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Odor tracking in flying *Drosophila* requires visual reafference and compass neurons

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Flying *Drosophila* critically depend on high-contrast visual surroundings to localize odor sources in still air, yet the neural mechanisms of visual integration for active odor tracking are unknown. We demonstrate that Ellipsoid body-Protocerebral bridge-Gall (E-PG) neurons—head direction cells in the central complex—work in concert with self-generated visual motion signals to maintain a stable heading metric during olfactory navigation in flight. Using a magnetic tether system and a digital “visual clamp,” we show that removing the visual feedback generated by a fly’s own turns (reafference) causes the animal to lose its heading within an odor plume. Thus, olfactory and mechanosensory signals alone are insufficient for plume stabilization. E-PG neurons have been shown to store visual changes in heading during flight. Genetically hyperpolarizing E-PG neurons significantly compromised the flies’ ability to both acquire and maintain heading toward a food odor. Notably, silencing these neurons did not disrupt basic visual reflexes, such as optomotor gaze stabilization or object tracking, indicating a specific role in odor-directed visual navigation rather than visual flight control. While odor was found to modulate the frequency and amplitude of turns independently, E-PG neurons are essential for directing the orientation of corrective saccades toward the plume center. These results establish that visual reafference engages the internal visual compass and associated control circuits to sustain a spatial working memory of heading changes between saccades, allowing flies to maintain a straight course and navigate effectively toward an invisible odor source in flight in still air.

visual memory | multisensory | central complex | navigation

Flying insects, such as *Drosophila*, require olfactory cues to locate food and reproductive sites, sometimes over vast distances (1). Odor plumes in nature can be intermittent and filamentous, so odor detection alone does not reliably indicate the source location because the structure of the odor plume is determined by wind conditions, which may rapidly shift direction or be entirely still (2). In wind, a flying fly tends to “cast and surge,” making cross-wind turns if the odor signal is lost (cast), and speeding upwind while the odor is detectable (surge) (3, 4). In fact, because wind can determine the direction of an odor signal, odor activates central brain circuits that encode the relative wind direction for this purpose (5). The absence of wind poses a challenge to olfactory navigation that can be solved by either measuring the static odor gradient and orienting up it (chemotaxis), or by changing the rate of turns as the odor intensity changes (chemokinesis). Walking flies use both strategies (6, 7). In flight, rapid turns—termed “saccades” for their functional similarity to gaze stabilizing eye movements—are more frequent and larger in still air than wind (8), emphasizing the importance of this motor behavior for odor tracking. Flying through still air, a food odor carried by advection tends to provoke a “sink and circle” behavior composed of decreased altitude (sink), coupled with bouts of unidirectional saccades (circle) (8, 9). Saccade direction is influenced by the static odor gradient across the two antennae, generating differential neural activation across the antennal lobes (7, 10).

Olfaction is essential in many ways and yet is insufficient on its own to enable odor localization during flight. Rather, *Drosophila* have a peculiar dependence upon vision to localize an odor source. In free-flight within a visually featureless, low contrast 1-meter arena, flies fail to target the advecting odor source in still air, yet they have no trouble approaching the source when the arena walls are covered with a high contrast pattern of vertical edges (9). Follow-up work tested flies tethered on a magnetic pivot, free to steer in the horizontal (yaw) plane during flight. A slow, effectively windless, plume of food odor elicits small amplitude low frequency saccades directed back and forth across the plume. However, if the high contrast visual background is switched to a uniform grayscale, the animals no longer balance saccade direction to maintain their heading toward the

Significance

A crucial brain function that remains largely mysterious is building robust working memory by integrating stimuli across sensory modalities. For flying *Drosophila*, visual feedback is required to localize an odor source. A group of cells residing in the navigation center of the fly brain [Ellipsoid body-Protocerebral bridge-Gall (E-PG) neurons] have been shown to function like an internal compass, providing a reference frame for downstream circuits that can encode directional memory of heading changes akin to mammalian head-direction cells. Here, we show that self-generated visual motion signals work with E-PG compass neurons so that the direction of exploratory turns can be stored and recalled to maintain the animal’s heading within an odor plume during flight.

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plume, instead randomly exploring the circular arena as if there were no odor present (11, 12). The same visual dependence is observed with thirsty flies seeking water vapor in flight (13). Computational models indicate that subtle modulation of visual control parameters is sufficient to enhance odor localization in flight in still air (9, 14), yet the functional role and neural mechanisms of visually guided plume tracking are unknown.

In flies and other insects, allocentric heading changes are encoded and stored by compass neurons that project from a toroidal neuropile called the Ellipsoid Body to the Protocerebral Bridge and Gall (15), thereby named “E-PG” neurons. During flight under head-fixed virtual reality conditions, a revolving bump of E-PG neural activity dynamically tracks the displacement of the visual scene as a fly alters its wing kinematics to steer back and forth in yaw, much like the needle on a compass except with each individual calibrated to its own “north” (16, 17). In the rigid tether paradigm, genetic inactivation of E-PG cells eliminates idiosyncratic menotaxis in response to a celestial-like cue, suggesting an important role for E-PGs in navigation and dispersal (18). For a head-fixed fly, the time course and magnitude of bump displacement faithfully follows its voluntary course changes in flight. Most notably, segments of straight flight interspersed with spontaneous turns are tracked by phase-shifts in bump position, thereby providing a stable metric of heading direction even for turns separated by several seconds or more (17). We reasoned that high contrast stationary surroundings provide visual feedback about self-induced displacements and that these reafferent visual signals engage compass neurons to record heading changes, directing saccades into an invisible plume in still air. Here we show that removing visual reafference (19)—visual input generated by the fly’s volitional turns—causes flies to lose the plume, and that flies with silenced E-PG neurons are unable to locate an odor plume nor follow a food odor plume they have already encountered.

Results

Magnetically Tethered Flies Maintain their Heading into a Food Odor Plume. Flies were presented with a visual panorama projected from a digital projector, reflected from first-surface mirrors, to form a geometrically perspective corrected image that subtended 360° in azimuth and 70° in elevation (Fig. 1A). A small nozzle supplied saturated vapor at 14 mL/min flow rate, drawn away from beneath the fly with gentle suction. The fly is tethered to a steel pin and suspended within a frictionless magnetic pivot that allows free rotation in the yaw plane (Fig. 1B). A photoionization detector was used to sample the plume gradient inside the flight arena, which indicates the highest concentration occurs near the odorant nozzle, but that trace odor stimuli occur across the azimuth (Fig. 1C). The fly’s heading is measured using a high speed infrared video camera from below. To eliminate self-generated image motion during free-yaw turns, the yaw position of the fly is subtracted from the orientation of the stimulus in the next frame. In effect, this iteratively revolves the visual display in register with the fly’s yaw turns, removing the expected visual reafference but leaving any proprioceptive sense of body rotation intact (Fig. 1D). We performed an offline validation of the spatial and temporal precision of the clamp by using DeepLabCut (20, 21) to track body angle from the raw offline video footage. We then compare the offline tracking results to the original online heading measurement and resultant image motion dynamics (Fig. 1E). Hardware projection error amounted to half of the acceptance angle of a single photoreceptor and less than one fifth of the impulse response time of an optomotor reflex (*Materials and Methods*). Visual stimuli were composed of either a symmetric 30°

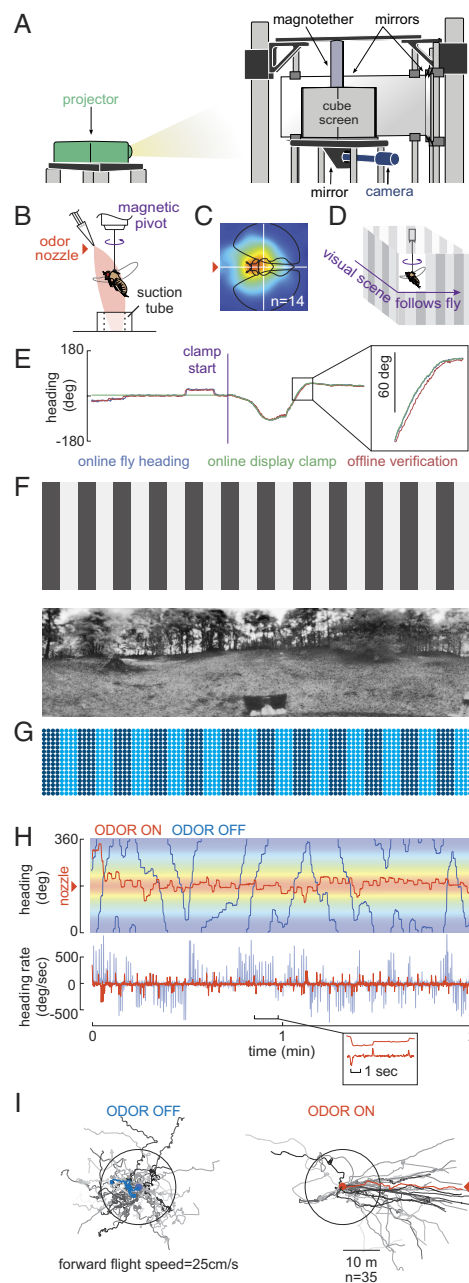


Fig. 1. Flies actively center a food odor plume on a free-yaw visual flight simulator. (A) Digital projection onto mirrors forms a perspective corrected display on four sides of an acrylic cube lined with a diffuser. The display image was 1,280 × 800 pixels, each subtending 0.4°. (B) Saturated odor vapor (apple cider vinegar, ACV) is delivered from a nozzle 3 mm from the fly’s head and drawn away with suction. A magnetic pivot allows the fly or revolve 360°. (C) The spatial odor gradients were collected from a 12 × 12 grid using a photoionization detector. The grid was sampled 14 times and values were averaged at each point and plotted in pseudocolor. The fly silhouette is drawn to scale and the center of rotation indicated with the white crosshair. (D) The fly’s body angle is tracked in real time with infrared video and used to yoke the rotation of the visual display so that the visual scene remains stable relative to the fly’s body axis as the fly freely steers in yaw. (E) Time series plot of example fly heading, online display dynamics of the visual clamp, and offline fly heading tracked with DeepLabCut to validate online stimulus dynamics. Inset shows a zoomed-in segment indicative of the difference between fly heading and display movement, which was on average 2.6° for 50% of all trials. (F) Two visual stimuli were programmed with the digital projection system, a 30° wavelength square grating and a natural scene. (G) Data in Figs. 1H and 4 were collected on a legacy system based on a cylindrical array of 24 × 96 blue pixels, subtending 3.75°, drawn to scale. (H) Data from a single fly for a 2 min trial in ACV and using the blue LED display. (I) For 35 flies tested as in G, simulated flight paths were reconstructed assuming a fixed forward flight speed. Each trajectory started from the same initial orientation. The circle indicates 10 m radius.

wavelength squarewave grating or asymmetric natural panoramic image (Fig. 1*F*). Two of the experiments below were performed using a 96 × 16 pixel cylindrical array of blue LEDs with 3.75° pixels equipped with identical magnetic tether and odor plume apparatus (Fig. 1*G*) (13).

