli (Fig. 1e, Supplementary Video 5 and Extended Data Fig. 1a). Visual stimuli evoked robust calcium transients in a subset of microglomeruli, but only when the localized stimuli were in specific spatial locations around the fly (Fig. 1f). We computed receptive fields for responsive microglomeruli and found that most receptive fields are centred in the ipsilateral visual hemifield (Fig. 1g–i, Extended Data Fig. 1b and Methods). Finally, LTR microglomeruli are clustered retinotopically and principal component analysis based on receptive field centres indicates that they form a spatial map with axes that are almost parallel to the fly’s visual field (Fig. 1j, k and Extended Data Fig. 1c–e).

We next examined the anatomical relationship between the LTR and individual classes of ring neurons that send arborizations to the region19–21. We studied dendritic arborization patterns of ring neurons targeted by EB1-GAL4, which labels R2 ring neurons required for pattern memory9, and c232-GAL4, which labels R3/R4d neurons required for spatial memory2 (Supplementary Videos 1 and 2 for R3/R4d; Supplementary Videos 3 and 4 for R2). In agreement with past anatomical work22, different ring neuron classes arborize in specific contiguous parts of the LTR (Extended Data Fig. 2a, b). Each ring neuron in these classes extends dendrites into a single microglomerulus in the LTR, and sends axonal arbors throughout a class-specific ring of the ellipsoid body (Fig. 2a, Extended Data Fig. 2c–e, see also23–25).

To understand whether different types of ring neurons have distinctive visual response properties, we mapped receptive fields for dendritic microglomeruli of R3/R4d and R2 ring neurons (Extended Data Fig. 3a–c and Extended Data Fig. 3d–f, respectively). We found visual responses in ~7/40 c232-GAL4-labelled microglomeruli—corresponding to ~7/20 R4d microglomeruli (Extended Data Fig. 3a)—and ~14/20 of R2 microglomeruli labelled by EB1-GAL4. Receptive fields for R2 and R4d neurons cover large parts of the visual field, with highest density near the midline of the ipsilateral visual field (Extended Data Fig. 3g, h). In summary, R4d and R2 microglomeruli appear to have similar visual response properties and overlapping receptive fields, but with peak sensitivity in different parts of the visual field (Extended Data Fig. 3i–k).

We next probed the fine structure of microglomerular receptive fields using sparse white noise stimuli (Fig. 2b, see Methods). Reverse correlation of microglomerular responses revealed prominent inhibitory subfields in the receptive fields (Fig. 2c for R2; Fig. 3a for R4d). The spatial scales of receptive field structure we observe is within the range for visual features that evoke innate responses in flies, and that are used for visual pattern learning in tethered flies22. To test the validity of these white-noise-based receptive fields, we used them to predict responses to novel bar stimuli (Fig. 2d, see Methods). The predicted responses captured much of the temporal and spatial variation in the data (Fig. 2e), with high correlations between estimated and actual responses (Extended Data Fig. 4a, b).

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Noting that the receptive field structure of ring neuron inputs resembles those of simple cells in mammalian primary visual cortex\(^{13}\), we next ask if these neurons share other response properties. Indeed, when we presented flies with a series of moving oriented bars, we found strong orientation tuning in microglomerular response patterns (Fig. 2f, g). As expected for receptive field structures with both excitatory and inhibitory lobes, microglomeruli also showed orientation tuning when presented with bars of opposite contrast, that is, dark bars on a bright background (Extended Data Fig. 5a, b), as are often used in fly behavioural studies\(^{1,2}\). Examining orientation tuning across the population of microglomeruli, we observed a strong preference for vertically oriented bars (Fig. 2h and Extended Data Fig. 4h, i for R2; Extended Data Fig. 4c, k, l for R4d; Fig. 2k and Extended Data Fig. 4g, n, o for the pan-neuronal line). Receptive fields tuned to vertical orientations are distributed across the visual field (Fig. 2i, j for R2; Extended Data Fig. 4c, d for R4d; Extended Data Fig. 4f for the pan-neuronal line). A fraction of neurons also shows direction-selectivity (Fig. 2j and Extended Data Fig. 4j for R2; Extended Data Fig. 4e, m for R4d; Fig. 2l and Extended Data Fig. 4p for the pan-neuronal line).

We next assessed the degree of stereotypy in response properties of ring neuron dendrites of different flies. We found strong correlations across flies in receptive field structure for R4d (Fig. 3a, b, Extended Data Figs 6 and 8), and R2 (Fig. 3c, Extended Data Figs 7 and 8).

Ring neurons and the ellipsoid body have often been ascribed a role in complex visuomotor tasks\(^{13-15}\). We examined the possible motor function of ring neurons by assessing potential correlations between neural activity and locomotion in tethered flies walking on an air-supported ball\(^{14}\) or flying\(^{15}\), in darkness or in the presence of a bright stripe moving left or right in front of the fly (Fig. 4a, see also Methods and Supplementary Videos 6 and 7). Although some R3/R4d neurons did show occasional correlations with locomotion when flies walked in the dark, and responses from visually stimulated animals showed occasional modulation during walking, the changes were within the expected variability of visual responses in stationary flies (Fig. 4b–d). Responses were also insensitive to walking direction. Overall, LTR visual responses could be modelled accurately without taking walking state into account (Extended Data Fig. 9a–e and Extended Data Fig. 10). By contrast, responses to visual stimuli were consistently diminished during tethered flight (Fig. 4e, f, Extended Data Fig. 9f–h and Supplementary Video 7), but showed no obvious correlations with flight direction as assessed by differences in wingbeat amplitude envelope. Thus, ring neuron LTR responses were modulated by motor state, but not in a manner consistent with a direct role in motor coordination, and in a markedly different manner than in the optic lobe\(^{15,16}\).

