Context-dependent representations of movement in *Drosophila* dopaminergic reinforcement pathways

Aryeh Zolin, Raphael Cohn, Rich Pang, Andrew F. Siliciano, Adrienne L. Fairhall and Vanessa Ruta

Dopamine plays a central role in motivating and modifying behavior, serving to invigorate current behavioral performance and guide future actions through learning. Here we examine how this single neuromodulator can contribute to such diverse forms of behavioral modulation. By recording from the dopaminergic reinforcement pathways of the *Drosophila* mushroom body during active odor navigation, we reveal how their ongoing motor-associated activity relates to goal-directed behavior. We found that dopaminergic neurons correlate with different behavioral variables depending on the specific navigational strategy of an animal, such that the activity of these neurons preferentially reflects the actions most relevant to odor pursuit. Furthermore, we show that these motor correlates are translated to ongoing dopamine release, and acutely perturbing dopaminergic signaling alters the strength of odor tracking. Context-dependent representations of movement and reinforcement cues are thus multiplexed within the mushroom body dopaminergic pathways, enabling them to coordinately influence both ongoing and future behavior.

Neuromodulators confer flexibility to neural circuits, endowing animals with the ability to adapt to a complex and ever-changing world. Across invertebrates and vertebrates alike, the neuromodulator dopamine influences diverse facets of behavior over different timescales. Dopamine has canonically been studied for its role in representing reward and stimuli predictive of reward, providing a teaching signal that drives learning. However, loss of dopaminergic signaling also leads to pronounced motor and motivational deficits, highlighting a central role in both coordinating and invigorating an animal’s actions. Dopamine, therefore, subsumes multiple seemingly disparate functions, shaping ongoing behavioral performance and modifying future behavior through learning.

How can a single neuromodulator exert such a broad influence on behavior? The diverse roles of dopamine could arise from functionally specialized and anatomically segregated circuits that encode either reinforcement, motor or motivational signals. Alternatively, the same dopaminergic pathways could contribute to multiple forms of behavioral modulation. Indeed, recent recordings revealed that midbrain dopaminergic neurons responsive to rewards also display motor-associated activity as animals perform a learned task. Whether ongoing motor correlates in dopaminergic neurons simply encode motor kinematics, convey a motivational signal to promote reward-seeking behavior or reflect the expectation of reward remains enigmatic. Moreover, whether the multiplexed activity of dopaminergic neurons indicate that the same pathways can both rapidly shape online behavioral performance and instruct learning is unclear.

In this study, we took advantage of the concise circuitry of the mushroom body—the associative olfactory center of *Drosophila*—to gain insight into the dual representation of reward and motor activity by dopaminergic pathways. The mushroom body is richly innervated by dopaminergic neurons (DANs) whose segregated axonal projections target discrete, non-overlapping compartments that tile the length of the mushroom body's output lobes. DANs innervating different compartments convey the reinforcement signals that instruct learning, with distinct subsets activated in response to either reward or punishment. Each compartment of the mushroom body lobe is also innervated by the dendrites of 1–3 mushroom body output neurons (MBONs). Local dopamine release within a compartment can, therefore, tune the strength of synaptic connections between the Kenyon cells that encode odor identity and the MBONs that bias behavior, enabling the same olfactory cue to drive either approach or avoidance depending on an animal’s past experience. Recent studies have also revealed that the activity of specific mushroom body DANs correlates with the spontaneous motor actions of an animal. The mushroom body thus offers the opportunity to explore motor-related signaling in a simplified dopaminergic circuit with a defined role in reinforcement learning.

To gain insight into the nature of motor-associated activity in DANs, we devised methods to record from the mushroom body as animals actively pursued an appetitive odor plume in a virtual olfactory environment, allowing us to directly compare ongoing dopaminergic activity during spontaneous and purposive behavior and disambiguate whether their signaling relates to motor kinematics or represents a context-dependent cue related to the task. We found that the same DANs responsive to reinforcement cues that instruct learning provide a rich representation of the moment-by-moment actions of an animal. Dopaminergic correlates of behavior, however, are not invariant but depend on the navigational strategy used for odor tracking, such that DANs preferentially represented the behavioral variables most...
relevant for pursuit. Together, our results suggest that rewards and pur-
positive actions are encoded through similar patterns of DAN activity and dopamine release, highlighting how the same neuromodulatory pathways can contribute to multiple forms of behavioral modulation over diverse time scales, conveying motivational signals to rapidly shape current behavior as well as instructive signals to modify future behavior through learning.

Results

Mushroom body DAN activity correlates with locomotion. To explore how dopaminergic pathways in the Drosophila mushroom body coordinately represent reward and movement, we monitored their activity in head-fixed animals walking on an air-supported, freely rotating ball (Fig. 1a and Extended Data Fig. 1a). We expressed a synthetically localized calcium indicator GCaMP (sytG-
CaMP6s) under the tyrosine hydroxylase and dopa-decarboxylase drivers (TH/DDC), allowing us to visualize calcium influx in DAN axon terminals innervating the different compartments that tile the mushroom body lobes (Fig. 1a,b). We focused on the population of DANs targeting the γ lobe (γ2–γ5), as these respond to positive and negative contextual cues and instruct short-term associations20,22,24. Starved animals walking in the dark, in the absence of any overt stimuli, alternated between spontaneous bouts of locomotion and quiescence and, when presented with a droplet of 1 M sucrose, immediately ceased walking and consumed the sugar, allowing us to compare the activity evoked by reward and locomotion within the same trials (Fig. 1c).

As previously described, ingestion of sucrose evoked bi-directional changes in the γ lobe dopaminergic population31, activating the γ4 and γ5 DANs while suppressing the γ2 and γ3 DANs. Before and after sugar consumption, fluctuations in DAN activity were also observed that appeared to be temporally aligned to an animal’s locomotion (Fig. 1c). The relative magnitude of these reward and motor-associated signals differed across DAN subsets. Although γ4 DANs were robustly activated by both sugar ingestion and locomotion, γ2 and γ3 DANs were activated only during walking and inhibited by reward (Fig. 1c and Extended Data Fig. 1b). In contrast, sugar-evoked responses in the γ5 compartment were far larger than any motor-associated fluctuations (Fig. 1c and Extended Data Fig. 1b). To characterize the locomotor-related activity of DANs, we therefore focused on the γ2, γ3 and γ4 subpopulations.

The dual representation of locomotion and reward within a compartment could arise either from heterogeneous classes of DANs that target the same compartment or from the multiplexed activity of individual neurons. Recent connectomic analyses indicate that the γ4 DANs can be divided into distinct subclasses based on their patterns of pre-synaptic connectivity29, raising the possibility that they comprise a functionally diverse population. Using inter-
sectional genetic drivers, we recorded from two morphologically distinct γ4 DAN subpopulations that could be distinguished by the axonal tract that they follow to innervate the lobes (Extended Data Fig. 1d,e; MB312B, upper commissure; MB316B, lower commissure). Although both subpopulations exhibited robust motor-associated activity, MB316B+γ4 DANs also responded to sugar ingestion (Extended Data Fig. 1f–h). To further explore the multiplexed activity of the MB316B+γ4 DANs, we used constrained non-negative matrix factorization (CNMF)29 to identify clusters of correlated pixels within the γ4 compartment, which presumably correspond to the individual axonal boutons of different neurons. Individual pixel clusters displayed both movement- and reward-related signals, an observation further corroborated by imaging of single MB312B+ and MB316B+soma (Extended Data Fig. 2). Distinct DAN subclasses innervating the γ4 compartment thus appear to be functionally specialized, with a population selectively tuned to ongoing movement and a subnet conveying multiplexed signals about locomotion and reward.

To assess whether the motor-associated activity of DANs can drive dopamine release, we expressed the genetically encoded dopamine sensor dLight1.3b34 in Kenyon cells, which are the primary targets of DANs in the mushroom body (Fig. 1b,d). During bouts of locomotion, we observed rapid fluctuations in dLight signaling along the length of Kenyon cell axons that adhered to the compartmental organization of the γ lobe (Fig. 1b, Extended Data Fig. 1c and Supplementary Video 1). Dual recordings of DANs expressing the red calcium sensor jRGECO35 and Kenyon cells expressing dLight showed that dopamine release was highly correlated with DAN activity in the γ4 compartment, with similar signals evoked by locomotion and reward (Fig. 1e,f). Despite the heterogeneity of γ4 DAN subclasses, we were unable to identify any functional topography or compartmental subdomains in either the activity of the broader DAN population labeled by TH/DDC or dLight signaling. Indeed, multiple clustering algorithms defined each compartment as a relatively homogenous functional unit (Fig. 1b and Extended Data Fig. 1c). Examination of the Drosophila connectome reveals that different DAN subclades have highly intermingled pre-synaptic terminals within the γ4 compartment32 (Extended Data Fig. 1e), suggesting a potential basis for the spatially homogenous patterns of dopamine release evoked by both locomotion and reward.