To highlight the nature of plume tracking behavior, a plume of either saturated water vapor (odor OFF) or saturated apple cider vinegar (ACV, odor ON) was presented continuously for 2-min trials. The visual panorama consisted of a stationary 30° grating on the LED display (Fig. 1*G*). In the absence of ACV, an exemplar animal explores the 360° arena with rapid body saccades, characterized by spikes in angular velocity, which punctuate periods of stationary, fixed flight heading. When the odor plume is switched on, the saccades become smaller, less frequent, and are oriented to maintain the flight heading centered near the nozzle (Fig. 1*H*). For these tethered flies that can change only their azimuthal heading, we simulated 2D flight trajectories v from yaw body angle θ assuming a moderate flight speed $r = 25$ cm/s (3, 4). Flight vectors were calculated for each time point with the following formula:

$$v = \langle r * \cos(\theta), r * \sin(\theta) \rangle = r.$$

We tested 35 flies before and after odor exposure (Fig. 1*I*). In the absence of odor, the simulated flight trajectories are short, curved, or circular, and project in all directions from the starting point. By contrast, after being presented with the odor plume, flies balance the distribution of right and left saccades in order to maintain their heading toward the odor nozzle for 2 min, equivalent to roughly 30 m at nominal flight speed of 25 cm/s (Fig. 1*I*).

Free-Yaw Flies Use Visual Reafferece to Maintain a Constant Heading Within a Plume. Prior work using the free-yaw magnetic tether system has demonstrated the importance of visual feedback in odor tracking behavior. For flies that begin test trials within the odor plume, switching from a high contrast grating to an equiluminant grayscale background significantly reduces their ability to maintain their heading in the plume, and this visual dependence occurs both to track attractive apple cider vinegar and anti-track aversive benzaldehyde (11–13, 22). These results suggest that visual feedback generated during self-motion is somehow being integrated and stored, sometimes for several seconds, in order to direct saccades back into the plume. Note that vision is not the only known modality providing directional cues for navigation (23). However, when other directional cues are intact, except for visual reafferece, we posit that removal of visual contrast eliminates the brief turn-direction memory required to return to a given heading direction. Alternatively, a spatially uniform visual panorama could trigger kinesis-like changes in saccade rate or amplitude that are independent of the control of heading direction.

To test these alternatives, we removed visual reafferece normally generated during turns by digitally yoking or clamping yaw rotation of the visual display to the fly's body axis in free-yaw conditions (Fig. 1*E*). Thus, during a leftward saccade, the fly experiences normal changes in yaw inertia and odor intensity, but loses the expected rightward image displacement. We used the symmetrical striped grating background, which contains no spatial features that might impose a preferred heading.

Flies were drawn into the ACV plume with bar oscillation before each trial. Each fly was tested both without the visual clamp (Fig. 2*A*, *Left*), and with the clamp engaged at the 20 s mark (Fig. 2*A*, *Right*; from here onward, time series data are plotted vertically to facilitate visual inspection of left–right steering asymmetries across the midline odor plume). Inspection of the raw trajectories indicates that flies disperse from the midline plume when the clamp is activated (Fig. 2*A*, *Right*), drifting laterally

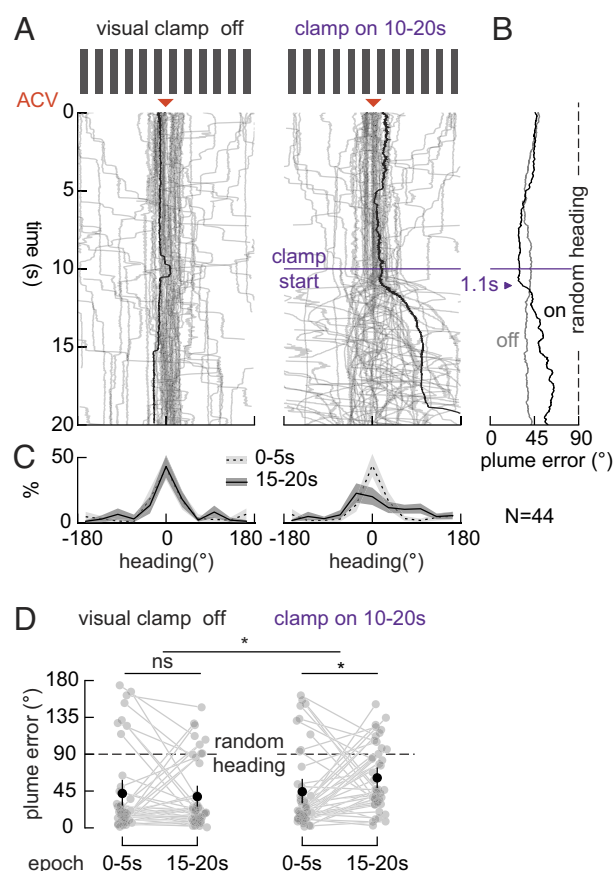


Fig. 2. Removing visual reafferece compromises odor tracking in flight. Flies were tested with a 30° symmetrical grating under continuous ACV delivery. (A) Raw flight trajectories for each fly in gray; one example trajectory is plotted with a dark line. Visual clamp remained off for the full trial (Left) or was activated at the 10 s mark (Right). Each fly was tested once in both conditions. The orange arrowhead indicates location of odor nozzle delivering ACV. (B) The absolute angular distance from the plume averaged in time across the population. 90° plume error is equivalent to chance heading relative to the plume. (C) The distribution of heading across the population for two epochs: 0 to 10 s (dashed line) and 30 to 40 s (solid line). Shaded envelope indicates 84% CI, where nonoverlapping indicates $\alpha < 0.05$. (D) Average plume error across the test trial for each fly, within subjects comparisons across the first and last epoch of the trial. Error bars indicate 95% CI. Asterisks indicate level of significance of the one-tailed bootstrap test (Materials and Methods) $*P < 0.05$.

from the odor nozzle when they would normally fixate. We computed the plume error in time as the angle between the fly's heading and the odor nozzle (Fig. 2*B*). Plume error of 90° would be equivalent to chance heading relative to the odor nozzle. Next, to examine how accumulating plume error impacts the flies' heading changes, we compared the distribution of azimuthal flight heading for 10 s epochs at the start ($t = 0$ to 5 s) and end ($t = 15$ to 20 s) of each trial (Fig. 2*C*). Engaging the visual clamp significantly increased plume error by 16° on average ($P < 0.05$; Fig. 2*D*), which led to an effect size of 20° more than the no-clamp test ($P < 0.05$; Fig. 2*D*). The proportion of trajectories that centered the plume was reduced significantly by the clamp onset (Fig. 2*B*, *Right*). However, note the nonzero probability of transit near the plume even with the visual clamp engaged, suggesting some amount of visually independent chemotaxis, at least over the 10 s time course of the visual clamp test. These results present direct evidence that when the visual feedback experienced during normal closed-loop flight is disabled, olfactory and mechanosensory feedback signals alone are insufficient to stabilize heading into an odor plume. Rather, visual reafferece generated specifically by self-motion is crucial.

Plume Tracking Requires Compass Neurons. We next sought to test whether odor navigation required the use of E-PG compass neurons known to track visual heading changes, at least during body-fixed flight. We crossed a split-Gal4 line that selectively labels E-PG neurons (18) with UAS-Kir2.1, an inward rectifying potassium channel that hyperpolarizes neurons. F1 genetic controls carry a promoterless (empty) split-Gal4 at the same insertion driving UAS-Kir2.1, thereby obviating the need for parental controls with different genetic background.

The experimental design was similar to the visual clamp experiment above. However, since the angular domain of action of the E-PG activity bump can “jump” between symmetric stripes (24), we tested flies with both a periodic grating and a natural panorama. We compared the azimuthal distributions of flight heading during the initial 10 s epoch, when flies had been visually positioned near the plume, to the final 10 s epoch, when flies would be expected to be actively maintaining their heading. The empty > Kir control animals track the plume well within both the artificial grating and natural visual panoramas (Fig. 3A), with no obvious differences in the trajectory of average plume error (Fig. 3B). The fraction of flies maintaining their heading directly at the plume center was lower for the grating than for the natural scene (Fig. 3C 30 s→40 s, see nonoverlapping CI at 0°). On average, plume error increased roughly 15° over the course of the

experiment, but this was not statistically significant ($P > 0.05$) and there were no significant effects of switching between visual backgrounds for the genetic control flies (Fig. 3D). The results indicate that both the grating and natural scene are effective at facilitating plume tracking, although centering performance is improved with the natural scene, at least close to the odor nozzle.