Behavioural genetics studies in *Drosophila* have suggested that the central complex is required for a wide range of important sensorimotor functions. However, in the absence of physiological recordings from the region in flies, it has been challenging to determine its role in the diversity of behaviours that it has been implicated in. We studied the visuomotor responses of ring neurons, which provide input from the LTR to the ellipsoid body of the central complex. Analogous neurons in other insects respond to polarized and unpolarized light\(^{17-19}\), and to mechanosensory stimulation\(^{20}\), one of the sensory modalities that we did not explore but which may partly account for unresponsive
neurons in our study. We found that R2 and R4d ring neurons are visually responsive, and these responses are not significantly modulated by walking state. Although visual responses are diminished during flight, they do not vary systematically with wingbeat patterns associated with turns. It is possible that outputs of these ring neurons within the ellipsoid body rings could be more sensitive to motor actions, but our physiological results in their dendrites during behaviour are inconsistent with a major role for these ring neurons in motor coordination in the fly. Further, the high degree of stereotypy that we observe in ring neuron receptive field characteristics across flies indicates that, rather than directly performing motor coordination, these neurons probably provide downstream central complex circuits with similar behaviourally relevant visual feature sets on which to base motor decisions. As a striking example, the strong vertical tuning preference we observe in the LTR may partly underlie the tendency of flies to fixate on vertical stripes. For instance, the strong vertical tuning preference we observe in the LTR may partly underlie the tendency of flies to fixate on vertical stripes. For instance, when a red or yellow bar is presented in the lower left or upper right quadrant, respectively, R2 neurons were strongly excited. Similarly, when a red or yellow bar is presented in the lower right or upper left quadrant, respectively, R4d neurons were strongly excited. These findings suggest that ring neurons may play a role in orienting the fly towards vertical or horizontal stimuli.

Figure 2 | Ring neurons are tuned to specific visual features and orientations. a, Multicoloured FLP-out of R2 neurons showing three cell bodies on each side along with their colour-matched microglomeruli (green, light green and purple at left; two green and one red at right). Red and yellow asterisks mark two cell bodies (one on each side, lateral) and arrows of like colour their respective LTR microglomeruli (medial). All neurons send processes throughout ellipsoid body rings. Scale bar, 30 μm. b, Example frame of white noise stimulus used for receptive field mapping using reverse correlation. c, Sample receptive fields of R2 microglomeruli. Red subfields show excitatory responses >30% of maximum; blue subfields show inhibitory responses <30% of minimum of mean-subtracted weighted average. See Extended Data Fig. 8 for all receptive fields. d, Bright bars with four different orientations used as test features. e, Modelled and actual (black) ΔF/F changes of an R2 microglomerulus in response to differently oriented bars (fly 2 in Extended Data Fig. 7). In red is shown the trial used for fitting parameters (i), and the tests (ii–iv). f, Orientation tuning curves for R2 neurons (two-trial average, fitted in red). The measurement 90° corresponds to a back-to-front movement of the vertical bar. 270° to front-to-back movement of the vertical bar. Error bars represent standard deviation. g, Polar plots of orientation tuning data and fits (red) for data shown in f. Numbers italicized in bold indicate ΔF/F (%) of microglomeruli of R2 neurons coloured by azimuth and elevation of centre of their excitatory receptive fields, measured using horizontally moving bars as described in Extended Data Fig. 1 (two-trial average). h, Direction selectivity of same microglomeruli (two-trial average). i, Preferred orientation (collapsed to 0°–180°) (κ) and direction selectivity (Φ) of microglomeruli in pan-neuronal line (both four-trial average). See Methods for analysis details. Scale bars for h and k, 5 μm.

Figure 3 | Ring neuron LTR microglomeruli show stereotyped receptive field properties across flies. a, Subset of receptive fields measured in R4d neurons across seven flies aligned by similarity (see Extended Data Fig. 8b for full set). Numbers below receptive fields are cross-correlations with top receptive field in the column as template. b, Histogram of cross-correlation values calculated for R4d neuron receptive fields with best-matched template. c, Histogram of cross-correlation values for R2 neurons (n = 6 flies, see Extended Data Fig. 8a for receptive fields).
The retinotopic bias, structure of excitatory and inhibitory subfields, orientation tuning and direction selectivity we see are reminiscent of those seen in calcium imaging studies in simple cells in mammalian visual cortex\(^5\), providing an interesting example of how evolutionarily distant visual systems with different types of eyes nonetheless use similar feature sets to process visual scenes. From Hubel and Wiesel’s distant visual systems with different types of eyes nonetheless use broadly distributed tuning preferences in such areas.