Differential encoding of behavior across compartments. Although information about ongoing locomotion could be globally broadcast across the brain37, we observed that the DANs innervat-
ing different compartments reflected distinct facets of motor activity that unfolded over different timescales. For example, y3 DAN activity consistently tracked the onset and offset of each bout of locomotion, independent of the heterogeneous kinematics of sponta-
neous movement (Fig. 2a–c). In contrast, y2 and γ4 DAN activity was highly variable even for bouts of walking that were indistin-
guishable by multiple behavioral metrics, both within and across individuals (Fig. 2a–c, Extended Data Fig. 3a–f and Supplementary Fig. 1). Conversely, although y3 DAN activity faithfully reflected walking bouts enduring for tens of seconds, it only weakly tracked the rapid fluctuations in an animal’s velocity during periods of continuous locomotion (Fig. 2a and Extended Data Fig. 4a). Rather, increases in forward velocity were associated with increased γ4 activity and decreased y2 activity, whereas increases in angular velocity (turning) were associated with increased y2 and γ3 activity and decreased γ4 activity (Fig. 2d–f and Extended Data Fig. 4a–c). However, as observed for movement initiation, these relationships were inconsistent even within the same individuals (Extended Data Fig. 4a). Linear filters describing the moment-to-moment relationship between DAN activity and forward or angular velocity, on average, accounted for only a fraction of the variance in DAN activity during locomotion, but their predictive power varied widely among flies (Fig. 2d–f). Filters generated from multiple DANs accounted for significantly more of the variance than any single dopaminergic compartment (Extended Data Fig. 4g), suggesting that the ensemble activity of heterogenous DANs carries more information about an animal’s ongoing behavior than any individual compartment.

During bouts of ongoing locomotion, the activity of different DANs was highly correlated on a sub-second time scale (Extended Data Fig. 4d–f). However, these intercompartmental relationships fluctuated rapidly (Extended Data Fig. 4d,h). Interestingly, the coordi-
inated activity of DANs during locomotion partially mirrored the responses of DANs evoked by sugar rewards or aversive shock35 (Extended Data Figs. 4a–f and 3e), highlighting how the rich inter-
connection between compartments37,46,47 might generate similar network states in response to both reinforcements and ongoing behavior.

Recording DAN activity during active odor pursuit. Motor cor-
relates in mammalian dopaminergic pathways have been proposed
to reflect an animal’s anticipation of reward or represent the motivation or vigor of goal-directed behaviors. The variable mapping between mushroom body DAN activity and apparently indistinguishable movements during spontaneous locomotion raised the possibility that these neuromodulatory pathways, likewise, do not simply encode the kinematics of locomotion but might also be shaped by an animal’s behavioral context. To explore this, we devised methods to record from DANs as flies were engaged in active pursuit of an appetitive odor plume.

A common navigational strategy employed by many insects, including *Drosophila*, is to reorient and increase their upwind velocity in response to an attractive olfactory cue, as this should lead them to the odor source. Indeed, flies freely walking in constant airflow displayed robust upwind tracking in response to a brief pulse of the food odor apple cider vinegar (ACV) (Supplementary Fig. 2a–e). To replicate odor-evoked tracking under the microscope, we used a closed-loop olfactory paradigm in which the heading of a tethered fly walking on an air-supported ball was yoked to the rotation of a tube carrying either a clean or odorized airstream, enabling an animal to control its orientation within an olfactory plume. To replicate odor-evoked tracking under the microscope, we used a closed-loop olfactory paradigm in which the heading of a tethered fly walking on an air-supported ball was yoked to the rotation of a tube carrying either a clean or odorized airstream, enabling an animal to control its orientation within an olfactory plume. (Fig. 3a and Supplementary Fig. 2g). Tethered flies could, thus, track an appetitive plume by reorienting and maintaining a steady
upwind heading, resulting in a net upwind displacement toward the fictive odor source (Fig. 3b and Supplementary Fig. 2d).

On average, the population of γ4 DANs was activated by brief pulses of ACV (Fig. 3d and Supplementary Video 2), consistent with their sensitivity to appetitive cues [14,21,26,31] (Fig. 1c,d and Extended Data Fig. 1b). However, odor-evoked responses were highly variable even between sequential odor presentations (‘trials’) within an individual animal (Fig. 3b,d). This variation appeared related to the concurrent behavioral response to the odor: γ4 DAN responses were strongest during trials when animals vigorously reoriented to track upwind and weaker when animals failed to alter their heading and continued to walk crosswind (Fig. 3b,f). Consequently, trial-by-trial, average γ4 DAN activity was correlated with the net distance an animal tracked upwind toward the fictive odor source (Fig. 3f and Extended Data Fig. 5a). γ2 DAN activity also depended on an animal’s behavioral response but in the opposite manner. These relationships were not apparent in clean air (Extended Data Fig. 5a–b), nor did they depend on differences in the efficacy of odor stimulation, as the magnitude of Kenyon cell responses was independent of the position of the odor tube (Extended Data Fig. 3h).

Thus, during active olfactory pursuit, mushroom body DANs neither solely represent the presence of an odor cue nor the kinematics of movement but, rather, reflect the behavioral response to the stimulus.

In the closed-loop paradigm, flies tracked toward the fictive odor source predominantly by altering their heading to reorient in the upwind direction, rather than increasing their forward walking speed (Fig. 3b, c and Supplementary Fig. 3a). Interestingly, the moment-to-moment correlation between γ4 DAN activity and these behavioral variables was differentially strengthened as animals transitioned from walking in clean air to odor pursuit, with a notable change in the γ4 DAN-[heading] filter (Fig. 3e). Likewise, across trials, an animal’s average heading, but not its average forward velocity, was correlated with the γ4 DAN response and inversely correlated with the γ2 DAN response (Fig. 3f). Motor variables thus appear to be distinctly encoded by DANs as animals actively track an odor plume.

**Context-dependent representations of odor-tracking behavior.** The emergence of novel behavioral correlates during olfactory...
Fig. 3 | DAN activity during active odor tracking. a, Schematic of experimental paradigm (top) where a tethered fly's heading is yoked to a motor controlling the position of an air tube rotating around the fly. Bottom: top-down view of a tethered fly showing the position of the air tube during upward and crosswind movement. b, Top: representative experiment depicting the fictive two-dimensional (2D) trajectory in response to ten presentations of ACV. Animals were presented with clean air for 30 s (black) and ACV for 10 s (orange). The hash mark indicates a ~20-s break in recording. Bottom: 2D trajectories (top row), |heading| (second row), forward velocity (third row) and γ DAN activity (bottom row) for trials in b, c. Upwind displacement during the odor trial plotted versus Δ[|heading|] (top) and Δ[forward velocity] (bottom) averaged throughout the odor presentation. Best fit line and Pearson coefficient ($r$) indicate where the relationship is statistically significant ($P < 0.001$ with Bonferroni correction; Supplementary Table 2). Fisher ($F$) transformation indicates significant differences in correlation coefficients for upwind displacement – Δ[forward velocity] and upwind displacement – Δ[|heading|] relationships with $z = 8.38$. $n = 26$ flies and 143 odor presentations. d, γ DAN $ΔF/F_o$ for all odor presentations, aligned to odor onset. Thick lines indicate average γ DAN activity. Translucent lines represent individual odor presentations. $n = 26$ flies and 143 odor presentations. e, Linear filters predicting DAN activity using forward velocity ($V_o$, left) or |heading| ($|h|$, right) in the odor plume (colored lines) and in clean air (black lines) over a 4-s window (0 mark indicates odor onset). $n = 26$ flies and 143 odor presentations. f, Average γ DAN $ΔF/F_o$ versus normalized upwind displacement (left), average Δ[|heading|] (middle) and average Δ[forward velocity] (right) during odor. Best fit line and Pearson coefficient ($r$) indicate where the relationship is significant ($P < 0.001$ with Bonferroni correction; Supplementary Table 2). Fisher $r$-to-$z$ transformation indicates significant differences in correlation coefficients for γ4-Δ[|heading|] and γ4-Δ[forward velocity] relationship with $z = -6.03$. $n = 26$ flies and 143 odor presentations. NS, not significant.