Hyperpolarizing E-PG neurons with Kir2.1 strongly compromised flies’ ability to track the odor plume. Visual inspection of the raw trajectories suggests broad dispersion of flies’ heading away from the odor nozzle during the time course of the experiment (Fig. 3E). Plume error accumulates throughout the trial approaching chance level (Fig. 3F). The increased tracking error is evident in the heading histograms comparing the first and last 10-s epochs, which show that centering probability is reduced significantly between epochs for EPG > Kir flies (Fig. 3G, notice that the CI do not overlap around $x = 0^\circ$). On average, plume error increases 37° and 39° between the start to the end of the experiment for the grating and natural scene, respectively, conferring a high level of statistical significance (Fig. 3H).

Silencing E-PG neurons significantly impairs, but does not eradicate, plume centering behavior, as evidenced by a significant number of trajectories centered at 0° (Fig. 3G). Because flies were encouraged to start close to the source of the plume, these experiments specifically tested whether they could maintain their

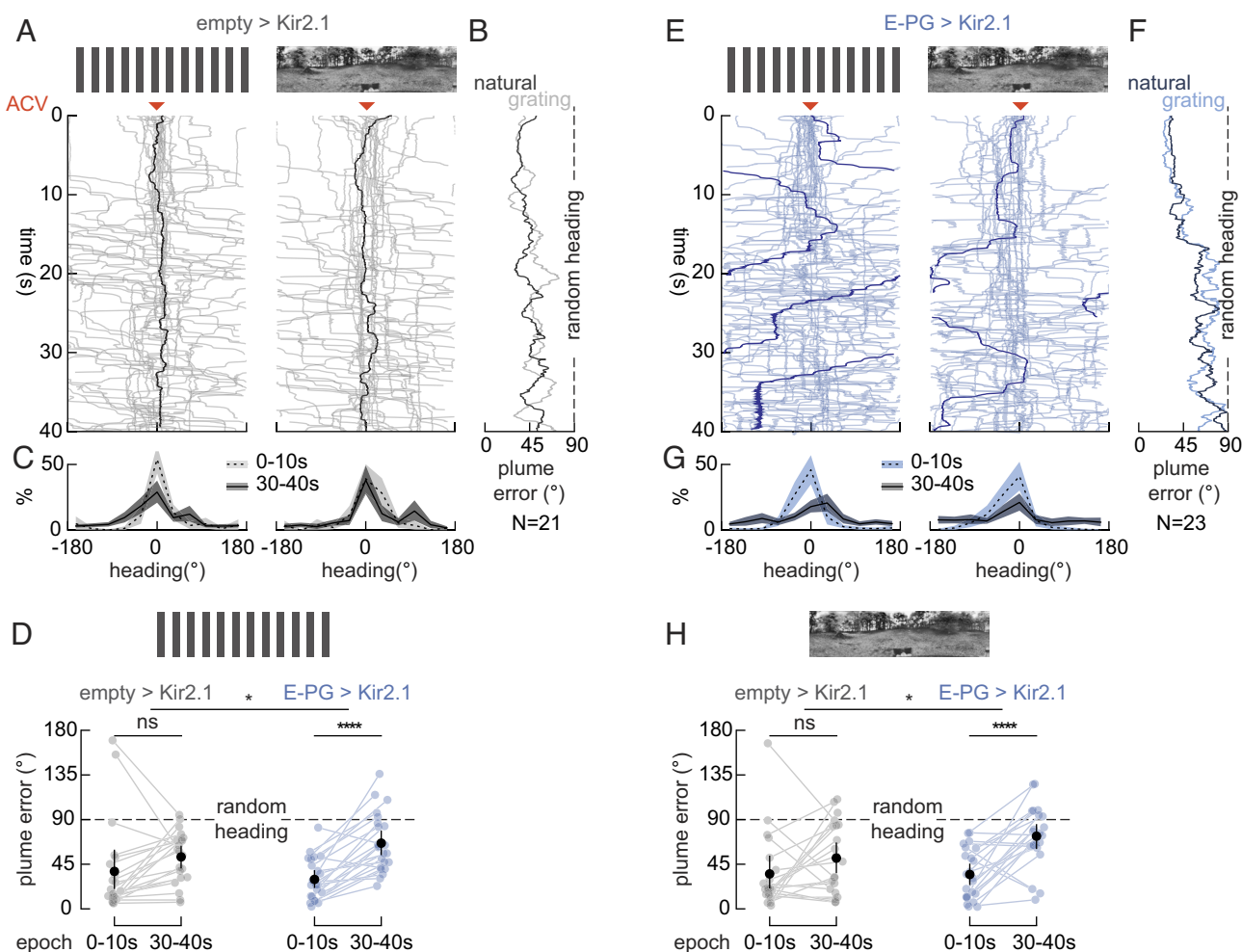


Fig. 3. Compass neurons are required for flies to maintain normal plume centering behavior in flight. All panels are formatted the same as in Fig. 2. (A) Empty-split-Gal4 controls are driving expression of UAS-Kir2.1; shorthand is empty > Kir2.1. Raw flight trajectories in light gray, with one exemplar in dark line. Flies were tested with both a 30° symmetrical grating (Left) and natural scene (Right). The orange arrowhead indicates location of odor nozzle delivering ACV. (B) Plume error averaged across the population as in Fig. 2. (C) Heading histograms with 84% CI, as in Fig. 2. (D) Plume error across the trial for each fly (dot), tested as in Fig. 2. (E–H) Same plots as A–D but for E-PG-split-Gal4 driving expression of UAS-Kir2.1 shorthand E-PG > Kir2.1.

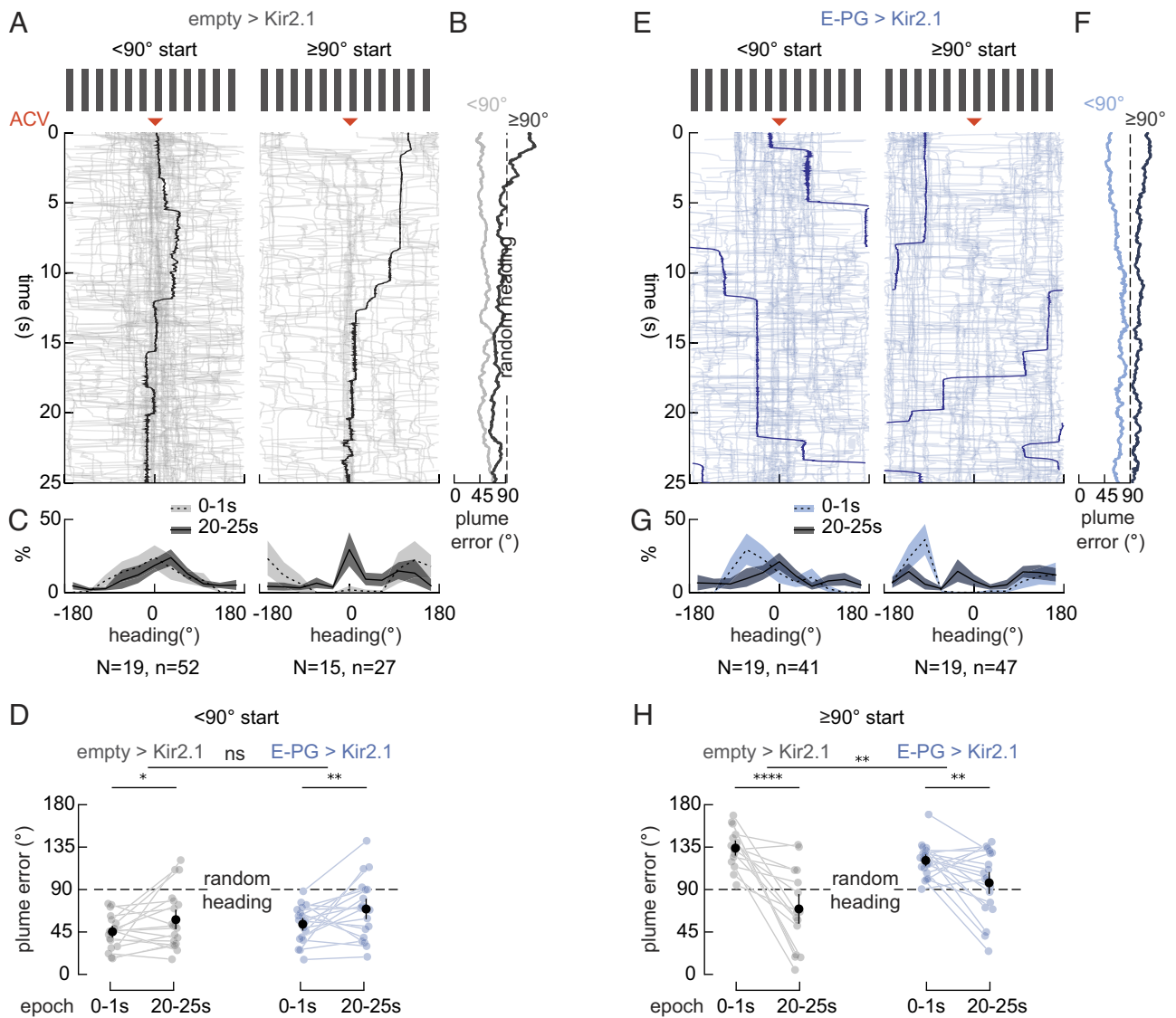


Fig. 4. Compass neurons are required for flies to acquire the plume in flight. All panels are formatted the same as in Figs. 2 and 3. (A) Flies were sorted into those that started trials near the odor nozzle (Left) and those that started far from the nozzle (Right). The orange arrowhead indicates location of odor nozzle delivering ACV. Example individual traces highlighted in dark line. (B) Average plume error as in Figs. 2 and 3. (C) Heading histograms divided for the first 1 s (dashed line) and last 5 s (solid line). (D) Plume error comparisons within subjects for flies starting trials near the odor nozzle. Error bars indicate 95% CI. Asterisks indicate level of significance of the one-tailed bootstrap test (Materials and Methods) * $P < 0.05$, ** $P < 0.01$ (E–G) Same plots as A–C but for E-PG-split-Gal4 driving expression of UAS-Kir2.1. (H) Plume error comparisons within subjects for flies starting trials away from the odor nozzle. Difference in means was tested with bootstrap comparisons resampled 100,000 times. Error bars indicate 95% CI. ** $P < 0.01$, **** $P < 0.0001$. Empty > Kir2.1 $N = 21$ flies; E-PG > Kir2.1 $N = 22$ flies.

heading in the food odor plume, not whether they could actively acquire it. To test for plume acquisition and simultaneously replicate the results, we performed the next experiment using a separate genetic cohort on a separate flight arena apparatus housed in a different lab room. This apparatus has been used in several other projects from the lab (10–12, 22) using identical olfactometer hardware, but is equipped with a 16×96 wrap-around dot matrix LED display that cannot present high-resolution natural scenes. We used a high contrast 30° squarewave grating typical of other studies from our lab. All aspects of this experiment were similar to those in Fig. 3, except that flies were not visually guided into the plume.