The studies of receptive field modulation show significant shift towards lower responses during flight stimuli during flight. Walking was calculated as (\(F/F\))walk. Azimuthal position of visual stimuli shown in e. c, Distributions of R4d neuron visual responses during walking and non-walking conditions are not significantly different (n = 14 flies; \(\text{trials}_{\text{walking}} = 1,722\); \(\text{trials}_{\text{non-walking}} = 42\); mean\(_{\text{walking}} = 0 \pm 44.3\%\); mean\(_{\text{non-walking}} = 1.1 \pm 49.7\%\); \(P = 0.45\)). Percentage decrease in \(F/F\) during walking was calculated as \((\Delta F/F)_{\text{walk}} = (F/F)_{\text{non-walking}} / (F/F)_{\text{walking}}\). Same as c for R4d neurons (n = 8 flies; \(\text{trials}_{\text{walking}} = 2,015\); \(\text{trials}_{\text{non-walking}} = 245\); mean\(_{\text{walking}} = 0 \pm 28.1\%\); mean\(_{\text{non-walking}} = -2.2 \pm 24.4\%\); \(P = 0.37\)). e, Subset of simultaneously recorded R4d microglomeruli during flight. Boxes indicated with an asterisk show diminished responses to identical visual stimuli during flight. Percentage decrease in \(F/F\) during flight (n = 13 flies; \(\text{trials}_{\text{flying}} = 759\); \(\text{trials}_{\text{non-flying}} = 481\); mean\(_{\text{flying}} = 0 \pm 42.2\%\); mean\(_{\text{non-flying}} = 31.2 \pm 72.3\%\); \(P = 6 \times 10^{-5}\)). Percentage decrease in \(F/F\) during flying was calculated as \((\Delta F/F)_{\text{flying}} = (F/F)_{\text{non-flying}} / (F/F)_{\text{flying}}\). All P-values show two-sample Kolmogorov–Smirnov test.

**METHODS SUMMARY**

Calcium imaging experiments to measure receptive fields were performed with UAS-GCaMP3; c232-GAL4, UAS-GCaMP3; EBI1-GAL4, and pJFRC7–20XUAS-GaMP5.003 (VX00005)/R57C10-GAL4 flies. We used pGP-JFRC7–20XUAS-IVS-GaMP6f 15.693 (attP40)/R57C10-GAL4 flies for pan-neuronal orientation tuning experiments and pGP-JFRC7–20XUAS-IVS-GaMP6f 15.641 (attP40)/c232-GAL4 for orientation tuning experiments with dark bars. c232-GAL4 and EBI1-GAL4 were gifts from M. Heisenberg and T. Lee, respectively.

For calcium imaging experiments, female flies were mounted in custom stainless-steel shim holders with designs similar to those previously described\(^{14,15}\). The dissection procedure, LED visual arena and two-photon imaging set-up were similar to those used in previous experiments\(^4\). All data analysis was performed in MATLAB (MathWorks).

**Online Content** Any additional Methods, Extended Data display items and Source Data are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Author Contributions Both authors designed the study and wrote the manuscript. J.S. carried out the experiments and data analysis.

Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to V.J. (vivek@janelia.hhmi.org).
METHODS

Fly stocks. All experiments were performed with female flies, with ages chosen based on expression levels of relevant fluorescent proteins. At least six animals were used for any single condition tested—specific sample sizes are noted for each set of experiments and were chosen based on the level of variability observed in initial experiments. Flies were randomly picked from their housing vials for all experiments. We used all data that passed a quality threshold based on the observed health of the fly during an experiment and the signal-to-noise ratio of the imaging signal.

Calculated imaging experiments to measure receptive fields were performed with UAS-GaMP6x:232-GAL4, UAS-GaMP3:EBe1-GAL4, and pJFRC7–20XUAS-GaMP6x (VK00005)/B5C10-GAL4 flies. We used pGP-JFRC7–20XUAS-IVS-GaMP6x 15.6.45 (attP40)/R57C10-GAL4 flies for pan-neuronal orientation tuning experiments (2–5 days old) and pGP-JFRC7–20XUAS-IVS-GaMP6x 15.6.45 (attP40)/R57C10-GAL4 for orientation tuning experiments (1–2-weeks old) with dark bars. c232-GAL4 and EBe1-GAL4 were gifts from M. Heisenberg and T. Lee, respectively.

To label dendrites and axons in different ring neurons, EBe1-GAL4 and c232-GAL4 were each crossed to pJFRC7–3XUAS-IVS-Syr-GFP (attP18)y2, pJFRC18–10XUAS-DenMark (attP40)y2,3 and pJFRC19–10XUAS-IVS-myr-TopHat2(VK00005) (gift of B. Pfeiffer and R. Harris, unpublished).

To compare the expression patterns of EBe1-GAL4 and c232-GAL4 to the pan-neuronal line R57C10 in the same fly, EBe1-GAL4 and c232-GAL4 were each crossed to pJFRC7–13XXL.3ExAop2-IVS-myr-GFP (su(Hw)attP8)y2, pJFRC21–10XUAS-IVS-mCD8–RFP (attP18)y2, Sc/Cyo; R57C10-Exa-pA65 (attP2)y2.

For stochastic single cell labelling of EBe1-GAL4 and c232-GAL4 with three colours a ‘flip-out’-based approach (Nern et al., manuscript in preparation) was used. In brief, heat-shock induced expression of FLP recombinase was used to excise FRT-flanked interruption cassettes from UAS reporter constructs carrying HA, V5 and Flag epitope tags, and stained with epitope-tag specific antibodies. This labelled a subset of the cells in the expression pattern with a stochastic combination of the three labels.

Anatomy: fly dissections, immunohistochemistry and confocal imaging. Confocal stacks were recorded with a 10 × 0.63 objective on a Zeiss confocal microscope. Dissections and staining were performed as previously described4,5. The primary antibody mixture consisted of: 1:1,000 sheep anti-GFP (AbD Serotec), 1:1,000 rabbit anti-DSRed (Clontech), 1:100 rat anti-HA (for Syt/DenMark/HA staining, Roche), and 10% normal donkey serum (Jackson ImmunoResearch) in PBS–TX.