pursuit suggests that DAN representations of movement might depend on behavioral context. To examine this possibility, we altered the experimental conditions to induce flies to rely on a distinct navigational strategy to track toward the fictive odor source. By increasing the speed of the airflow, we observed that animals walked upward almost continuously for several meters, even in the absence of odor (Fig. 4a), consistent with evidence that high airflow triggers positive anemotaxis in many insect species43. During these extended bouts of anemotaxis, the moment-to-moment correlations between γ2 and γ4 DAN activity and a fly’s heading were...
Fig. 4 | Mushroom body DAN activity–behavior correlations depend on a fly’s navigational strategy. a, Representative experiment showing DAN activity and behavior under high-airflow conditions. Top: fictive 2D trajectory during a 5-min trial with a 60-s presentation of ACV (orange). Second row: expanded view of the above trajectory, 20-s period centered at odor onset; [heading] (third row), forward velocity (fourth row) and γ DAN activity (bottom row) during that same 20-s period. b, Upwind displacement plotted as a function of average Δ[heading] (top) and average Δforward velocity (bottom) during odor presentation. Pearson coefficient (r) indicates where the relationship is significant (P < 0.0001 with Bonferroni correction; Supplementary Table 2). Fisher r-to-z transformation indicates significant differences in correlation coefficients with z = –7.43. Fisher r-to-z transformation also indicates significant differences in correlation coefficients for upwind displacement – Δforward velocity across the high- and low-airflow contexts with z = –3.89 but no significant differences in the correlation coefficients for upwind displacement – Δ[heading]. n = 22 flies and 52 odor presentations. c, Linear filters predicting DAN activity using forward velocity (Vf, left) or [heading] (h, right) in the odor plume (colored) and in clean air (black, dashed) over a 4-s window (0 mark indicates odor onset), n = 22 flies and 52 odor presentations. d, Average γ DAN ΔVf/Fc plotted as a function of upwind displacement (left), average Δ[heading] (middle) and average Δforward velocity (right) during odor presentation. Pearson coefficient (r) indicates where the relationship is significant (P < 0.01 with Bonferroni correction; Supplementary Table 2). Fisher r-to-z transformation indicates significant differences in correlation coefficients for γ4–Δ[heading] and γ4–Δforward velocity relationships across the high- and low-airflow contexts with z = –1.77 and z = –3.42, respectively. n = 22 flies and 52 odor presentations. NS, not significant.

strengthened compared to when animals walked in more circuitous paths in clean air under low-airflow conditions (Extended Data Fig. 5g). Distinct behavioral correlates, therefore, arise as animals pursue a straight upwind trajectory, irrespective of whether tracking was elicited by high airflow or an appetitive odor.

Tethered flies walking upwind in high airflow responded to ACV by maintaining their current heading and transiently increasing their forward velocity (Fig. 4a, b, Extended Data Fig. 5f and Supplementary Fig. 3b). As observed in the low-airflow context, we found that, as animals transitioned from walking in clean air to odor pursuit, the correlations between DAN activity and different behavioral variables changed, both in the structure of their moment-to-moment relationships captured by the linear filters (Fig. 4c) and in their averaged activity over the course of an odor trial (Fig. 4d). Notably, whereas, in the low-airflow context, the average γ4 DAN activity during the odor stimulus was predominantly correlated with changes in an animal’s heading (Fig. 3f), in the high-airflow context, it was more strongly correlated with an animal’s forward velocity (Fig. 4d). These distinct relationships were apparent even if behavioral data were subsampled to have equivalent variance in low- and high-airflow regimes (Extended Data Fig. 5h,i). Despite the differential encoding of motor actions in low and high airflow, γ4 DAN activity remained well-correlated with an animal’s net upwind displacement in the odor plume across experimental conditions (Figs. 3f and 4d). Context-dependent correlations between DAN activity and behavior thus emerge as flies engage in different odor-tracking strategies, with the behavioral metrics most relevant to odor pursuit selectively strengthened in each.

A simple model for odor pursuit. We sought to understand how the rapid remapping of DAN activity to behavior, captured by the changing linear filters, might give rise to the longer timescale relationships that emerge during olfactory navigation. We found that simply applying the best fit linear filter to the experimentally measured behavior (Fig. 5a) could largely reproduce the trial-by-trial relationships among DAN activity, an animal’s specific motor actions and the net distance it tracked toward the odor source (Fig. 5b,c). For example, the filters fit to γ4 DAN activity as animals walked in odor in low airflow gave rise to a strong correlation between the average γ4 DAN activity and a fly’s heading but a weaker correlation with its forward velocity, replicating the experimentally observed relationships (Fig. 5b). Conversely, the filters fit to γ4 DAN activity in high airflow predicted a strong correlation with the average forward velocity of an animal tracking the odor plume but not its heading (Fig. 5c). These relationships did not depend on the behavioral kinematics of odor pursuit, as applying the low-airflow filters to the high-airflow behavioral data replicated the DAN–behavior correlations displayed in the low-airflow context (Fig. 5e and Extended Data Fig. 6b,d). Likewise, the high-airflow filters were sufficient to reproduce the experimentally observed relationships when applied to the low-airflow data (Fig. 5d and Extended Data Fig. 6a,c), reinforcing that these context-dependent correlations depend principally on the distinct structure of the linear filters across conditions. Moreover, these relationships were not present if we used filters fit to DAN activity when animals walked in clean air (Extended Data Fig. 6e,f). The context-dependent restructuring of ongoing DAN–motor correlates thus naturally give rise to the longer timescale
relationships that we observe, ultimately strengthening the representation of actions that subserve odor navigation.

To further illuminate how the relationships between DAN activity and behavior unfolded over an odor trial, we calculated a cross-correlation matrix at various temporal offsets relative to the odor stimulus. This analysis supports our observations that the moment-to-moment correlations between DAN activity and distinct behavioral variables were differentially strengthened as an animal entered the odor plume (Extended Data Fig. 7). Unexpectedly, the activity of some DANs became significantly correlated not only with an animal’s current behavior but also with its prospective tracking throughout the odor presentation (up to 8 s into the future; Extended Data Fig. 7c,d). This anticipatory activity could not be explained by the possibility that animals maintain invariant trajectories within the odor plume, at least for the low-airflow context, where the auto-correlation of their heading was predictive for less than 3 s and was similar in both clean air and odor (Extended Data Fig. 7b). A nested model quantifying how much the past, present or future behavior contributed to predicting DAN activity further reinforced that DANs carry prospective signals (Extended Data Fig. 8). In the low-airflow context, although a fly’s heading before odor onset had a negligible contribution to DAN activity, including current or future heading significantly improved the model’s ability to predict γ4 activity in the initial epoch of an odor response (Extended Data Fig. 8a, b). In contrast, in the high-airflow context, an animal’s forward velocity, but not its heading, was the primary predictor of DAN activity in odor (Extended Data Fig. 8c). The anticipatory signaling of the mushroom body DANs further underscores that these pathways are not simply reporting on an animal’s instantaneous experience but also appear to reflect context-dependent computations spanning multi-second timescales.

Satiety state coordinately modulates DAN activity and behavior. Our functional evidence that DANs preferentially correlate with the actions relevant to odor pursuit suggests that they might, like mammalian reinforcement pathways, reflect goal-directed behavior. Given that hunger represents a critical and conserved regulator of motivational drive, we explored how satiety state coordinately alters DAN activity and odor pursuit by measuring both in the low-airflow context before (starved) and after (fed) consumption of sucrose. Once sated, tethered animals walked with lower velocity and more frequently failed to reorient and steer upward in response to ACV (Fig. 6a, b and Supplementary Fig. 3e), mirroring the diminished odor attraction of fed freely moving flies (Supplementary Fig. 2f)\(^4,44\). The dampened behavioral attraction to ACV in fed flies was accompanied by a corresponding attenuation of DAN responses (Fig. 6c).

Nevertheless, on a trial-by-trial basis, γ4 DAN responses remained significantly correlated with the change in an animal’s heading in the odor plume (Fig. 6a,d), indicating that population was still activated on the trials when a fed fly did reorient to walk upward toward the odor source. Indeed, fillers fit to DAN activity in starved or fed animals similarly predicted the longer time scale DAN–movement relationships observed in either satiety state (Extended Data Fig. 9). Thus, γ4 DANs remained preferentially correlated with contextually relevant behavioral variables irrespective of an animal’s hunger state. Although metabolic signaling pathways are thought to directly impact onto DANs\(^13,45\), our results suggest that satiety state-dependent changes in DAN activity, at least in part, arise from the behavioral differences in starved and fed flies, underscoring the tight connection among internal state, locomotor activity and motivational drive.

DAN activity influences ongoing behavior. The rapid context-dependent signaling of mushroom body dopaminergic pathways suggests that, beyond their established role in learning and memory, DANs might also acutely shape behavior. To test this possibility, we selectively expressed the light-gated anion channel, GtACR1, or the cation channel, CsChrimson, in distinct subsets of DANs, allowing us to transiently inhibit or activate these populations during odor tracking in freely moving animals (Fig. 7a). To minimize the potential confounds of behavioral modulation due to learning, we examined odor pursuit in nominally naïve animals in response to a single brief episode of optogenetic illumination (Fig. 7b). The protocerebral anterior medial (PAM) DANs innervate multiple mushroom body compartments, including γ4, and their activation is sufficient to instruct appetitive odor associations\(^14,20,24,28\). Optogenetic inhibition of the entire cluster of PAM DANs, or just the MB312\(^+\) population of γ4 DANs, consistently suppressed attraction to ACV in starved flies (Fig. 7a–c, Extended Data Fig. 10a and Supplementary Fig. 4a). Silencing the protocerebral posterior lateral (PPL) DANs that convey negative reinforcement during learning\(^10b,12,26\) had no effect on odor tracking. Conversely, optogenetic activation of PAM DANs increased the proportion of fed flies that tracked upward toward an odor and even promoted upward tracking in the absence of an odor stimulus (Fig. 7d, Extended Data Fig. 10b and Supplementary Fig. 4b,c). Acute manipulation of DANs, therefore, bi-directionally modulates olfactory approach behaviors, in accord with more chronic perturbations\(^4,44\), with inhibition of PAM DANs suppressing the strong attraction of starved animals and their activation reversing the behavioral indifference of fed flies.