In this dataset, flies were tested repeatedly allowing us to separate responses into two groups based on whether they started less than or greater than 90° from the nozzle. Control flies show such fast responses to the odor that we had to reduce the first epoch down from 0–5 s to 0–1 s in order to properly capture the initial

heading distributions. The empty > Kir flies that started within 90° of the nozzle remained centered at the plume with no significant change in heading distributions from the start and end of the trial, whereas those that started from the periphery were able to center it (Fig. 4A), highlighted by the convergence of plume error for the two groups (Fig. 4B). By contrast, the E-PG > Kir flies tended to maintain a high error throughout the trial after starting in the periphery and did not center it (Fig. 4E–G). Quantifying plume error across the population confirmed that starting at the plume can bias the measurements. For flies starting near the plume, E-PG silenced flies indeed perform nearly as well as controls, with roughly similar increases in plume error over the course of the experiment (Fig. 4D). However, for those flies that are challenged to acquire the plume, the empty > Kir flies quickly reduce their plume error, significantly below chance, whereas E-PG silenced flies continue to perform at chance level (Fig. 4H). These results demonstrate that hyperpolarizing E-PG neurons

compromises both plume acquisition and persistent centering. Together with the visual reafference results in Fig. 2, these indicate that visual input to working memory maintained by the local activity bump within E-PG compass neurons is crucial for flies to navigate an odor plume in still air.

Silencing Compass Neurons Does Not Disrupt Visual Flight Control Systems. Central complex circuitry has been shown to coordinate goal directed navigation behavior in insects as diverse as flies, locusts, butterflies, dung beetles, cockroaches, and bees (25). How can we be sure that disrupting the excitability of E-PG neurons does not compromise basic flight stability in a manner that makes it impossible to assess goal directed navigation? To explore this possibility, we performed a set of control experiments on E-PG > Kir flies to assess wide-field optomotor gaze stabilization and small-field bar tracking—two robust visual behaviors that do not rely on spatial memory or idiosyncratic goal navigation.

We first tested gaze stabilizing optomotor reflexes using a 30° grating rotating at constant speed. We find that E-PG > Kir flies perform as well as controls, tracking image motion with constant velocity smooth turns interspersed with occasional saccades that is typical of wild-type (WT) flies (26). The steady-state average responses of the two experimental groups overlap nearly perfectly (Fig. 5A). Next, we assessed dynamic steering responses to brief impulses of wide-field image motion. Using an m-sequence to approximate white noise, we used reverse correlation to derive the optimal linear filter linking optic flow to changes in flight heading (27). The estimated filters for body heading changes in magnetically tethered free-yaw flies show onset delay of ~50 ms and time-to-peak of ~150 ms, which closely agrees with measurements from the wing steering kinematics of WT rigidly tethered flies; there were no significant differences between empty > Kir and E-PG silenced flies (Fig. 5B).

Next, we tested bar tracking behavior. In free-flight and on a magnetic tether, *Drosophila melanogaster* responds to the movement of vertical edge objects resembling plant stalks by integrating the movement error to a threshold for executing an orientation saccade (26, 28). Here, we presented a randomly textured vertical bar revolving at constant speed superimposed upon a similarly textured stationary visual background. In response to this small-field motion stimulus, flies tend to pursue the bar using saccadic changes in visual heading while fixating the visual surroundings in between saccades. The trajectory of average flight heading following the bar did not differ between empty > Kir and E-PG > Kir flies (Fig. 5C). To quantify pursuit, we computed the performance index (29), the ratio of the fly's angular displacement to that of the visual stimulus. $PI = 1$ signifies perfect bar tracking, $PI = -1$ signifies perfect tracking in the opposite direction and $PI = 0$ signifies no response to the bar. E-PG silenced flies did not show weakened PI values, and in fact were better at bar tracking at least in the counterclockwise direction (Fig. 5D). Finally, we computed saccade rates during small-field bar motion trials. Here, E-PG silenced flies show significantly higher saccade rates, which we attribute to rigorous bar pursuit reflexes (Fig. 5E). These control experiments indicate that silencing compass neurons has no obvious impact on gaze stabilizing reflexes or object approach preferences. Rather, compass silencing causes deficits specifically associated with multimodal navigation.

Plume-Centering Saccades Require Compass Neurons for Directional Control, but not for Frequency or Amplitude Modulation. The control variables that maintain flight heading in *D. melanogaster* include reducing retinal slip to fly straight between rapid course changing saccades. Key saccade control

parameters include amplitude, direction, and rate, which have been shown to be modulated by odor during flight (12, 13). We tested whether these variables are influenced by inactivating compass neurons. Fig. 6 shows saccade peak velocity plotted at the azimuthal retinal position at which each saccade started. Note that we performed the analysis described below for each of the two visual treatments, natural panorama and striped grating, and found that the results were statistically indistinguishable, so we pooled data across the two visual background treatments. Saccades are spatially localized in two quadrants of the plot indicating that when flight heading is oriented to the left of the plume (negative “saccade start position”) they tend to execute saccades to the right (positive “saccade peak velocity”), and vice versa (Fig. 6A). To highlight the separation of saccade direction toward the plume, the data are replotted, zoomed-in on the central 180° azimuthal region (Fig. 6B). The average saccade start position is separated by 36° in azimuth, straddling the zero-position of the odor nozzle (Fig. 6A and B empty > Kir2.1). Therefore, effector control flies make saccades directed toward the plume center. By contrast, the saccades evoked by E-PG blocked flies are not, on average, directed toward the plume. In fact, we observed a peculiar side-bias for the population of E-PG > Kir saccades, executed from the left side of the plume (Fig. 6A and B E-PG > Kir2.1), which may be indicative of the disrupted directional control of saccades upon silencing E-PG neurons. We did not observe similar bias in other behavioral measurements.

We also compared intersaccade intervals (ISI) across experimental conditions, because ISI has been shown to increase when a fly is inside a plume of any attractive odor so far tested (11). Increased ISI would help keep the fly oriented within the plume by pausing turns, a phenomenon observed in free flight within a linear wind tunnel (3). We parsed the ISI values into two groups: those located within $\pm 30^\circ$ of the plume, and those that occurred more than 30° from the nozzle. There were no differences in the mean ISI (or peak velocity) on either side of the odor nozzle, so we reflected and pooled data to compute population averages. Mean ISI is significantly larger for saccades elicited near the plume center than in the periphery of the arena, and this change occurs for both control and E-PG silencing flies (Fig. 6C), although it is significantly larger in control flies. We performed the same analysis on the distribution of peak angular speed—absolute value of peak angular velocity—for the two experimental treatments. The peak angular speed of saccades evoked when the fly is headed into the ACV plume is significantly smaller than for saccades executed in the periphery of the arena, and this effect persists for flies with silenced E-PG neurons (Fig. 6D), although they were generally faster overall. Odor therefore reduces both saccade rate and amplitude independently from visual compass activity, indicating that some plume tracking control variables are under multi-sensory control (e.g., saccade direction, intersaccade optomotor gain, odor activated object approach) whereas others are not (e.g., saccade interval, saccade amplitude).

Discussion

Prior behavioral work in *Drosophila* found that flies cannot accurately locate an attractive food source unless there is sufficient visual contrast or texture in the scene (9, 11, 12). Our results here using the visual clamp and genetic inhibition of the E-PG heading compass help to explain why. The visual clamp experiment showed that when all other sensorimotor feedback is active, removal of visual reafference alone severely hinders the fly's ability to locate and track an attractive odor plume, ACV (Fig. 2). Likewise, genetic hyperpolarization of the E-PG heading compass neurons eliminated ACV tracking, by specifically removing the fly's ability