Fly preparation for receptive field mapping. Receptive field measurements were performed using a preparation described previously24, but with the behavioural apparatus removed to maximize the fly's visual field. The fly was briefly anesthetized on ice and transferred to a cold plate held at 4 °C. The proboscis of the fly was fixed by either pressing it into the head and fixing it with wax, or by stretching it with a pair of tweezers mounted on a micromanipulator and then fixing it with a mixture of wax and collagen. We additionally removed either the front legs or all legs for receptive field measurements. The fly was glued to a pin and positioned in the holder with a micromanipulator and fixed in the holder with UV gel1. An opening was cut into the head to obtain optical access to the brain. To stop brain movement due to pulsation of muscle M16, we cut the muscle or the nerves innervating the muscles with dissection needles. The fly holder (including the micromanipulator) was then transferred to the microscope and mounted using magnetic mounts. Flies were dark adapted for 5 to 10 min before recordings started.

Fly preparation for imaging during walking. For walking experiments, an air-supported bell was positioned under the fly with a three-axis micromanipulator as described previously24 and the walking velocity of the fly was monitored using a camera system.

Fly preparation for imaging during tethered flight. For flying fly experiments, a holder similar to those described previously24,25,26 was used. The holder was made out of two pieces of stainless steel shim (thickness 1.27 μm). The shim is cut using a laser mill and then folded into its pyramidal shape. One piece of the holder is glued onto a liquid chamber similar to the one used for walking behaviour using epoxy4,4. After removing the front legs of the fly to prevent it from grabbing the holder, the fly is glued to a pin and inserted into the holder with a micromanipulator. The second piece of the holder is then inserted to close the pyramidal shape around the fly’s head.

The setup for combined imaging and flight behaviour was similar to one previously described24. The fly was illuminated with infrared light from below. The wing beat of the fly was recorded using a mirror placed beneath the fly and a camera (Basler 602L, operating at 100 or 150 Hz frame rate)13.

Two-photon imaging. Calcium imaging was performed using a custom-built two-photon microscope controlled with ScanImage26. Fluorescence was detected using a photomultiplier tube (H7422PA-40, Hamamatsu). We used an Olympus ×40 objective (LUMPlanFL/IR, numerical aperture 0.8) and typically adjusted the power to below 20 mW at the back aperture of the objective. We imaged at a frame rate of 6.7 Hz. Focal planes were selected based on the anatomy and visual responses of microglomeruli. We focused on microglomeruli because this approach allowed us to distinguish different neurons in a labelled population (such distinctions are difficult to make in the ellipsoid body, where axons of different neurons arborize in close proximity).

Visual stimulation: LED arena. Visual stimuli were presented using a curved visual display24 that was covered with a colour filter to prevent cross-talk between fluorescence detection and visual stimulation as described4. Additionally, to avoid reflections of stimuli from the curved surface of the display, we covered the display with a diffuser (transfacer paper). Under such low-contrast conditions, we found that the signal-to-noise ratio (SNR) of calcium responses when stimulated by dark on bright-background stimuli (for example, dark bar on bright surround) was low. This motivated our preference for bright on dark-background stimuli, which produced higher SNR calcium responses (comparison in Extended Data Fig. 5).

For receptive field measurements the display spanned 270° in azimuth and 120° in elevation. The top left and top right corners of the display (three square panels in each corner, each with a size of 30° by 30°) could not be seen by the fly (because they were occluded by the fly holder) and were excluded from the display. For behavioural experiments, a similar but smaller display was used, spanning 210° in azimuth and 90° in elevation.

Visual stimulation: flashing dots for receptive field mapping. Excitatory receptive fields for c232-GAL4 and EBe1-GAL4 flies were measured with stationary bright square dots (7.5° by 7.5°) appearing randomly in the visual field of the fly for 1 s followed by a dark period of 1 s. The measurements were repeated until the entire visual field covered by the display was stimulated once. The display was sampled with a spatial resolution of the stimulus (7.5°). Measurements were performed in 8 blocks of 140 s each, presenting a total of 468 stimuli covering the entire display.

Visual stimulation: horizontally moving bars for receptive field mapping. We used bright bars (15° in elevation, 7.5° in azimuth) that moved at a speed of 30° s⁻¹ left and right (in azimuthal direction) in the fly’s field of view.

Two repetitions with stimulation in one direction were followed by two repetitions of movement in the opposite direction. To map responses over the entire visual field spanned by the display, the stimulus was shifted in steps of 15° in elevation from the top-most part of the visual display towards the bottom part and the left and right moving stimulation was repeated in each row.

Visual stimulation: white noise. For white-noise stimulation7,37,38 we subdivided the display into squares of 11.25° by 11.25°. Each stimulus frame consisted of 20 randomly selected bright squares whilst the remaining squares remained dark (in a few trials for one fly we used 30 squares). The stimulus appeared for one second followed by a dark period of one second. We presented 60 frames of random stimulation in a trial of 140 s. For c232-GAL4 flies we used an average of 33 ± 20 trials in 7 flies (59, 61, 29, 21, 21, 14 and 26 trials, respectively). For EBe1-GAL4 flies we used an average of 41 ± 13 trials in 6 flies (38, 25, 39, 63, 35 and 47 trials, respectively). The colour scale (Fig. 2, Extended Data Fig. 8) was adjusted to give equal weight to excitatory and inhibitory subfields (mean set to 0).