Discussion

In this study, we took advantage of the mushroom body’s concise circuit architecture to explore the nature of movement-related activity in a population of DANs that instruct associative learning. Although motor signals have been previously observed in the mushroom body DANs\(^26,29–31\), by developing methods to examine their activity as an animal was actively engaged in odor tracking, we gain new insight into these motor correlates and how they...
contribute to adaptive behavior. Although DANs provide a rich representation of a fly’s ongoing behavior, several lines of evidence suggest that these motor correlates do not simply reflect the kinematics of movement. First, the relationship between DAN activity and indistinguishable motor actions is not fixed but can rapidly change to reflect alterations to an animal’s context. Indeed, distinct DAN–motor correlations emerged as animals transitioned from walking in clean air to active odor pursuit, such that the representation of actions most relevant to odor tracking in a particular context were selectively strengthened (Fig. 8). As a consequence, γ4 DAN activity consistently correlated with an animal’s net upwind displacement toward the appetitive odor source on a given trial, irrespective of the specific navigational strategy used for tracking. Second, our analyses suggest how these moment-to-moment correlations give rise to longer timescale relationships, even extending beyond immediate behavior, such that DANs carried information about an individual’s prospective tracking within the odor plume. Finally, optogenetic
Fig. 6 | DAN responses and odor-tracking behavior are altered by satiety state. 

**a.** Top: schematic depicting that DAN activity and behavior are measured in the same flies before (starved) and after (fed) consumption of a sucrose meal. Middle: representative experiment showing the 2D trajectory of a fly walking under low-airflow conditions over a 5-min period in response to multiple presentations of ACV (orange) before (starved, left) and after (fed, right) a sucrose meal, with inset zooming into behavior. Bottom: comparison of indicated trajectories and γ DAN activity before and after feeding. Behavioral responses to ACV are diminished once animals are fed. Upwind displacement (left), average [heading] (middle) and average forward velocity (left) during odor presentation before (black) and after (maroon) feeding. Fisher r-to-z transformation indicates no significant differences in correlation coefficients for rz function of average |heading| (left) and average forward velocity (right) during odor presentation before (black) and after (maroon) feeding. 

**b.** Behavioral responses to ACV are altered after feeding. Paired two-sided t-test with Bonferroni correction, *P < 0.05; Supplementary Table 2. n = 10 flies and 102 odor presentations (49 before feeding and 53 after feeding). 

**c.** The relationships between γ DAN activity and behavior in different satiety states. Average z-score-normalized DAN activity plotted as a function of average [heading] (left) and average forward velocity (right) during odor presentation before (black) and after (maroon) feeding. Fisher r-to-z transformation indicates no significant differences in correlation coefficients for rγ [heading] relationships across starved and fed animals with z = –0.51. Pearson correlation coefficient (r) indicates where the relationship is statistically significant (*P < 0.02 with Bonferroni correction; Supplementary Table 2). 

**d.** Scatter plots of DAN–motor correlates are dynamically tuned to the actions that subserve goal-directed and purposive behavior (Fig. 8), such as seeking a food source, suggesting a fundamental connection between movement and motivation within these dopaminergic pathways.

**Layered multiplexing of reward and motor signals.** Our work supports emerging evidence that mushroom body DANs exhibit functional complexity beyond simply signaling reward or punishment. DANs innervating distinct compartments differentially reflect both external reinforcement cues and motor variables, giving rise to a rich and distributed representation of an animal’s ongoing experience through correlated patterns of activity across the population. The *Drosophila* connectome reveals how functional specialization within different DAN subsets might arise. Individual DANs display divergent patterns of synaptic connectivity, integrating from an array of interneurons emanating from additional higher-order nurseries, feedback from MBONs innervating different compartments and select ascending sensory pathways. The mushroom body DANs are, thus, wired into a highly interconnected network that integrates from multiple brain centers, poised to convey pre-processed signals that reflect an animal’s external environment, internal state, motor actions and motivations. The complexity of inputs to the DANs further suggests how their activity might be rapidly remapped to different behavioral actions depending on the context in which they are performed, either by inheriting context-dependent signals from their diverse pre-synaptic partners or reweighting these inputs in a context-dependent manner. Likewise, the highly recursive and integrative wiring of DANs suggests how both ingestion of a sucrose reward and tracking toward an appetitive odor source can generate similar patterns of network activity.

Mirroring these multifaceted and complex functional representations, we found that the same DANs that instruct olfactory associations also acutely influence odor pursuit. These observations suggest that, in *Drosophila*, diverse forms of dopaminergic modulation might be subserved by the same neuronal pathways. Recent work suggests that mammalian striatal DANs display similar heterogeneous and multiplexed activity in which representations of behavior and motivational cues are coupled with teaching signals.
Diverse computational roles for dopamine. An important remaining question is whether and how the targets of the mushroom body DANs—Kenyon cells and MBONs—differentiate between dopamine signals evoked by external reinforcements or those arising from behavior. Although subsets of γ4 DANs appear functionally specialized, their axonal projections are intermingled throughout the compartment and form similar numbers of synapses with γ lobe Kenyon cells\(^2\). This synaptic organization suggests that ongoing release of dopamine during locomotion might engage the same synaptic plasticity mechanisms that drive memory formation, allowing both rewards and self-generated actions to similarly modulate behavior. Although the goal of reinforcement learning is to shape an animal’s long-term decision-making policies\(^47\), short-term associations are required to solve the credit assignment problem and extract the causal relationship between an animal’s actions and subsequent rewards. The combination of ongoing DAN activity during odor pursuit and rapid dopamine-dependent plasticity of the γ lobe offers a potential mechanism for storing such an eligibility trace of a fly’s recent actions. For example, Kenyon cell-to-MBON synapses activated by an odor during locomotion could be rendered sensitive to an ensuing reward, generating a short-term association useful for updating a fly’s decision-making policy during odor...
navigation. Such a mechanism could underlie recent evidence for history-dependent odor-tracking behavior44 or facilitate pursuit of an odor plume. Indeed, a simulated mushroom body network optimized for different tasks, including odor navigation, revealed highly distributed DAN activity patterns that co-varied with multiple task-relevant variables, mirroring the multifaceted representations that we experimentally observe11.

Optimal behavioral policies might not only emerge through learning but could also be hard-wired into the nervous system through evolution to assure appropriate adaptive behavior. The mushroom body's position at the nexus of sensory circuits conveying odor signals and the efferent MBON pathways that bias behavior makes it an ideal substrate to allow dopaminergic signaling to modulate olfactory behavior, whether based on an animal's past experience or its current context. Our data add to growing evidence that the mushroom body plays a role beyond associative learning45,46, suggesting that dopaminergic modulation might act through multiple mechanisms that function over distinct timescales. Consistent with this notion, the Drosophila connectome reveals that DANs synapse directly onto MBONs15,10, providing a parallel route of communication that is mechanistically distinct from associative plasticity. Interestingly, the heterogeneous subclasses of y4 DANs differentially synapse onto the dendrites of the three MBONs innervating the y4 compartment32, suggesting an additional mechanism to expand the computational capacity of a single compartment and to shape moment-to-moment behavior.

Conservation of neuromodulatory mechanisms. Our work supports the basic correspondence of dopaminergic systems in insects and mammals, despite their separation by several hundred million years of evolutionary divergence. Dopamine acts upon circuits that display a fundamentally different architecture across these distant phyla yet can give rise to similar forms of learning and adaptive behavior. Whether the analogous roles of dopamine in insects and mammals reflect the basic conservation of neuromodulatory pathways or convergent evolution is not clear31. One possibility is that dopamine's evolutionary ancient role as a modulator of motor circuits32 predisposed dopaminergic systems to acquire more specialized functions as brain complexity evolved, linking reward pursuit and reinforcement. Alternatively, the ubiquity of dopamine as a modulator of associative circuits could reflect similar computations arising repeatedly, potentially taking advantage of conserved receptor signaling pathways and molecular hardware. In either case, the coupling of reinforcement signals and locomotor representations in the same neuromodulatory system across divergent taxa suggests an inherent connection between them. By representing and invigorating motor actions and assigning value to sensory stimuli through learning, dopaminergic pathways allow animals to flexibly adapt their behavior over different timescales, acutely shaping moment-by-moment movements to achieve short-term goals and effecting persistent changes in behavior through learning.

Online content
Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41593-021-00929-y.

Received: 17 May 2020; Accepted: 1 September 2021; Published online: 25 October 2021

References
1. Bargmann, C. I. & Marder, E. From the connectome to brain function. Nat. Methods 10, 483–490 (2013).
2. Schultz, W., Dayan, P. & Montague, P. R. A neural substrate of prediction and reward. Science 275, 1593–1599 (1997).
3. Watabe-Uchida, M., Eshel, N. & Uchida, N. Neural circuitry of reward prediction error. Annu. Rev. Neurosci. 40, 373–394 (2017).
4. Waddell, S. Reinforcement signalling in Drosophila; dopamine does it all after all. Curr. Opin. Neurobiol. 23, 324–329 (2013).
5. Da Silva, J. A., Tecuapetla, F., Paixão, V. & Costa, R. M. Dopamine neuron activity before action initiation gates and invigorates future movements. Nature 554, 244–248 (2018).
6. Howe, M. W. & Dombeck, D. A. Rapid signalling in distinct dopaminergic axons during locomotion and reward. *Nature* 535, 505–510 (2016).