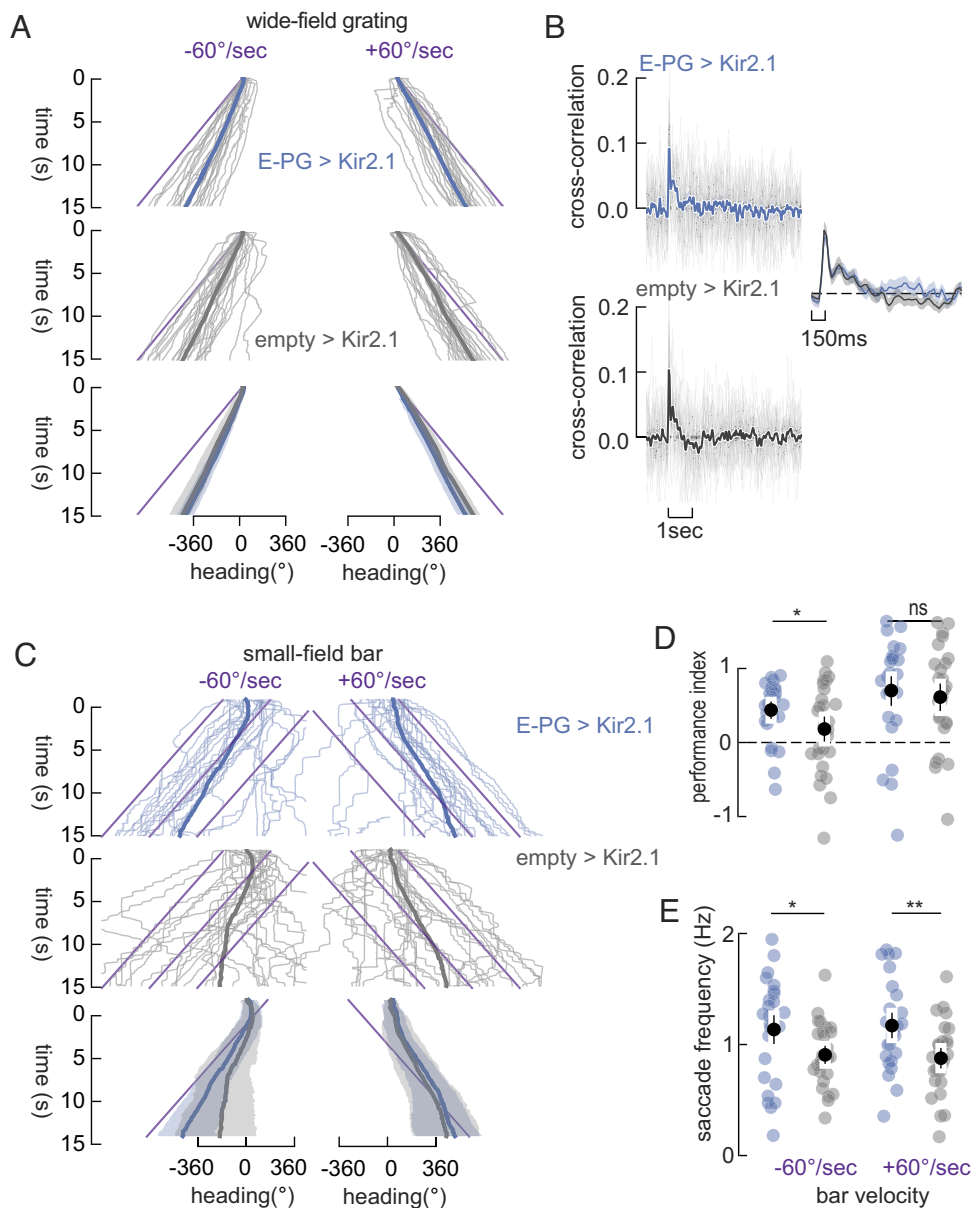


Fig. 5. Basic visual flight control is not disrupted by silencing compass neurons. (A) Steady-state smooth optomotor stabilization test. A high-resolution grating of 30° wavelength stripes (Fig. 1A) was revolved at constant velocity. Individual fly responses plotted with thin lines. Average of the population in thick lines. Shaded envelope indicates 95% CI (Bottom). Stimulus path indicated in purple lines. (B) Impulse response test. A 30° grating was moved along an m-sequence trajectory to simulate spectrally white oscillation frequencies. Individual fly responses plotted with thin lines. Average of the population in thick lines. Inset replots and overlays the average impulse responses for the two groups. Shaded envelope indicates 95% CI. (C) Bar tracking behavior test. A randomly textured 30° bar was moved across a similarly random textured stationary background at constant speed. Stimulus indicated in purple lines. Individual fly responses plotted with thin lines. Average of the population in thick lines. Shaded envelope indicates 95% CI (Bottom). (D) Performance index equals the ratio of fly azimuthal displacement to bar displacement. For each fly (colored dot) and population means (black dot). (E) The rate of saccades during each trial, for each fly (colored dot) and population means (black dot). Difference in means was tested with bootstrap comparisons resampled 100,000 times. Error bars indicate 95% CI. * $P < 0.05$, ** $P < 0.01$. Empty > Kir2.1 $N = 21$ flies; E-PG > Kir2.1 $N = 22$ flies.

to perform body saccades back toward the plume (Figs. 3 and 4). Other parameters of saccade control such as the ISI and saccade amplitude scale proportionally with the odor intensity gradient and are not dependent on the E-PG heading compass (Fig. 6). We further demonstrated that genetic inhibition does not disrupt canonical visuomotor reflexes like the smooth optomotor response and saccadic bar tracking (Fig. 5). Our results suggest that E-PG cells are essential to the fly's ability to localize an odor in flight specifically because they track changes in visual heading. Therefore, flies searching for an odor in a scene lacking visual contrast struggle because E-PG compass fidelity depends greatly on visual cues flight (23), particularly in flight.

There are several odor modulated visual behaviors that contribute to olfactory search and localization behavior during flight in flies. First, a fundamental visual reflex—the gaze stabilization by optomotor response that minimizes retinal slip to enhance visual detection, operates in flies as a linear transformation with high gain, and does not adapt to varying feedback conditions (26, 30–32)—is strengthened by food odor in *Drosophila* (33). Computational modeling has demonstrated that even a small increase in odor modulated optomotor responses, iterated over many ISIs, is sufficient to bring a fly closer to an odor source in flight (9, 14). This helps explain how flies maintain a forward heading when odor evokes an upwind surge (4, 34, 35). At least

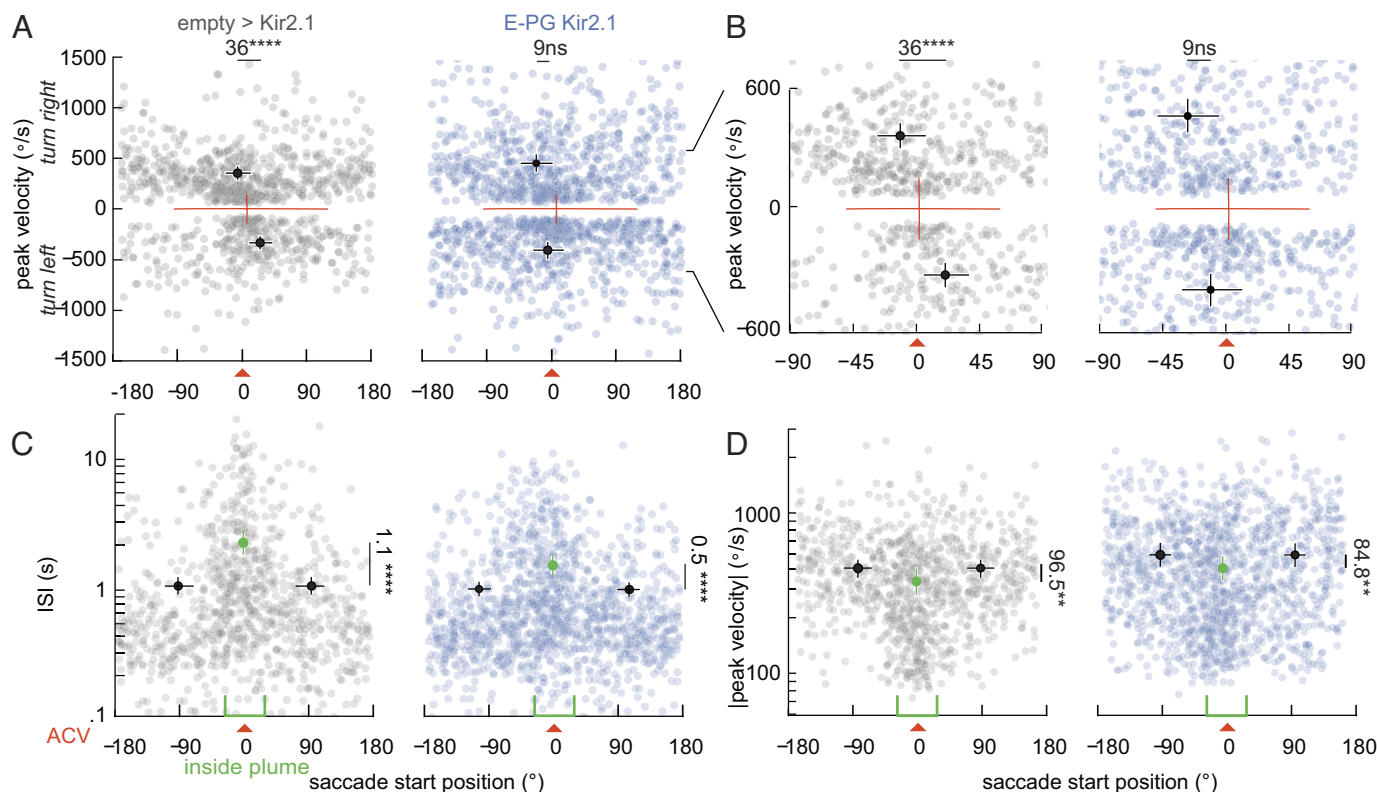


Fig. 6. Compass neurons are required for normal saccade orientation toward the plume, but not for lower saccade rate within the plume. (A) For each saccade in the dataset, the azimuthal angle at the start of each saccade is plotted against its signed peak angular velocity (i.e., positive values indicate rightward saccades). Orange cross hair indicates the zero axis intercepts. The orange arrowhead indicates location of odor nozzle delivering ACV. Dark dots indicate population means; error bars indicate 95% CI. Difference in means is indicated numerically at the top of each plot. (B) Data replotted from panel A but zoomed into a smaller range of axis values to highlight that flies oriented to the left of the plume made right directed saccades and vice versa. (C) For each saccade, the time interval to the prior saccades is plotted against the azimuthal position of fly heading at the start of the saccade. Mean values of saccades within $\pm 30^\circ$ of the odor nozzle (green X-axis marks, green symbols) were compared to those greater than 30° (black symbols). Assuming bilateral symmetry, to compute peripheral means, data were reflected over the azimuth and pooled. Difference in means is indicated numerically at the right of each plot. (D) For each saccade, the absolute value of peak velocity is plotted against the azimuthal position of heading at the start of the saccade. Mean values are computed from two azimuthal groups as in panel C. In all panels, the difference in means is indicated with a solid line spanning the mean points. In all panels, difference in means was tested with bootstrap comparisons resampled 100,000 times. ** $P < 0.01$, **** $P < 0.0001$. Empty > Kir2.1 $n = 997$ saccades from $N = 21$ flies; E-PG > Kir2.1 $n = 1,418$ saccades from $N = 22$ flies.

one wide-field directionally selective motion detecting neuron, residing within the visual pathway that is implicated in optomotor control, shows increased visual responses when visual stimuli are paired with food odor (36).