To validate white-noise-based responses, we stimulated the fly with bright bars of 56.25 degrees by 18.75 degrees oriented either vertically, horizontally or rotated by 45° or 45° degrees with respect to the vertical direction. To stimulate the receptive fields of all microglomeruli in an unbiased way, the bar stimuli were presented at a random position on the display and the display was sampled with a resolution of 11.25° in both elevation and azimuth.

Visual stimulation for measuring orientation tuning curves. For orientation tuning curve measurement we used a stimulus that extended across the entire display, from 120° in azimuth for vertical orientation (90°) to 270° azimuth for horizontal orientation. The width of the bar was 15° and it was moved at a speed of 75° s⁻¹. The angle was changed incrementally in steps of 11.25° starting with either horizontally or vertically oriented bars for one direction of movement, and then repeated in the opposite direction of movement. To obtain tuning curves with dark stimuli we inverted the contrast of bright and dark and removed the diffuser.

Visual stimulation during walking and flying experiments. For walking and flying experiments we used a vertical bright bar spanning 90° in elevation and 15° in azimuth. The bar moved horizontally at a velocity of 15° s⁻¹. The bar stayed stationary for 10 s after moving in one direction for 17 s and then resumed moving in the opposite direction for 17 s.

Data analysis. All data analysis was performed in MATLAB (MathWorks). All errors and error bars shown are s.d. All P-values shown are based on t-tests, unless otherwise noted.

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Frame alignment and movement correction. Data recorded using two-photon imaging were aligned in the xy plane on a frame-by-frame basis. Data were thresholded to distinguish arborizations in the lateral triangle from background. All above-threshold pixels were set to the same value. Frames were aligned by cross-correlating each thresholded frame to a single frame at the beginning of the measurement. Multiple trials were aligned by cross-correlation of trial-averaged frames thresholded as above.

Calculation of fluorescence changes. The baseline for calculating \( \Delta F/F \) was selected by averaging the 10 preceding frames with lowest intensity in each trial or by using the baseline at the beginning of the experiment. Due to the low baseline intensity of GCaMP6, background fluorescence was not subtracted in measurements with GCaMP6. Calcium traces recorded from behaving flies were smoothed with a third order Savitzky–Golay filter over 7 frames for comparisons with behavioural data.

Regions of interest selection. For receptive field measurements in stationary flies, regions of interest (ROIs) corresponding to microglomeruli were selected manually in videos of \( \Delta F/F \). Overlapping parts of ROIs were excluded from further analysis.

For experiments in behaving flies, regions of interest were selected using visually supervised k-means clustering (Extended Data Fig. 10a–d). We used a subset of three trials (2,820 frames) for clustering-based selection of ROIs. We then used correlation-based k-means clustering between the calcium traces in all thresholded pixels. The number of clusters was selected based on an estimate of the number of microglomeruli. If not all microglomeruli could be separated into different clusters, the number of clusters was increased in a second run. We then set a threshold to remove clusters that were smaller than a certain number of pixels (60). We additionally removed clusters that had an average cross-correlation value lower than a threshold (0.2). We further split anatomically disconnected regions of the same cluster and again removed those parts that were smaller than a size threshold (30 pixels). In a final check, the remaining ROIs were overlaid with the frames of the calcium video that showed the largest response in this ROI, and ROIs that did not correspond to microglomeruli were removed manually.

Receptive field mapping. Receptive fields (RFs) were smoothed using a Gaussian filter (4 pixels with a standard deviation of 1 pixel by default; 5 pixels with a standard deviation of 4 pixels for white noise measurements).

For RF measurements (for example, in Fig. 1), we combined responses to left- and right-moving stimuli (each averaged over two trials). The onset of the calcium response was correlated with the bar’s movement into the RF. Due to the size of the RF, a bar moving from the right side of the fly towards the left side entered the RF from the right side and induced a calcium onset starting at the right side of the (excitatory part of the) RF. Similarly, a bar entering the receptive field from the left side induced a calcium onset starting at the left side of the RF. Due to the faster onset response than off-response of GCaMP, the onset of the RF was better defined than the offset. To find the centre of the excitatory RF measured with moving bars, we defined the RF centre as the weighted centroid of the average of responses to thresholded left- and right-moving stimuli (Extended Data Fig. 1b). This was equivalent to delimiting the RF by its calcium onset response. This procedure made the location of computed RF centres invariant to the kinetics of the calcium indicator.

We used the following parameters to characterize the excitatory parts of RFs measured with single stationary dots (measured at 50% of the maximum \( \Delta F/F \) response of each RF) (see Extended Data Fig. 7c): area, major axis (of an ellipse that has the same normalized second central moments as the region as determined with the MATLAB ‘regionprops’ function), minor axis, eccentricity, orientation (the angle between the horizontal x axis and the major axis of the ellipse, 0 corresponding to horizontal orientation), retinotopic correlation (the correlation coefficient between the centre of the RF, determined as the weighted centroid of the RF area and the centre of the corresponding ROI), and movement.

Receptive field display. To display microglomerular RFs, we coloured light- emitting diode (LED) arena positions in proportion to the \( \Delta F/F \) response elicited by the stimulus presented in that position. For moving stimuli, the calcium response in each pixel was determined as the average over all response frames that were recorded while the stimulus was at that position. Calcium responses were interpolated to account for mismatches between frame rates and movement of stimuli. For stimulation with stationary stimuli, the \( \Delta F/F \) values shown in RF plots correspond to the maximum \( \Delta F/F \) during the stimulation period. To prevent cross-talk between sequentially presented stimulus frames due to the slow decay of the calcium response (which extended beyond stimulus presentation) we only considered the calcium increase and not the calcium decay in assigning \( \Delta F/F \) values to stimulus frames.