7. Panigrahi, B. et al. Dopamine is required for the neural representation and control of movement vigor. *Cell* 162, 1418–1430 (2015).

8. Salamone, J. D. & Correa, M. The mysterious motivational functions of mesolimbic dopamine. *Neuron* 76, 470–485 (2012).

9. Beierholm, U. et al. Dopamine modulates reward-related vigor. *Neuropsychopharmacology* 38, 1495–1503 (2013).

10. Parker, N. F. et al. Reward and choice encoding in terminals of midbrain dopamine neurons depends on striatal target. *Nat. Neurosci.* 19, 845–854 (2016).

11. Engelhard, B. et al. Specialized coding of sensory, motor and cognitive variables in VTA dopamine neurons. *Nature* 570, 509–513 (2019).

12. Yves, K., Jérôme, F., Clément, R. & Christian, L. Context-dependent multiplexing by individual VTA dopamine neurons. *J. Neurosci.* 40, 7489–7509 (2020).

13. Coddington, L. T. & Dudman, J. T. Review learning from action: reconsidering movement signaling in midbrain dopamine. *Neuron Act. Neuron* 104, 63–77 (2019).

14. Berke, J. D. What does dopamine mean? *Nat. Neurosci.* 21, 787–793 (2018).

15. Watabe-Uchida, M. & Uchida, N. Multiple dopamine systems: weal and woe dependence in insect flight decisions during odor tracking. *Cell. Rep.* 8, 1–21 (2011).

16. Aso, Y. et al. The neuronal architecture of the mushroom body provides a logic for associative learning. *eLife* 3, e04577 (2014).

17. Claridge-Chang, A. et al. Writing memories with light-addressable reinforcement circuitry. *Cell* 139, 405–415 (2009).

18. Liu, C. et al. A subset of dopamine neurons signals reward for odor memory in *Drosophila*. *Nature* 488, 512–516 (2012).

19. Aso, Y. et al. Specific dopaminergic neurons for the formation of labile aversive memory. *Carr. Biol.* 20, 1445–1451 (2010).

20. Yamagata, N. et al. Distinct dopamine neurons mediate reward signals for short- and long-term memories. *Proc. Natl Acad. Sci. USA* 112, 578–583 (2015).

21. Aso, Y. et al. Three dopamine pathways induce aversive odor memories with different stability. *PLoS Genet.* 8, e1002768 (2012).

22. Aso, Y. & Rubin, G. M. Dopaminergic neurons write and update memories with cell-type-specific rules. *eLife* 5, 1–15 (2016).

23. Aso, Y. et al. Mushroom body output neurons encode valence and guide memory-based action selection in *Drosophila*. *eLife* 3, e04580 (2014).

24. Burke, C. J. et al. Layered reward signalling through octopamine and dopamine in *Drosophila*. *Nature* 492, 433–437 (2012).

25. Hige, T., Aso, Y., Modi, M. N., Rubin, G. M. & Turner, G. C. Heterosynaptic plasticity underlies aversive olfactory learning in *Drosophila*. *Neuron* 88, 985–998 (2015).

26. Cohn, R., Morante, I. & Ruta, V. Coordinated and compartmentalized neuromodulation shapes sensory processing in *Drosophila*. *Cell* 163, 1742–1755 (2015).

27. Waddell, S. Neural plasticity: dopamine tunes the mushroom body output network. *Carr. Biol.* 26, R109–R112 (2016).

28. Handler, A. et al. Distinct dopamine receptor pathways underlie the temporal sensitivity of associative learning. *Cell* 178, 60–75 (2019).

29. Berry, J. A., Cervantes-Sandoval, L., Chakraborty, M. & Davis, R. L. Sleep facilitates memory by blocking dopamine neuron-mediated forgetting. *Cell* 161, 1656–1667 (2015).

30. Aimon, S. et al. Fast near-whole–brain imaging in adult *Drosophila* during responses to stimuli and behavior. *PLoS Biol.* 17, e2006732 (2019).

31. Sjö, K. P. et al. Valence and state-dependent population coding in dopaminergic neurons in the fly mushroom body. *Carr. Biol.* 30, 809277 (2020).

32. Li, F. et al. The connectome of the adult *Drosophila* mushroom body provides insights into function. *eLife* 9, e26576 (2020).

33. Pnevmatikakis, E. A. et al. Simultaneous denoising, deconvolution, and demixing of calcium imaging data. *Neuron* 89, 285–299 (2016).

34. Patriarchi, T. et al. Ultrafast neuronal imaging of dopamine dynamics with designed genetically encoded sensors. *Science* 356, eaat4422 (2018).

35. Sui, H. et al. Sensitive red protein calcium indicators for imaging neural activity. *eLife* 5, e12727 (2016).

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

© The Author(s), under exclusive licence to Springer Nature America, Inc. 2021
Methods

Fly husbandry. Flies were maintained on cornmeal-agar-molasses medium at 23–25 °C and 60–70% relative humidity, under a 12 h light/dark cycle, and transferred to vials containing only a Kimwipe soaked in 2 ml of water (or 0.2 mM all-trans-retinol water for optogenetic experiments) 18–24 h before all experiments. For optogenetic experiments, flies were grown on retinoid-free sugar and yeast-based food in complete darkness. One- to two-day-old females were transferred to conventional food containing 0.4 mM all-trans-retinal (Sigma-Aldrich no. R2500) and placed in the dark for ≥24 h.

Fly stocks and genotypes. All experiments were performed using 1–7-day-old females. For detailed fly stock sources and genotypes, see Supplementary Table 1.

Fly tethering and dissection. For in vivo imaging of neural activity, flies were prepared as described previously7 with minor modifications. Briefly, 3–7-day-old females were anesthetized using CO2 (<30%) and tethered to a specially designed holder dish. The fly was held in place with UV-curable glue (Loctite) applied to each eye and thorax, and the proboscis was glued in an extended position. Care was taken to avoid glue contacting the antennae or aristae. After a less than 2-h recovery period, the dish was filled with external saline (108 mM NaCl, 5 mM KCl, 2 mM CaCl2, 8.2 mM MgCl2, 4 mM NaHCO3, 1 mM NaHPO4, 10 mM sucrose, 5 mM HEPES sodium salt, pH 7.5 with osmolarity adjusted to 275 mosm), and the cuticle covering the dorsal portion of the head was removed. Muscle 16 and obstructing trachea were removed, with care taken to keep the antennae and antennal nerves intact.

Two-photon functional imaging. All functional imaging experiments were performed on an Ultima two-photon laser scanning microscope (Bruker) equipped with galvanometers driving a Chameleon Ultra II Ti:Sapphire laser. Emitted fluorescence was detected with either photomultiplier tube or GaAsP photodiode (Hamamatsu) detectors. Images were acquired with an Olympus ×40, 0.8 numerical aperture objective at 512 pixels × 512 pixels resolution. For fast-scanning volumetric imaging in vivo (Extended Data Fig. 2g), the laser was directed through an 8-kHz resonant scanning galvanometer, and the objective was controlled by a piezoelectric Z-focus.

Image processing. All image processing was performed using custom scripts in MATLAB or Fiji/ImageJ (National Institutes of Health). Compartment (DAN subpopulation)-specific activity was computed by averaging all fluorescence signal within a manually defined mushroom body lobe compartment. All DAN>sytGCaMP6s signals are normalized (divided by MB247(KC)>dsRed fluorescence within the same region of interest (ROI). Data from all other functional imaging experiments are plotted as raw fluorescence on an arbitrary scale or normalized as indicated.

Tethered locomotion. For tethered locomotion experiments, a spherical treadmill was designed based on previous studies53. Briefly, a 6.35-mm diameter ball was shaped from LAST-A-FOAM FR-4618 (General Plastics) by a custom-made steel rotation was measured by a rotary encoder (CUI Devices, AMT10 Series) that was connected to an airflow. The ball was recorded at 60–61 fps using a Point 6.75 mm in diameter with a 1-mm channel drilled through the bottom and shaped from LAST-A-FOAM FR-4618 (General Plastics) by a custom-made steel rotation was measured by a rotary encoder (CUI Devices, AMT10 Series) that was connected to an airflow. The ball was recorded at 60–61 fps using a Point

Closed-loop arena. The heading of the fly, as calculated by FicTrac, was transmitted to an Arduino Mega via serial port. Custom Arduino code was used to translate heading into tube position controlled by motors described below. The closed-loop air delivery system was custom designed using OnShape (www.onshape.com) and 3D printed using VisiJet Crystal material at XHD resolution in a 3D Systems ProJet 3510 HD Plus. O-ring outside dimension (OD) and inside dimension (ID) gland surfaces were designed with excess material for printing and then manually modified with a lathe for improved RMS (surface) finishing. 360° tube rotation was driven by a bipolar stepper motor (Pololu, item no. 1206) controlled through a A4988 Stepper Motor Driver Carrier (Pololu, item no. 2980) coupled by a Dust-Free Timing Belt XL Series. 1/4” width (McMaster-Carr, 1679K121, trade no. 130 x 1025) to the rotating tube system, which rotated mounted on an Ultra-Corrosion-Resistant Stainless Steel Ball Bearing (3/4” diameter, 1–3/8” OD, McMaster-Carr, 5908K19). Air channel was kept airtight using oil-resistant O-rings (1/16” fractional width, dash no. 020, McMaster-Carr, 24181T126). Motor rotation was measured by a rotary encoder (CUI Devices, AMT10 Series) that was used to correct for skipped steps.