In addition to enhancing optomotor stability, odor encounters also engage robust visual fixation and centering of objects during flight. In free-flight, after encountering a food odor, flies approach a dark object on the arena floor that they ignore in clean air (4). A similar phenomenon is observed in tethered flight under visual closed-loop feedback conditions, in which food odor increases flies' tendency to actively steer toward vertical bars and small spots (37, 38). It is noteworthy that the distribution of saccade direction with respect to visual features is qualitatively similar to the orientation functions toward olfactory features presented here (9, 39), highlighting shared motor control variables for flight navigation toward salient sensory features.

Visual reflexes in flies are fast—the full cascade of visual processing and musculoskeletal mechanics required to evoke a steering response by the wings in response to an impulse in image velocity occurs within 100 ms (27). Olfactory motor responses are slower—a corrective turn into an odor plume during flight occurs with an onset delay of ~ 190 ms and a corrective turn back into the plume is delayed 450 ms from loss of the odor signal (4). By contrast, the time intervals between saccades are at least 10 times longer than these sensory reflexes. In free-flight, the distribution

of ISI peaks at roughly 700 ms and tails off at 1.1 s (40). Our measurements of ISI inside the plume are longer still, ranging 300 ms to 10 s (Figs. 1 H, Inset and 6C). After accounting for those neural processing delays, and notwithstanding some added sluggishness due to free-yaw tethering, that delay often leaves multiple seconds during which a fly is maintaining a straight heading and must retain a working memory of heading change from the prior saccade. By integrating rotational and directional cues, the E-PG ring attractor network covers these extended behavioral timescales by maintaining a short-term directional memory (41). During flight under head-fixed virtual closed-loop flight conditions with a naturalistic visual scene, the activity bump of E-PG neurons maintains a faithful representation of an animal's stationary heading for up to 10 s between turns (17).

We posit that visual reafference acts with E-PG signals to guide saccades toward an odor plume. However, during voluntary saccades, premotor efference copy commands hyperpolarize directionally selective motion detecting neurons that coordinate gaze stabilizing head movements (42, 43). This mechanism is thought to enable flies to suppress the perception of visual motion during rapid body saccades (44) in much the same way that our own eye saccades are invisible to us. This suppressive effect on motion perception occurs only for voluntary changes in heading, not for reactions to external visual perturbations such as might occur after a gust of wind (45). By contrast, during head-fixed flight, the

E-PG bump does not follow steering commands in the dark, and therefore it would appear that premotor commands alone are insufficient to spin the compass (41). It is unknown whether proprioceptive feedback from naturalistic body rotation during free-yaw saccades, perhaps originating from gyroscopic sense organs, might supply mechanosensory input that dynamically tunes the E-PG heading compass (41, 46).

Mechanosensory input to the compass could provide an alternative explanation for our results—that visual reafference acts separately from nonvisual mechanosensory input to the heading compass, with each experiment coincidentally disrupting odor tracking behavior via distinct neural mechanisms. However, visual reafference is likely to be interacting with odor-activated directional memory because plume centering can persist long after switching off an odor but not after switching off visual feedback. This is especially evident when presenting a plume of ethyl butyrate, a liquid ester that smells like pineapple and evokes strong plume centering in our apparatus. However, unlike ACV and other food odors, switching off an ethyl butyrate plume does not rapidly terminate plume centering behavior. Instead, flies continue to direct saccades back-and-forth toward the “phantom plume” for up to 25 s, unless visual feedback is removed by switching the visual display from a high contrast striped grating to a zero-contrast gray (11). If proprioceptive inputs were sufficient to accurately update the E-PG compass, then flies should be able to maintain heading in the phantom plume even within visually featureless surroundings. Note that the visual dependence of plume tracking is not specific to olfaction, with water-deprived flies also depending upon visual reafference to localize a plume of water vapor (13). For this project, we chose to use ACV because it is a more naturally occurring attractant, thus commonly used in fly olfaction studies, and yet still depends on visual feedback for accurate tracking (12).

For a walking fly, proper E-PG activity does not require visual input. Even in darkness the compass tracks changes in walking direction, likely using proprioceptive feedback from the legs; however, in the light visual cues are weighted more than walking signals when these are in conflict (16). Regardless of how multisensory signals are balanced for mapping compass dynamics to body movement, the compass is crucial not only for odor navigation during flight, as we show here, but also for odor navigation during walking (6). A class of neurons intrinsic to the fan-shaped body (FSB) temporarily stores a fly's heading direction upon encountering food odor presented within a wind stream, and this information is used to guide upwind navigation during walking (47). Moreover, the FSB depends on inputs from the E-PG heading compass to transform signals from a body- (egocentric) to world-centric (allocentric) coordinate system. If FSB circuits could be activated by odor to store visual heading direction instead of wind direction, then this is precisely the sort of mechanism that could underlie visual navigation to an odor source in still air.

In conclusion, our working model of visual-olfactory integration mechanisms for plume navigation consists of three components: 1) odor-modulated optomotor gain helps a fly maintain a straight course between saccades, 2) odor-amplified visual object saliency draws the animal's attention and engages saccades toward otherwise neutral features, and 3) the E-PG compass tracks visual displacements in order for premotor circuits to balance the direction of saccades that maintain a stable flight heading in still air. Our results motivate additional research questions. 1) How does odor evoke a state change from exploration to visual heading-controlled tracking, in which the compass neurons balance saccade valence? 2) Does odor activate a motor search rhythm to probe the plume boundary? The corollary would be to simply fly straight in a plume. 3) How does the plasticity of heading

circuitry facilitate olfactory navigation (48)? Exploring these questions likely involves examining the FSB circuits that convey the optic flow that encodes flight travel direction independently from heading direction (49) and are activated by odor to store wind direction information and mediate persistent upwind walking (5).

Materials and Methods

Fly Stocks. Fly lines of adult female *D. melanogaster*, 3 to 6 d posteclosion.

D. melanogaster: SS00096-SplitGAL4 ($P\{w[+mc]=GMR19G02-bBPp65ADZpUw\}$ in attP40).

D. melanogaster: Empty-SplitGAL4 ($P\{w[+mc]=BP-p65ADZp\}$ in attP40) and $P\{w[+mc]=BP-ZpG4DBD\}$ in attP2).

D. melanogaster: +;+;UAS-eGFP-Kir2.1 ($pJFRC49-10XUAS-IVS-eGFPKir2.1$ in attP2).

D. melanogaster WT strain originating from a wild caught female in 2008.

Flight Experiments. Each fly was removed from food and water for at least 1 h before testing. Flies were tethered as described previously (50, 51). Each fly was placed in the flight arena, and tested with a 30° grating revolving at constant speed in each direction. Visual inspection confirmed whether the fly could freely rotate and smoothly follow the stimulus at least one full revolution. At the start of each experimental trial, a 15° white vertical bar projected onto a solid black background was oscillated in front of the nozzle for 5 s to orient the fly toward the plume. Random block statistical design was used, and stimulus variables were presented within subjects for each experiment.

Magnocube. Recent work has demonstrated that coordinated photoreceptor mechanical contraction and intraretinal movements impart significantly higher spatial acuity than previously thought in *Drosophila* (52, 53). We adopted the high-resolution projector display and graphics library of Cabrera and Theobald (54) and this system has been described previously (50). A digital projector and first-surface mirrors projects an image into the vertical sides of a 4" × 4" × 4" perspex cube lined with gray rear projection material. The projector (TI DLP LightCrafter 4500 EVM) produced frames 1,280 × 800 pixels each subtending 0.4° at 120 Hz. The magnetic tether was set up as described above, positioning the fly at the center of the cube. From this position, the display subtends the fly's visual field by 360 horizontally and 70 to 90 vertically, decreasing from the center of each panel to the corners. This discrepancy is accounted for by programmatically restricting the vertical subtended angle to 70° all around the azimuth and correcting for perspective. 12 concentric IR LEDs illuminate the magnet behind the fly while the fly sits in their shadow, cast by the bottom magnet, so the lights do not illuminate the fly directly. Instead, we use the shadow of the fly, which contrasts greatly against the diffuse and reflected IR light. The brightness of the LEDs and camera exposure times were customized to minimize the influence of motion blur and shot noise.

Real-Time Tracking. To track the flies in real-time, we added custom Python code to the Holocube library. Holocube uses pygame (<https://pygame.org/>) and OpenGL (<https://www.opengl.org/>) to render perspective-corrected viewports of 3D graphics and a range of psychophysical stimuli. Holocube runs on an event loop at about 60 Hz in our system and our code adds a function to each loop during the acquisition phase of each stimulus. When a new fly is added, they are shown a rotating dot field that flips direction every 8 s to both assess the subject's responsiveness and tune the apparatus. As this runs, the experimenter adjusts the position of the camera using micromanipulators until the center of rotation aligns as close as possible to the center of the camera. The tracking algorithm relies on thresholding each frame to separate the fly (except for their wings) from the background. A graphical user interface (GUI) allows the experimenter to adjust this threshold while watching the fly respond to the widefield rotation in real-time. This GUI also allows the user to adjust the size of two concentric rings at the center of the frame, which are used for measuring the fly's heading. Due to the way the flies are tethered, their abdomen sticks out a bit further from the center of rotation than their head. So, the outer ring first determines the tail angle based on the circular mean of the angles corresponding to the thresholded values. Then, after omitting the tail angle $\pm 90^\circ$, the inner ring determines the head angle by the same process. This is repeated every loop and allows very rapid heading calculations (up to at least 480 Hz) because it converts a typically 2D process into a mostly 1D one.