Retinotopy. To assess retinotopy, we calculated the correlation coefficient between microglomerulus centres and centres of their RFs. This was compared to the correlation coefficient obtained after randomly shuffling correspondences.

Additionally, we performed principal component analysis (PCA) on the \((x, y)\) values of RF centres. The first PC gave us the direction of maximum variation of RF centres, which we consider to be the primary retinotopic axis (in the fly’s visual field) for the RF population.

Orientation tuning. To measure orientation tuning (Fig. 2), single 15°-wide bars spanning the entire visual display were presented, and their orientation changed in steps of 11.25°. Responses from multiple trials were then averaged, and tuning curves were fit using the sum of two circular Gaussians:

\[
dI = a_1 e^{-k_1 (\cos(\theta) - \theta_1)^2} + a_2 e^{-k_2 (\cos(\theta) - \theta_2)^2}
\]

in which \(a_1\) and \(a_2\) are amplitudes, \(\theta_1\) and \(\theta_2\) are the maximum angles and \(k_1\) and \(k_2\) are width parameters.

The preferred orientation for each microglomerulus was the maximum of its fitted tuning curve.

Orientation selectivity. The orientation selectivity index (OSI)\(^{16}\), was computed as the difference between the response in the preferred direction, \(\Delta F/F_{\text{max}}\), and the direction orthogonal to it (preferred direction ± 90°), \(\Delta F/F_{\text{ortho}}\), normalized by the sum of the responses in the two directions:

\[
\text{OSI} = \frac{\Delta F/F_{\text{max}} - \Delta F/F_{\text{ortho}}}{\Delta F/F_{\text{max}} + \Delta F/F_{\text{ortho}}}
\]

Direction selectivity. The direction selectivity index\(^4\) (DI) was calculated as the difference between responses in the preferred direction, \(\Delta F/F_{\text{max}}\) and anti-preferred direction (preferred direction + 180°), \(\Delta F/F_{\text{opposite}}\), normalized by their sum:

\[
\text{DI} = \frac{\Delta F/F_{\text{max}} - \Delta F/F_{\text{opposite}}}{\Delta F/F_{\text{max}} + \Delta F/F_{\text{opposite}}}
\]

The sign of the DI was defined as positive for front-to-back movement and negative for back-to-front movement.

White-noise-based receptive fields and response predictions. RFs were reconstructed by thresholding calcium traces at 30% \(\Delta F/F\) and averaging over all frames that induced a response larger than this threshold, weighted by peak calcium response. Only the rising slope of the calcium response was considered. The displayed RFs are the weighted averages of stimulus frames and the mean value (background) is subtracted. Max is the maximum of the weighted average after subtracting the mean value, and min is the minimum of this average. The mean value is set to 0. To predict responses to oriented bars (Fig. 2), we multiplied the mean-subtracted white-noise-based RF with stimulus values in each pixel and summed over all pixels. We convolved the result with a calcium response function. The amplitude and time constants of the calcium response function were fitted using response bars of one orientation and then used in the prediction of responses to the remaining three orientations.

The calcium response function, \(c_{\text{r}}\), used in predicting responses to oriented stimuli and in the analysis of calcium responses recorded during behaviour (see below) is given by:

\[
c_{\text{r}}(t) \sim (1 - e^{-t/\tau_b}) (1 - e^{-t/\tau_d})
\]

where \(\tau_b\) is the onset time and \(\tau_d\) and \(\tau_d\) are the rise and decay times of the calcium indicator.

Walking behaviour analysis. Ball movement was recorded with a sampling rate of 4 kHz and velocities were calculated with a time base of 250 ms. Velocities were then averaged over all velocity values in each two-photon imaging frame. Average velocities were calculated by detecting epochs in which the fly was moving and then averaged over this period. Owing to strong walking activity in behaving animals, there were few recordings that allowed us to compare microglomerular responses in walking and stationary conditions (Extended Data Fig. 10e–n).

Flight behaviour analysis. The wing angle was detected by first manually identifying the wing hinge in both wings. The wing was then detected by first subtracting the background recorded while the fly was not flying, smoothing with a mean filter, and setting a threshold for detecting the wings in two ROIs surrounding the wings. The wing angle was then defined as the angle between the wing hinge and the tip of the wing. Flight was typically intermittent (Extended Data Fig. 10b, p).

Model for fitting responses of visual microglomeruli during behaviour. For describing the calcium responses of visual microglomeruli during walking behaviour we fitted the responses with a model consisting of a single excitatory and inhibitory Gaussian function. Model fitting was only performed for microglomeruli that responded with a single peak during one passage of the stimulus on the display, not for bilateral receptive fields. The initial position of each Gaussian, the (common) standard deviation and the amplitude were used as fit parameters.