Odor stimulation and airflow. Odor stimulation was achieved by directing a continuous stream of either 100 ml min−1 (low-airflow conditions) or 400 ml min−1 (high-airflow conditions) of clean air through a 2-mm-diameter tube made of VisiJet Crystal material directed at the fly’s antennae. Then, 10%–20% of the total airstream was diverted through the headspace of a 500-ml bottle containing water. At a trigger, a custom-built solenoid valve controller system redirected the odor stream from the water bottle to a bottle containing the odorant (ACV, HCl).

Sugar feeding. For recordings involving sucrose, flies were tethered for imaging and locomotion as described above. Early during recovery from anesthesia, flies would often fully extend their proboscis before regaining other motor functions, and, at this point, glue was applied to the lateral sides of the proboscis, with care taken to avoid glue contacting the distal proboscis, mouth parts, antennae and aristae. As in all other tethered experiments, muscle 16 and obstructing trachea were removed, with extra care taken to keep the antennal nerves and the esophagus intact. This approach allowed for flies to ingest sugar through their proboscis with minimal motion of the brain relative to the imaging plane. As a control for movement artifacts in experiments recording GCaMP activity from all mushroom body DANs, the stable fluorophore diRed was expressed in the anatomically overlapping population of Kenyon cells, and all DAN>sytGCaMP6s signals were normalized (divided by) MB247(KC)>dsRed fluorescence within the same ROI. In addition, this channel was monitored for movement artifact, and trials that demonstrated changes in activity during sugar feeding beyond the range observed during spontaneous locomotion were excluded from analysis. For CNMF analysis of MB312B and MB316B flies (Extended Data Fig. 2a–d), flies were positioned with a mounted micro-manipulator (Scientifica). Red food coloring was added to the sucrose, and the fly abdomens were inspected after each experiment to confirm sucrose ingestion.

Freedly moving fly behavior and optogenetic perturbations. Behavior in freely moving flies was assayed in a custom-built apparatus as described previously9.

Data analysis and presentation. Unless otherwise noted, all analyses, visualizations, representations, normalizations and averaging of behavior and DAN activity were performed or generated using custom scripts in MATLAB and/or ImageJ. The following MATLAB functions were used to generate the parenthetical variables/images: imagesc (heat maps), movcorr (running correlations), hist (histograms) and kmeans (k-means clustering). All figures, illustrations, schematics and cartoons were created using Adobe Illustrator CC.

To facilitate visual comparison of neural activity and behavior (Figs. 1c–e, 2a–c, 3a–b, and 4a; Extended Data Figs. 1f, 2a, 3a–c, 4a–c and 8a; and Supplementary Fig. 3a–c), the behavioral variables of net motion and/or forward velocity were convolved by the bi-exponential rise and decay function of GCaMP6s (rise \( t_{1/2} = 173 \) ms and decay \( t_{1/2} = 550 \) ms), GCaMP4D (rise \( t_{1/2} = 50 \) ms and decay \( t_{1/2} = 150 \) ms), dLight (rise \( t_{1/2} = 10 \) ms and decay \( t_{1/2} = 100 \) ms) or jRGECO (rise \( t_{1/2} = 40 \) ms and decay \( t_{1/2} = 200 \) ms) and then multiplied by the mean of the top 10% of unconvolved behavioral variables for each trace (translated from radians per second into millimeters per second according to the radius of the treadmill). In other analyses, the behavioral data were smoothed using a 3-point moving average so that smoothing out the fine temporal structure of the signal could lead to the loss of relevant information within the data and introduce spurious temporal shifts. Behavioral variables were captured at 60–61 frames per second, whereas neural activity (fluorescence) was captured at 10 frames per second. Owing to electronic delays within the imaging system, however, frames were captured over a range of 110 ms (≤30°) and then converted into one distinct time series, a standardized and regular time series was generated for each trace containing 100 ms time bins (0, 100, 200, 300, etc.). All behavioral or fluorescence data captured between these standardized cutoﬀ points were averaged and assigned to the lower bound of the time bin. This allowed for neural and behavioral data to be aligned and meaningfully shifted.

Comparisons of movement- and reward (sucrose)-related DAN activity and neurotransmitter release (Extended Data Fig. 1b) were made by averaging normalized (\( \Delta F/F \)) with \( F_i \) = median trial fluorescence) sytGCaMP6s or dLight1,3b fluorescence during bouts of movement or during ingestion of 1 M sucrose solution manually identified by concurrent digital video recording.

CNMF analysis was performed as follows. Frames with large motion artifacts were manually removed from the imaging stack, which was then motion corrected using non-rigid motion correction (NoRMCorr)19. ROIs were extracted using CNMF20. Greedy and graph non-linear multidrig initialization methods were used to extract ROIs corresponding to DAN pre-synaptic terminals and mushroom body compartments.

Anatomic reconstructions of y4 DAN subpopulations (Extended Data Fig. 1d) and y4 DAN pre-synaptic boutons (Extended Data Fig. 1e) were performed as follows. Using the female adult hemibrain, we analyzed the y4 DAN subtypes PAM07, PAM08a, PAM08b, PAM08c, PAM08d and PAM08e. We screened driver lines corresponding to y4 DAN subtypes using NeuroBridge21. y4 DANs could be coarsely separated into neurons whose contralateral processes cross the midline over an upper commissure, which is covered, in part, by MB312B, or lower commissure, which is covered, in part, by MB316B. Pre-synaptic terminals corresponding to upper and lower commissure y4 DANs were analyzed using...
Instances of movement initiation (Fig. 2b, Extended Data Fig. 3a–f) and plotted using navis (https://github.com/schlegelp/navis).

Supplementary Figs. 1–4 were identified in silico using a custom MATLAB script that relied on a manually set cutoff to find inflection points in net motion after a prolonged pause (≥2 s) followed by sustained movement (≥3 s). Triggered average of parameters of behavior at the start of movement were normalized by dividing them by their average value during a given trial. Each individual behavioral trace was then aligned to generate the average, standard deviation and 95% confidence interval and plotted on an arbitrary scale on an aligned set of axes. Relationships between DAN activity and different behavioral variables and relationships among different DANs during individual bouts of movement initiation were calculated by averaging the ΔF/F₀ signal over the period of t = 1 s to t = 2.5 s relative to the onset of movement and averaging the behavioral variables (normalized as above by dividing by their average value) over the same interval. Neurobehavioral variables such as forward velocity and [angular velocity]: t = 1 s to t = 3 s; [lateral velocity]: t = 0 s to t = 1 s. Proportion of time moving after start was calculated over a 10-s period after initiation of locomotion.

Principal component analysis of behavioral variables during movement onset (Supplementary Fig. 1) was performed on the concatenated net motion, forward velocity, [angular velocity] and [lateral velocity] across a 4-s time window centered on instants of movement initiation (4 variables × 4-s time window × 10-Hz sampling = 160 initial variables per movement onset). All behaviors were z-scored over the 4-s window before concatenation.

To identify bouts of movement and periods of quiescence, custom MATLAB scripts used manually set cutoff values and the non-convolved net motion recordings to perform an in silico identification of periods of walking and periods of not walking during trials. Walking/still designations were then manually verified, and time points of transition were stored to be used in subsequent analyses.

Probability of accurate walking state prediction (Fig. 2c) was calculated as follows.

Epochs of movement and quiescence were identified as above. All data from a single animal were concatenated, and 20% of time points were randomly set aside (using the MATLAB function randperm), whereas the remaining 80% were used to train a multinomial logistic regression model (mnml) to predict locomotor state from compartmentalized DAN activity. The model was then tested on the previously allocated 20% of time points. During testing, the model produced both a prediction of locomotor state and a probability of accuracy. Predictions were compared with actual locomotor state, and the probability of accuracy of correct predictions was averaged (with incorrect predictions assigned a value of 0). This process was repeated 100 times for each animal, and the mean probability of accurate predictions was averaged across all 100 repetitions.

Increases in forward velocity and [angular velocity] (Extended Data Fig. 4b,c) were calculated as follows.