However, unintentional translations run the risk of being misinterpreted as rotations, muddying the results, and side asymmetries due to off-center tethering run the risk of shifting the measured angle away from the true angle by some offset. So, we built an offline DeepLabCut (20, 21) model to validate the results of this online tracking algorithm. We found that the mean difference between the real-time and validated offline tracking across trials amounted to 0.04° (5.58 SD). The 50th percentile of the distribution of mean error was 2.6°, and the average frame interval was 17 ms (2.26 SD). Thus, the real-time tracking operated faster than the onset delay of the visuo-motor impulse response in flight (27), and the spatial error amounted to less than the acceptance angle of a single ommatidium (55). All of the code for is freely available on github (56).

Visual Clamp. We modified our digital projection system to deliver high-resolution open-loop “clamped” visual stimuli to the free-yaw fly to subtract expected visual reafference. This system has been described previously (50). Briefly, using the same real-time tracking described above, this system subtracts its generated motion by the next frame. To do this for each event loop, the system sets the angular coordinate of the background, specifically about the vertical z axis, equal to the heading measured from the most recently captured image from the camera. Because the real-time tracking and display system generated negligible spatiotemporal error (mentioned above and in Fig. 1E), setting the background coordinate system equal to the fly's frame of reference virtually opens the visual feedback loop, effectively eliminating visual reafference.

It is worth noting that the display's center of rotation matches the fly's center of rotation at the tether point, not the center of rotation of the eye. Thus, when the display is stationary and the fly rotates, an object appearing near visual midline would dilate and contract slightly as the fly rotates toward and away. The radius from the fly's head to the thorax is no more than 0.5 mm and the radius of the arena is 50.8 mm. The greatest ratio of the subtended angles (maximum/minimum) is 1.02, so there is a 2% distortion in the size of frontal vs. rear objects. For the stripes of the 30° grating, this means true perceived stripe widths ranging from 14.85 to 15.15°, a range of 0.3°, between stripes in front and behind the fly, respectively. That is an order of magnitude less than the acceptance angle of a single photoreceptor and two orders of magnitude less than the nominal pattern wavelength. These distortions are sufficiently small to be negligible. Furthermore, with the clamp activated, because the center of fly rotation is the center of the arena, then the minimally distorted size of whatever is in front of the fly maintains the same angle as the fly rotates. For instance, a 15° vertical bar projected in front of the fly would actually subtend 15.15° because the fly head is a little bit closer than the center of the arena. As the fly rotates, the bar rotates with it and therefore keeps that maximum subtended angle. This system does not fully erase the perception of all visual movement, but it does cause strong disruption to reflexive visual stability demonstrated within the data we present here, as well as those presented previously using this system (50), and a similar approach using an LED based system (31).

Odor Stimuli. A gas multiplexer (Sable Systems) was used to switch mass flow regulated air (Sable Systems MFC-2) through a bubbler of apple cider vinegar or water at the rate of 14 mL/min. The odor port delivering the headspace of the odorant was positioned 3 mm away from the head of the fly. A 5 mm diameter tube below the fly provided a vacuum flow 30 mL/min to clear the odor plume. To measure the spatial distribution of the plume, we used a miniature photoionization detector (mini-PID Aurora Scientific, Ontario, Canada). The detector probe was placed at each point of a 12 × 12 horizontal grid at the plane of the fly's antennae. Ethanol, which has an ionization-potential of 10.62 eV, was used as a tracer gas. The odor signal peaks near the fly's head, but is detectable throughout the full azimuth of the free-yaw fly, which is evident in the sharply directed “anti-tracking” focused 180° from the plume that we have observed in response to aversive benzaldehyde (22).

Saccade Analysis. Saccade statistics were extracted in multiple phases based on the procedure in (26) and detailed in our freely available custom analysis script written in Python. Angular velocity was calculated as the gradient of the fly headings after applying a 15 Hz Butterworth low-pass filter. Saccades were identified in the heading data by applying a local maximum detector (scipy.signal.find_peaks) to the angular speed. The parameters of this peak-finding function were manually tuned to include as many saccades as possible while excluding large deviations

indicative of tracking errors [for 60 fps, distance: 15 frames, width: 2 frames, prominence: (1, 30) rads, wlen: 15 frames]. This function also provides the left and right bases (adjacent local minima) of the segment containing the peak, offering starting points for measuring the beginning and end of each saccade. Each saccade is processed individually, considering the segment defined by the left base – 10 frames and right bases + 10 frames in order to roughly measure the distribution of velocities before and after the supposed saccade. Using each saccade-specific distribution of pre- and postsaccade angular velocities, we define the beginning and end of the saccade as the duration when angular velocity exceeds 2 SD from the mean. Previous methods, which relied solely on angular speed, were insufficient to identify saccades in the visually clamped condition because visually clamped flies often perform saccades superimposed on substantial constant-velocity drifts.

The results were visually inspected for a random sample of about 10 flies in each of the experiments involving saccade analysis to validate this procedure. The beginning and end time points were used to calculate pertinent parameters to compare saccade statistics across genotypes and visual conditions. For each saccade, we define the following parameters: starting angle is the fly's heading at the start of the saccade, peak velocity is the velocity corresponding with the maximum angular speed during the segment after applying a 0-order cubic spline interpolation (scipy.interpolate.interp1d), and ISI is the amount of time since the end of the previous saccade. Given this definition of ISI, the ISI of the first saccade of in each test is undetermined.

Statistics. All of the statistics presented here amount to comparing means, accounting for within-subject covariances whenever possible. Because many of the variables tested (probability, angular error, normalized cross-correlation, tracking performance index, saccade frequency, peak velocity, and ISI) follow largely nonnormal distributions due to mathematical and physical boundaries, we used a bootstrapping approach (57) to test for statistical significance. To do this, the distributions of mean values were sampled with replacement in sets of size N for a total of 100,000 times where N is the sample size. Taking the average of each resampled set generates an empirical sampling distribution of the mean with 100,000 estimates. We then estimate the 95% CI by taking the 2.5 and 97.5 percentiles of the sampling distribution as the lower and upper bounds. Whenever we compare two distributions of mean values, if drawn from independent samples, we compare the sampling distributions elementwise and then calculate the null probability.

For instance, if we hypothesize that some mean value is greater for group A than B our null hypothesis is that $A_i \leq B_i$. To test this, we would calculate each sampling distribution independently and then store a 1 if $A_i \leq B_i$ and 0 otherwise, where A_i and B_i represent the i -th element in each array. Taking the pseudocount-corrected average provides the null probability:

$$P = \frac{1 + \sum_{i=0}^N A_i \leq B_i}{N + 1}$$

Note that simply taking the average could incorrectly suggest that a result is impossible rather than just not observed in our sample (58).

When comparing results within subjects, we take the within-subject differences first and then calculate P in much the same way, except that our null hypothesis is a comparison to 0 (for instance, A_0). When comparing variables with many values per subject—such as the histograms in Figs. 2–5 or the time series in Fig. 6—sampling is performed hierarchically to maintain the covariances in the resultant sampling distribution. For instance, a histogram was measured for each of N subjects from two different epochs (0 to 5 s and 15 to 20 s). This resulted in two sets of 11 probabilities, which were randomly sampled in their entirety 100,000 times, resulting in a matrix of size $11 \times 2 \times N \times 100,000$. To get the sampling distributions for each mean at each position and epoch, we take the mean along the 3rd dimension, reducing the size of the matrix to $11 \times 2 \times 100,000$. Finally, we get the 95% CI by taking the 2.5 and 97.5 percentiles along the last dimension, resulting in 2 matrices of size 11×2 . For histogram comparisons, we actually use 84% CI because separation of two such intervals indicates a significance level of 0.05, whereas 95% CI would represent an overly conservative estimate (59). All analysis code and data are available online (60).

Data, Materials, and Software Availability. All analyses were performed in Python, using a combination of established APIs (numpy, scipy, matplotlib, and statsmodels). Data and plotting routines are published on Open Science Framework (OSF): https://osf.io/mpx89/overview?view_only=56d0e2ea7ca443d3aff78755694cc4e8 (60).