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Since the inhibitory responses could not be directly observed in our calcium signals, we set all negative values to zero.

\[ rf^* = (e^{-(x-x_0)^2/(2\sigma_x^2)} - e^{-(x-x_1)^2/(2\sigma_x^2)}) \]

\[ rf = 0 \text{ if } rf < 0; \quad x_0 \text{ and } x_1 \text{ are the location of the excitatory and inhibitory receptive fields, respectively, and } \sigma_x \text{ is the width of the receptive field. The calcium signal } s(\Delta F/F) \text{ was then modelled as the convolution of the receptive field with the calcium response function, } crf, \text{ and a constant offset, } bg: \]

\[ s = crf \otimes rf + bg \]

Because the response in flying flies depended on the state of the fly, we used a model for the receptive field that additionally included the left and right wing angles as parameters:

\[ rf^{\text{flying behaviour}} = (e^{-(x-x_0)^2/(2\sigma_x^2)} - e^{-(x-x_1)^2/(2\sigma_x^2)}) \]

\[ rf = 0 \text{ if } rf < 0; \text{ behaviour } = a lw + b rw + c \text{ depends linearly, with the parameters } a, b \text{ and } c, \text{ on the left wing angle } lw \text{ and the right wing angle } rw. \]

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Extended Data Figure 1 | Pan-neuronal receptive fields for LTR microglomeruli. a, Selected two-photon imaging frames showing calcium response in the LTR with pan-neuronal expression of GCaMP5. Highlighted in red are the microglomeruli that were selected for receptive field reconstruction. Scale bar, 5 μm. b, Schematic of visual stimulation used, receptive fields (two-trial averages) measured with left-and right-moving visual stimuli and their intersection labelled at full width at half maximum (FWHM) intensity (white ellipse) with the weighted centroid (white asterisk) of the distribution. c, Centre of each receptive field plotted against the centre of its corresponding microglomerulus. d, Correlation coefficient of data in d ($r = 0.39$, $P = 0$) and correlation coefficient of same data after randomly permuting the microglomeruli ($n = 3,000$ permutations, mean = $(4.6 ± 0.076) \times 10^{-4}$). e, Principal component axes of receptive field centres (see Methods).
Extended Data Figure 2 | Ring neurons make dendritic projections to LTR microglomeruli and send axonal arbors to the rings of the ellipsoid body.
a. A frame of a confocal stack of the LTR labelled with antibody staining against GFP using a pan-neuronal driver line, R57C10-LexA and antibody staining against red fluorescent protein (RFP) of EB1-GAL4. d, dorsal; m, medial. 
b. A section of a confocal stack of the LTR labelled with GFP using a pan-neuronal driver line R57C10-LexA and RFP labelling of c232-GAL4. c, Multicolour FLP-out labelling of c232-GAL4 (see Methods). d, Antibody staining of c232-GAL4 expressing the dendritic marker, DenMark (red) and an axonal label, synaptotagmin-tagged GFP (green). Entire cell is outlined with membrane-tagged HA (blue) (see Methods). Top panel is merge of all colours. e, Same as d for EB1-GAL4.
Extended Data Figure 3 | Ring neurons respond with strong calcium transients to single stationary bright dots; receptive fields of ring neuron populations are spread non-uniformly across the largely ipsilateral visual field. a. Overlay of all regions of interest of R4d microglomeruli recorded in a fly during visual stimulation over an average of all frames in a calcium video (940 frames), and selected frames of the calcium video showing responses of individual microglomeruli. Scale bar, 5 µm. b. An example of the single dot stimulus appearing at the specified azimuth and elevation for 1 s followed by a dark period of 1 s (coloured dot indicates both the stimulus and the subsequent off period) (top). ΔF/ΔF0 for microglomerulus 3 in a responding to the stimulus (bottom). c. Excitatory receptive fields for all microglomeruli shown in a. d. Overlay of all regions of interest selected among R2 microglomeruli recorded during visual stimulation over an average of all frames (n = 940) in a calcium video, and selected frames of the calcium video showing responses of individual microglomeruli. Scale bar, 5 µm. e. Azimuth and elevation of the single bright dot presented for 1 s followed by a 1 s interval without stimulus (top). ΔF/ΔF0 for microglomerulus 1 in d responding to the visual stimulus (bottom). f. Excitatory receptive fields for all microglomeruli shown in d. g. Overlapping receptive fields of R4d neurons. Ellipses have the same normalized second central moments as the receptive fields shown in c thresholded at 30% ΔF/ΔF0. Intensity scale indicates how many receptive fields overlap in a given region. h. Same as g for R2 neurons shown in f. i–k, Average receptive fields measured across all flies with single dot stimulation for R4d neurons (green, n = 8 flies, 159 receptive fields) (i), for R2 neurons (purple, n = 12 flies, 219 RFs) (j) and for both (overlay) (k). Recordings from the right lateral triangle were flipped to the contralateral side for averaging. Although a large fraction of R2 and R4d ring neurons we imaged were visually responsive, there were ring neurons that did not respond in our experiments. Such neurons may have receptive fields in areas of the fly’s visual field that we did not sample (for example, very lateral areas), be tuned to visual dimensions that we did not explore (such as colour or polarization or complex shapes), or be selectively responsive to other sensory modalities (such as mechanosensation or thermosensation).
Extended Data Figure 4 | Ring neuron orientation preference and selectivity, direction selectivity and receptive field centre positions.