A custom MATLAB script isolated local maxima in forward or [angular] acceleration during bouts of movement, and the preceding point of acceleration = 0 was then identified, and velocity and neural activity were then aligned to these time points. To exclude instances when animals altered both their forward and angular velocities, epochs in which the acceleration of the alternative behavioral variable rose beyond a manually set cutoff point were excluded. For plotting, behavior was centered on these inflection points, and the average behavior over a time window from t = −2 s to t = 2 s. Neural activity was then aligned to the large number of weights in the four-DAN filter, with a random 80/20 fitting/held-out 20% fitting/test split of trials repeated 50 times. For each split, filter weights were chosen to be those that minimized the squared difference between the true and predicted behavioral variable, averaged over all time points in all trials used for fitting. Filters were evaluated by calculating the R² between the true and filter-predicted behaviors in the 20% of held-out trials.

Partial correlations (Extended Data Fig. 4f) were generated via custom Python scripts (the following way. To produce a correlation matrix between two variables x and y, conditioned on auxiliary variables z₁, ..., zₜ, we first computed the best fit linear predictions of x and y, respectively, using z₁, ..., zₜ, as the only predictors (in addition to an intercept term). The partial correlation between x and y given z₁, ..., zₜ was then computed as the Pearson correlation between (x − z₁ ... zₜx) and (y − z₁ ... zₜy).

Both the net neural signal and the other DAN activities were included in the auxiliary variables.

Position of tethered flies on a fictive two-dimensional plane was calculated by FiCtrac software based on the diameter of the foam ball upon which the fly was walking, and representations of fictive walking trajectories were generated using custom-written MATLAB scripts.

Neural activity and behavior at odor onset were identified using the aligned internal time stamps generated with Bruker software or FiCtrac software, respectively, and representations were generated using custom-written MATLAB scripts. For DAN activity, ΔF/F₀ was calculated, with F₀ defined as the average normalized (see ‘Image processing’ subsection) GCaMP fluorescence during a 10-s period immediately before odor onset. In air, controls were similarly calculated except with a 15-s temporal offset.

Individual odor responses were aggregated from trials across multiple flies. Every fly was presented with at least ten odor presentations, but trials in which FiCtrac failed to continuously track the fly’s movements throughout the period immediately preceding and through the entire odor presentation were excluded, as in these instances, the animal was not maintained in the closed-loop virtual reality configuration. As a result, the number of trials from any individual fly included in the dataset ranged from 3 to 12. We, therefore, aggregated responses across animals to examine the relationship between changes in DAN activity and changes in different behavioral variables.

Relationships between averaged DAN activity and behavior during an odor trial were calculated as follows.

Upwind displacement was divided by the individual fly’s average walking speed in the 10 s before odor presentation and then calculated as the change in fictive position along the axis of airflow from the start of the odor plume (t = 0 s) until the end of the odor plume (t = 10 s). Changes in forward velocity (Δv) and head angle (Δθ) were then calculated by subtracting the average forward velocity or [heading] during bouts of movement in the 10-s period preceding odor onset (t = −10 s to t = 0 s) from the average forward velocity or [heading] during bouts of movement occurring within the odor plume (t = 2 s to t = 10 s) to account for any delays in odor delivery. Using shorter or longer time bins gave similar results. y DAN ΔF/F₀ was averaged over (t = 2 s to t = 10 s) with F₀ = average y DAN ΔF/F₀ activity during bouts of movement from t = −10 s to t = 0 s before the onset of odor.

Relationships between averaged DAN activity and behavior as animals walked in clean air (Extended Data Fig. 5a,b,e,f) were calculated similarly except over the 20 s preceding an odor stimulus. Subsampling of odor responses (Extended Data Fig. 5g) was performed by serially excluding data below or above a manually set cutoff point until the variances of the behavioral variables in the two datasets were approximately equal.

Linear model (Figs. 3c, 4c, and 5 and Extended Data Figs. 6 and 9): Before filter fitting, we z-score-normalized DAN activity within each compartment relative to the entire 5-min trial. Forward velocity was normalized by dividing all time points by the median forward velocity computed during all identified walking periods in the trial. Absolute (unsigned) heading was measured in radians. We then considered either the 10 s before odor onset (in air) or the 10 s after odor onset (in odor). The average 10-s activity of each DAN subset across all trials was then subtracted from the DAN activity during each individual trial (either in air or odor) to account for any rapid rise or fall during odor onset and allow the filters to better explain how behavior gives rise to the fluctuations around the mean response. Using the collection of 10-s windows aggregated across all odor pulses and trials for either the ‘in odor’ or ‘in air’ period, we then fit 5 ± 5 linear filters predicting DAN activity from the two behavioral variables. Specifically, for each compartment, we assumed that DAN activity at time t, g(t) was a weighted variance explained by behavior across flies and trials without requiring us to hold out data for cross-validation (because many trials did not have sufficient data points to do so due to the limited amount of time the fly was walking).
sum of the forward velocity and absolute heading signals in a window extending 4 s before and 1 s after. Mathematically, we let the estimate 
\[ g(t) = w_h(t - 4) + w_v(t - 3.9) + ... + w_v(t - 0.9) + w_h(t + 1) + w_v(t - 4) + w_v(t - 3.9) + ... + w_v(t + 0.9) + w_h(t + 1) \]
where \( h \) and \( v \) are absolute heading and forward velocity, respectively. We chose all \( w \) values by minimizing the squared error \( (g(t) - g^2(t)) \) averaged across time, pulses and trials. This yielded 5 s of absolute heading and forward velocity weights for each DAN compartment in each period (‘in air’ and ‘in odor’). We repeated this process across all conditions (low-flow, high-flow and fed). Experimentally derived data were similarly normalized when directly compared to model-generated data.

Cross-correlation matrices (Extended Data Fig. 7) were generated in Python in the following way. For each 10-s odor pulse across all trials, all measured variables (DAN activity, heading, etc.) within the 20-s time window surrounding the pulse onset were selected and stored (that is, the window included the 10 s before the pulse and the 10 s of the pulse itself). For each variable, this generated an initial data matrix with the number of rows equal to the number of odor pulses across all trials and with 200 columns (20 s × the 10-Hz sampling). DAN activity was normalized for each pulse by subtracting and then dividing by the average fluorescence within this 20-s window. Forward velocity and heading were normalized by subtracting the forward velocity or heading, respectively, averaged over the first 10 s (the 10 s preceding the odor pulse onset). A correlation matrix between variables \( x \) and \( y \) across odor pulses was then generated by computing the Pearson correlations between \( x(t) \) and \( y(t) \) for all \( t \) spanning the 20-s window. \( P \) values were calculated as standard Pearson correlation \( P \) values using the scipy Python package.

The nested model analysis (Extended Data Fig. 8) used linear models, and significance levels for the increase in variance explained (as stepwise predictors were added) were computed using an F-test. Models were fit and analyzed using Python scripts, and results were plotted in MATLAB. Briefly, for a given condition (low or high airflow) and DAN compartment, each odor pulse was treated as a ‘trial’. Across trials, we sought to predict DAN activity during the initial phase of odor presentation (\( y_{1-4} \), time-averaged 1–4 s after odor onset) using combinations of the following predictors: [heading] time-averaged over the 10 s before odor onset (\( h_{1-4} \)), initial |forward velocity| (time-averaged from 1–4 s after odor onset, \( \Delta v_{1-4} \)), initial |heading| (time-averaged from 1–4 s after odor onset, \( \Delta h_{1-4} \)) and future |heading| (time-averaged from 7–10 s after odor onset, \( \Delta h_{7-10} \)). Windows were chosen based on the approximately 3-s heading auto-correlation time.

We then proceeded with a stepwise regression. For a given condition and DAN compartment, we first identified (using least squares regression) the best linear prediction of initial DAN activity \( y_{1-4} \) given \( h_{1-4} \). That is, we identified the parameter \( a \) in the model \( g = ah \), that minimized \( (y_{1-4} - \gamma)^2 \) averaged over all trials/pulses. We next added initial forward velocity as a predictor by identifying the best fit parameters \( a \) and \( b \) in the model \( g = ah + b \Delta v_{1-4} \) and recomputed the trial/pulse-averaged error. We repeated the procedure by sequentially adding in the remaining two predictors, \( \Delta h_{7-10} \) and \( \Delta h_{1-4} \), and recomputing the corresponding error. This stepwise regression allowed us to evaluate whether the predictive power gained by adding in each new predictor was significant by comparing the explained variances of the model with and without the candidate predictor using an F-test.

### Statistical analysis

All statistical analyses were performed using built-in MATLAB functions unless otherwise noted. Exact \( P \) values for every statistical test performed are listed in Supplementary Table 2.

- Pearson correlation coefficient and associated \( P \) values: corrcdf
- One-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison test: anova1 and multcompare
- Fisher r-to-z transformations were calculated using http://vassarstats.net/rdiff.html.

Two-tailed paired \( t \)-tests with Holm–Bonferroni post hoc correction: \( t \)-test

Two-sample \( F \)-test for equal variances: vartest2

### Reporting Summary

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

### Data availability

The data that support the findings of this study are available at https://github.com/rutalaboratory/Zolin_etal_2021 and upon request.

### Code availability

Code used for processing and modeling of the data is available at https://github.com/rutalaboratory/Zolin_etal_2021.