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1. M. H. Dickinson, Death valley, *Drosophila*, and the devonian toolkit. *Annu. Rev. Entomol.* **59**, 51–72 (2014).
2. J. Houle, F. van Breugel, Near-surface wind variability over spatiotemporal scales relevant to plume tracking insects. *Phys. Fluids* **35**, 055145 (2023).
3. S. A. Budick, M. H. Dickinson, Free-flight responses of *Drosophila melanogaster* to attractive odors. *J. Exp. Biol.* **209**, 3001–3017 (2006).
4. F. van Breugel, M. H. Dickinson, Plume-tracking behavior of flying *Drosophila* emerges from a set of distinct sensory-motor reflexes. *Curr. Biol.* **24**, 274–286 (2014).
5. A. M. M. Matheson *et al.*, A neural circuit for wind-guided olfactory navigation. *Nat. Commun.* **13**, 4613 (2022).
6. A. F. Siliciano *et al.*, A vector-based strategy for olfactory navigation in *Drosophila*. bioRxiv [Preprint] (2025). <https://doi.org/10.1101/2025.02.15.638426> (Accessed 1 March 2026).
7. Q. Gaudry, E. J. Hong, J. Kain, B. L. de Bivort, R. I. Wilson, Asymmetric neurotransmitter release enables rapid odour lateralization in *Drosophila*. *Nature* **493**, 424–428 (2013).
8. S. D. Stupski, F. van Breugel, Wind gates olfaction-driven search states in free flight. *Curr. Biol.* **34**, 4397–4411.e6 (2024).
9. M. A. Frye, M. Tarsitano, M. H. Dickinson, Odor localization requires visual feedback during free flight in *Drosophila melanogaster*. *J. Exp. Biol.* **206**, 843–855 (2003).
10. B. J. Duistermars, D. M. Chow, M. A. Frye, Flies require bilateral sensory input to track odor gradients in flight. *Curr. Biol.* **19**, 1301–1307 (2009).
11. P. Krishnan, B. J. Duistermars, M. A. Frye, Odor identity influences tracking of temporally patterned plumes in *Drosophila*. *BMC Neurosci.* **12**, 62 (2011).
12. B. J. Duistermars, M. A. Frye, Crossmodal visual input for odor tracking during fly flight. *Curr. Biol.* **18**, 270–275 (2008).
13. D. Limbani, G. L. Turner, S. M. Wasserman, Dehydrated *Drosophila melanogaster* track a water plume in tethered flight. *iScience* **26**, 106266 (2023).
14. F. J. Stewart, D. A. Baker, B. Webb, A model of visual-olfactory integration for odour localisation in free-flying fruit flies. *J. Exp. Biol.* **213**, 1886–1900 (2010).
15. T. Wolff, N. A. Iyer, G. M. Rubin, Neuroarchitecture and neuroanatomy of the *Drosophila* central complex: A GAL4-based dissection of protocerebral bridge neurons and circuits. *J. Comp. Neurol.* **523**, 997–1037 (2015).
16. J. D. Seelig, V. Jayaraman, Neural dynamics for landmark orientation and angular path integration. *Nature* **521**, 186–191 (2015).
17. S. S. Kim, A. M. Hermundstad, S. Romani, L. F. Abbott, V. Jayaraman, Generation of stable heading representations in diverse visual scenes. *Nature* **576**, 126–131 (2019).
18. Y. M. Giraldo *et al.*, Sun navigation requires compass neurons in *Drosophila*. *Curr. Biol.* **28**, 2845–2852.e4 (2018).
19. E. von Holst, H. Mittelstaedt, Das refferenzprinzip. *Sci. Nat.* **37**, 464–476 (1950).
20. A. Mathis *et al.*, DeepLabCut: Markerless pose estimation of user-defined body parts with deep learning. *Nat. Neurosci.* **21**, 1281–1289 (2018).
21. T. Nath *et al.*, Using DeepLabCut for 3D markerless pose estimation across species and behaviors. *Nat. Protoc.* **14**, 2152–2176 (2019).
22. S. Wasserman, P. Lu, J. W. Aptekar, M. A. Frye, Flies dynamically anti-track, rather than ballistically escape, aversive odor during flight. *J. Exp. Biol.* **215**, 2833–2840 (2012).
23. R. I. Wilson, Neural networks for navigation: From connections to computations. *Annu. Rev. Neurosci.* **46**, 403–423 (2023).
24. C. Dan, B. K. Hulse, R. Kappagantula, V. Jayaraman, A. M. Hermundstad, A neural circuit architecture for rapid learning in goal-directed navigation. *Neuron* **112**, 2581–2599.e23 (2024).
25. S. Heinze, Unraveling the neural basis of insect navigation. *Curr. Opin. Insect Sci.* **24**, 58–67 (2017).
26. J.-M. Mongeau, M. A. Frye, *Drosophila* spatiotemporally integrates visual signals to control saccades. *Curr. Biol.* **27**, 2901–2914.e2 (2017).
27. J. C. Theobald, D. L. Ringach, M. A. Frye, Dynamics of optomotor responses in *Drosophila* to perturbations in optic flow. *J. Exp. Biol.* **213**, 1366–1375 (2010).
28. F. van Breugel, M. H. Dickinson, The visual control of landing and obstacle avoidance in the fruit fly *Drosophila melanogaster*. *J. Exp. Biol.* **215**, 1783–1798 (2012).
29. G. Frigetto, M. A. Frye, Columnar neurons support saccadic bar tracking in *Drosophila*. *eLife* **12**, e83656 (2023).
30. M. F. Keleş, J.-M. Mongeau, M. A. Frye, Object features and T4/T5 motion detectors modulate the dynamics of bar tracking by *Drosophila*. *J. Exp. Biol.* **222**, jeb190017 (2019).
31. B. Cellini, M. Ferrero, J.-M. Mongeau, *Drosophila* flying in augmented reality reveals the vision-based control autonomy of the optomotor response. *Curr. Biol.* **34**, 68–78.e4 (2024).
32. L. F. Tammero, M. A. Frye, M. H. Dickinson, Spatial organization of visuomotor reflexes in *Drosophila*. *J. Exp. Biol.* **207**, 113–122 (2004).
33. D. M. Chow, M. A. Frye, Context-dependent olfactory enhancement of optomotor flight control in *Drosophila*. *J. Exp. Biol.* **211**, 2478–2485 (2008).
34. M. A. Frye, M. H. Dickinson, Motor output reflects the linear superposition of visual and olfactory inputs in *Drosophila*. *J. Exp. Biol.* **207**, 123–131 (2004).
35. V. Bhandawat, G. Maimon, M. H. Dickinson, R. I. Wilson, Olfactory modulation of flight in *Drosophila* is sensitive, selective and rapid. *J. Exp. Biol.* **213**, 4313–4313 (2010).
36. S. M. Wasserman *et al.*, Olfactory neuromodulation of motion vision circuitry in *Drosophila*. *Curr. Biol.* **25**, 467–472 (2015).
37. K. Y. Cheng, M. A. Frye, Odour boosts visual object approach in flies. *Biol. Lett.* **17**, 20200770 (2021).
38. K. Y. Cheng, R. A. Colbath, M. A. Frye, Olfactory and neuromodulatory signals reverse visual object avoidance to approach in *Drosophila*. *Curr. Biol.* **29**, 2058–2065.e2 (2019).
39. J. W. Aptekar, P. A. Shoemaker, M. A. Frye, Figure tracking by flies is supported by parallel visual streams. *Curr. Biol.* **22**, 482–487 (2012).
40. L. F. Tammero, M. H. Dickinson, The influence of visual landscape on the free flight behavior of the fruit fly *Drosophila melanogaster*. *J. Exp. Biol.* **205**, 327–343 (2002).
41. S. S. Kim, H. Rouault, S. Druckmann, V. Jayaraman, Ring attractor dynamics in the *Drosophila* central brain. *Science* **356**, 849–853 (2017).
42. A. J. Kim, J. K. Fitzgerald, G. Maimon, Cellular evidence for efference copy in *Drosophila* visuomotor processing. *Nat. Neurosci.* **18**, 1247–1255 (2015).
43. C. Busch, A. Borst, A. S. Mauss, Bi-directional control of walking behavior by horizontal optic flow sensors. *Curr. Biol.* **28**, 4037–4045.e5 (2018).
44. J. A. Bender, M. H. Dickinson, A comparison of visual and haltere-mediated feedback in the control of body saccades in *Drosophila melanogaster*. *J. Exp. Biol.* **209**, 4597–4606 (2006).
45. L. M. Fenk, A. J. Kim, G. Maimon, Suppression of motion vision during course-changing, but not course-stabilizing, navigational turns. *Curr. Biol.* **31**, 4608–4619 (2021). <https://doi.org/10.1016/j.cub.2021.09.068>.
46. Y. E. Fisher, Flexible navigational computations in the *Drosophila* central complex. *Curr. Opin. Neurobiol.* **73**, 102514 (2022).
47. N. D. Kathman, A. J. Lanz, J. D. Freed, K. I. Nagel, Neural dynamics for working memory and evidence integration during olfactory navigation in *Drosophila*. bioRxiv [Preprint] (2024). <https://doi.org/10.1101/2024.10.05.616803> (Accessed 1 March 2026).
48. Y. E. Fisher, J. Lu, I. D'Alessandro, R. I. Wilson, Sensorimotor experience remaps visual input to a heading-direction network. *Nature* **576**, 121–125 (2019).
49. C. Lyu, L. F. Abbott, G. Maimon, Building an allocentric travelling direction signal via vector computation. *Nature* **601**, 92–97 (2022).
50. M. Rimnieceanu, J. P. Curra, M. A. Frye, Proprioception gates visual object fixation in flying flies. *Curr. Biol.* **33**, 1459–1471.e3 (2023).
51. B. J. Duistermars, M. Frye, A magnetic tether system to investigate visual and olfactory mediated flight control in *Drosophila*. *J. Vis. Exp.* **21**, 1063 (2008).
52. L. M. Fenk *et al.*, Muscles that move the retina augment compound eye vision in *Drosophila*. *Nature* **612**, 116–122 (2022).
53. M. Juusola *et al.*, Microsaccadic sampling of moving image information provides *Drosophila* hyperacute vision. *Elife* **6**, e26117 (2017).
54. S. Cabrera, J. C. Theobald, Flying fruit flies correct for visual sideslip depending on relative speed of forward optic flow. *Front. Behav. Neurosci.* **7**, 76 (2013).
55. P. T. Gonzalez-Bellido, T. J. Wardill, M. Juusola, Compound eyes and retinal information processing in miniature dipteran species match their specific ecological demands. *Proc. Natl. Acad. Sci. U. S. A.* **108**, 4224–4229 (2011).
56. J. P. Curra, Code from "Proprioception gates visual object fixation in flying flies." GitHub. <https://github.com/jpcurrea/magnocube>. Deposited 12 November 2024.
57. D. Hinkley, "Bootstrap methods: Another look at the jackknife" in *Springer Series in Statistics*, C. N. Morris, Ed. (Springer, New York, 2008), pp. 178–206.
58. H. Goldstein, M. J. R. Healy, The graphical presentation of a collection of means. *J. R. Stat. Soc. Ser. A Stat. Soc.* **158**, 175 (1995).
59. B. Phipson, K. Gordon, Permutation *P*-values should never be zero: Calculating Exact *P*-values when permutations are randomly drawn behind a phipson. *Stat. Appl. Genet. Mol. Biol.* **9**, 1544–6115 (2010).
60. J. P. Curra, M. A. Frye, Data from "Odor tracking in flying *Drosophila* requires visual reafference and compass neurons." Open Science Framework. https://osf.io/mpx89/overview?view_only=49b32ad29b604c3697dd31b3f0bdeb6ca. Deposited 16 March 2026.