a, Correlation coefficients between predicted and measured responses for R2 neurons (histogram over all trials and receptive fields) (n = 6 flies). b, Same as a, for R4d neurons (n = 6 flies). c, Microglomeruli of R4d neurons coloured according to orientation preference and orientation selectivity (six-trial averages). d, Azimuth and elevation of the centre of the excitatory receptive fields of the neurons shown in c (two-trial averages, measured using moving bars as described in Extended Data Fig. 1 and Methods). e, Direction selectivity for neurons in c (six-trial averages). f, g, Microglomeruli shown in Fig. 2k, l labelled according to the azimuth and elevation of their excitatory receptive field centre (measured using moving stimuli as in Extended Data Fig. 1) (f) and their orientation selectivity (four-trial averages) (g). h–p, Distributions of preferred orientation, orientation selectivity and direction index for R2 (n = 10 flies), R4d (n = 12) and pan-neuronal (n = 11 flies) microglomeruli.
Extended Data Figure 5 | Comparison of orientation tuning curves measured using dark-on-bright and bright-on-dark bars. **a**, Orientation tuning curves (GCaMP6s) of three R4d microglomeruli measured using (top row) bright bars on a dark background (as in Fig. 2f) and dark bars on a bright background (exact contrast inversion of bright-bar stimulus). As suggested by excitatory–inhibitory structure of receptive fields (Fig. 2c, Extended Data Fig. 8), tuning curves measured with the two stimuli are qualitatively similar, but bright bars produce stronger responses (with our low-contrast, filtered LED array display, see Methods). **b**, Fitted tuning curves measured using dark and bright bars are highly correlated (mean $= 0.648 \pm 0.26$, \(n = 7\) flies).
Extended Data Figure 6 | Stereotypy of excitatory receptive fields across flies in R4d neurons. a, Excitatory receptive fields measured using single dot stimulation in one fly (fly1), and receptive fields recorded in other flies (n = 12) aligned by similarity. b, Cross-correlation coefficient between fly 1 and all other receptive fields in each column (mean = 0.74 ± 0.2).

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Extended Data Figure 7 | Stereotypy of excitatory receptive fields across flies in R2 neurons; summary of receptive field properties. a, Same as Extended Data Fig. 6a, measured in R2 neurons in 8 flies. b, Cross-correlation coefficient between fly 1 and all other receptive fields in each column for R2 neurons shown in a (mean = 0.75 ± 0.12). c, Table showing characteristics of excitatory receptive fields for R4d and R2 ring neurons measured with single dot stimulation calculated at half maximum ΔF/F. The retinotopic correlation coefficients are significantly different from the control distributions, with $P = 0.0283$ for R4d ($n = 8$ flies, 159 receptive fields) and $P = 0.0069$ for R2 ($n = 12$ flies, 219 receptive fields).
Extended Data Figure 8 | Stereotypy of white-noise-based receptive fields across flies in R2 neurons. a, Receptive fields measured using white-noise stimulation in 6 flies aligned according to similarity. We used an average of 41 ± 13 trials (6 flies, number of trials: 38, 25, 39, 63, 35 and 47). Each trial consisted of presentation of 60 stimulus frames (see Methods). b, Receptive fields measured using white-noise stimulation in 7 flies aligned by similarity (subset of this data are shown in Fig. 3). We used an average of 33 ± 20 trials (7 flies, number of trials: 59, 61, 29, 21, 21, 14 and 26).
Extended Data Figure 9 | Ring neuron visual responses in the LTR are diminished during flight but not walking.  

a, Microglomeruli selected by visually assisted k-means clustering in R2 neurons, a subset of which are shown in Fig. 4b. 

b, Average over $n = 24$ trials of responses of R2 microglomeruli to a bright bar ($15^\circ \times 90^\circ$) moving at $30^\circ \text{s}^{-1}$ shown during a period when the fly is walking more than 50% of the time (blue) and less than 50% of the time (red) during rightward moving stimulation (envelopes show s.d.). 

c, d, Correlation coefficient (mean $= 0.72 \pm 0.25$) between model (see Methods) and data for R2 microglomeruli recorded in $n = 8$ flies (c), and for R4d microglomeruli ($n = 14$ flies, mean $0.7 \pm 0.25$) that have unilateral receptive fields (d). 

e, Concatenation of seven epochs during walking behaviour with EBI-GAL4, UAS-GCaMP3 flies during stimulation with a left and right moving bar: data (blue) and model (red) fit (see Methods). 

f, Microglomeruli in R4d selected using visually assisted k-means clustering. 

g, Visual responses during flying and non-flying behaviour during stimulation with a bar moving from left to right (averaged over $n = 14$ trials for flying, $n = 14$ trials for non-flying), and during stimulation with a bar moving from right to left ($n = 14$ for flying and $n = 11$ trials for non-flying) for the fly shown in Fig. 4e (envelopes show s.d.). 

h, Correlation coefficient between model (see Methods) and data for R4d neurons with a linear dependence of the visual response on both wing angles (mean $= 0.65 \pm 0.27$) recorded in $n = 14$ flies.
Extended Data Figure 10 | Selection of microglomeruli using visually guided k-means clustering; walking and flight activity during behaving fly experiments. a, Cross-correlation matrix between all pixels above threshold (see Methods for details). b, Cross-correlation matrix sorted according to clusters found by k-means clustering. c, Resulting regions of interest. d, Overlay of resulting regions of interest and selected frames of calcium video. Regions of interest that do not correspond to microglomeruli (based on comparison with the calcium video) are removed. e–g, Velocities measured: forward (e), side (f) and rotational (h) for all c232-GAL4, UAS-GCaMP3 flies recorded during walking behaviour. h, Percentage of walking for all EBI-GAL4 UAS-GCaMP3 flies recorded. i–k, Velocities forward (i), sidewise (j) and rotational (k). l, Percentage of walking for all EBI-GAL4, UAS-GCaMP3 flies recorded during walking behaviour. m, Number of 130 s epochs recorded for each c232-GAL4, UAS-GCaMP3 walking fly. n, Number of 130 s epochs recorded for each EBI-GAL4, UAS-GCaMP3 walking fly. o, Percentage of time flying during recording for all c232-GAL4, UAS-GCaMP3 flies recorded during flight behaviour. p, Number of 130 s epochs recorded for each C232-GAL4, UAS-GCaMP3 flying fly.