### References

53. Green, J. et al. A neural circuit architecture for angular integration in *Drosophila*. Nature 546, 101–106 (2017).
54. Moore, R. J. D. et al. PicTrac: a visual method for tracking spherical motion and generating fictive animal paths. *J. Neurosci. Methods* 225, 106–119 (2014).
55. Pnevmatikakis, E. A. & Giovannucci, A. NoRMCorr: an online algorithm for piecewise rigid motion correction of calcium imaging data. *J. Neurosci. Methods* 291, 83–94 (2017).
56. Giovannucci, A. et al. CalmAn: an open source tool for scalable calcium imaging data analysis. *eLife* 8, e38173 (2019).
57. Meissner, G. W. et al. An image resource of subdivided *Drosophila* GAL4-driver expression patterns for neuron-level searches. Preprint at https://www.biorxiv.org/content/10.1101/2020.05.29.080473v1 (2020).
58. Clements, J. et al. neuPrint: analysis tools for EM connectomics. Preprint at https://www.biorxiv.org/content/10.1101/2020.01.16.909465v1 (2020).

### Acknowledgements

We thank S. R. Datta, B. Noro, A. Handler and members of the Ruta lab for valuable discussions and comments on the manuscript. We also thank C. Dan, V. Jayaraman and L. Tian for developing the dlight sensor flies and J. Petrillo and P. Stock for technical advice. Stacks from the Bloomington *Drosophila* Stock Center (NIH P40OD018537) were used in this study. This work was supported by the National Institutes of Health (R01NS113103 and DP2NS087942 (to VR) and T32GM007379 to the Well Cornell/ Rockefeller/Sloan Kettering Tri-Institutional MD-PhD Program (to A.Z. and A.S.)); by a Kavli Neural Systems Institute Fellowship (to A.S.); and by the Simons Collaboration on the Global Brain (to V.R. and A.F.).

### Author contributions

A.Z. performed DAN imaging and behavioral experiments, with assistance from R.C. R.C. designed and created the closed-loop system and wrote custom code for data analysis, with assistance from A.Z. R.P. performed analysis and modeling, with assistance from A.F. A.S. performed functional characterization of different DAN subsets and patterns of dopamine release. A.Z. and V.R. wrote the manuscript with input from all authors.

### Competing interests

The authors declare no competing interests.

### Additional information

Extended data is available for this paper at https://doi.org/10.1038/s41593-021-00929-y.

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41593-021-00929-y.

Correspondence and requests for materials should be addressed to Vanessa Ruta.

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

Reprints and permissions information is available at www.nature.com/reprints.
Extended Data Fig. 1 | Compartmentalized DAN activity and dopamine release coordinately represent reward and locomotion. (a) Schematic depicting experimental system and definition of the quantified parameters of locomotion. (b) Comparison of maximum DAN activity measured by sytGCaMP6s expressed in DANs (left) and dopamine release measured by dLight expressed in Kenyon cell (KCs) (right) in response to ingestion of a sucrose reward (R) or during locomotion (L). Signals normalized by subtracting the median fluorescence during the 5 min trial. Paired two-sided t-test with Bonferroni correction, p < 0.05 (*), see Supplementary Table 2. (c) Correlated and compartmentalized sytGCaMP6s activity in γ lobe DANs (top) and KC dLight expression reflecting dopamine release (bottom) during periods of spontaneous locomotion and sucrose ingestion. Multiple clustering algorithms identify each compartment as a relatively homogenous unit, with stronger correlations within than across compartments. Left: pixels color coded by k-means clustering analysis. Middle: pixels color coded by CNMF clustering analysis. Right: pixel-by-pixel cross-correlation (Pearson correlation coefficient) for the same animal. (d) Anatomic reconstructions of γ4 DAN subpopulations from hemibrain connectome. Upper and lower axonal commissures that DANs use to innervate the lobes highlighted in red and green, respectively. (e) Presynaptic distribution of DANs following upper (red) and lower (green) commissure within the γ4 compartment. (f) Overlay of forward velocity (black) and activity of either the MB312B + γ4 DANs (top, which follow the upper commissure, red) or MB316B + γ4 DANs (bottom, which follow the lower commissure, green) expressing GCaMP6f during locomotion and sucrose ingestion (maroon bar). (g) Average MB312B (top, upper commissure, red) or MB316B (bottom, lower commissure, green) responses aligned to the beginning of sucrose ingestion (maroon bar). N for MB312B = 6 animals, 10 sucrose presentations. N for MB316B = 5 animals, 14 sucrose presentations. (h) Heat map of maximum ΔF/F0 for MB312B (top, upper commissure) or MB316B (bottom, lower commissure) during locomotion (middle) or sugar ingestion (right) overlaid on GCaMP fluorescence (left) highlights that MB312B + DANs are active during locomotion but not reward ingestion while MB316B + DANs display multiplexed activity during both contexts.
Extended Data Fig. 2 | See next page for caption.
Extended Data Fig. 2 | Multiplexed and correlated activity in γ4 DAN subsets. (a) MB312B + γ4 DANs (upper commissure) expressing GCaMP6f fluorescence (left) with functionally correlated and spatially adjacent pixels clustered into single ROIs by CNMF analysis (middle). Right: representative ROIs whose activity is plotted in (c). Similar results observed in N = 6 animals. (b) Same as in (a) but for MB316B + γ4 DANs (lower commissure). Right: representative ROIs plotted in (d). Similar results observed in N = 5 animals. (c) Net motion (top row, black) aligned to the activity in representative CNMF-generated-ROIs from (a) (2nd, 3rd, and 4th rows, shades of green), total MB312B + DAN GCaMP activity (5th row), the average CNMF-generated-ROI activity (bottom row), and the activity in all ROIs (heatmap) from a representative experiment in a MB312B > GCaMP6f individual. Maroon bars indicate period of sucrose ingestion. (d) As in (c) but for MB316B + γ4 DANs (upper commissure). Maroon bars indicate period of sucrose ingestion. (e) Cytoplasmic GCaMP6f activity in MB312B + γ4 DAN soma (shades of green) in representative examples during sugar ingestion (left) and spontaneous movement (right) aligned to forward velocity (top row, black). Different shades of green indicate different γ4 DAN soma recorded from the same animal. Maroon bars indicate period of sucrose ingestion. (f) As in (e) but recording from MB316 + γ4 DAN soma. (g) Motor-associated signals across individual γ4 DANs is highly correlated. Cytoplasmic GCaMP6f activity in MB312B + γ4 DAN soma measured with volumetric imaging during spontaneous bouts of locomotion. For three flies: top row shows a representative bout of forward velocity (black), middle row shows cytoplasmic GCaMP6s fluorescence (shades of green indicate different γ4 DAN soma), and bottom row is heatmap depicting the cross-correlation (Pearson correlation coefficient) between GCaMP6s signals in different γ4 DANs during spontaneous locomotion in a 5 min trial.
Extended Data Fig. 3 | See next page for caption.
Extended Data Fig. 3 | Variability of DAN - behavior correlations. (a) Top: average motion (black) ± 95% confidence interval (CI, obscured by average line) as animals initiate locomotion. Bottom: heat map of ΔF/Fo in γ DANs aligned to movement initiation. Rows (bouts) ordered by average γ2 (left) or γ4 (right) ΔF/Fo. Dashed lines indicate 20% of trials with highest or lowest average ΔF/Fo. N=53 animals, 1060 starts. (b) DAN activity and parameters of locomotion during spontaneous movement initiation in which γ2 and γ4 were most differentially active. Left: average γ2 ΔF/Fo (top), motion (2nd row), acceleration (3rd), forward velocity (4th), and |angular velocity| (bottom) ± 95% CI as animals initiated locomotion. 20% of bouts of movement initiation with highest (dark) and lowest (lighter) average γ2 ΔF/Fo as indicated by lines in (a). Right: as left but for bouts of movement initiation with highest (lighter) and lowest (dark) average γ4 ΔF/Fo. N=212 bouts. (c) As in (b) but for flies walking in non-odorized air in closed-loop. N=91 bouts. (d) γ2 (top) and γ4 (bottom) DAN activity vs different behavioral variables. N=1060 bouts. All Pearson correlation coefficients are either weak (|r| < 0.18) or not significant (no Bonferroni correction). (e) Comparisons of average DAN ΔF/Fo during the onset of locomotion. Pearson correlation coefficient (r) indicated where relationship is statistically significant (p < 0.00001, Bonferroni correction, see Supplementary Table 2). N=1060 starts. (f) Pearson correlation coefficient between change in DAN activity and net motion during bouts of movement initiation for flies walking in clean air in closed-loop. (g) Filters predicting DAN activity from forward velocity (top) or |angular velocity| (bottom) in open loop (OL, as in Fig. 1f, light lines) or closed-loop (CL) in clean air ± 95% CI. OL: N=66 animals, 119 5-minute trials. CL: N=20 animals, 32 5-minute trials. (h) Comparison of γ Kenyon cell activity during presentation of apple cider vinegar from indicated angles. Average ΔF/Fo (dark line) ± 95% CI aligned to odor onset. Right: average ΔF/Fo, during odor presentation from indicated angles. N=16 animals, 3 odor presentations per orientation (total 144 odor presentations). One-way ANOVA followed by Tukey’s multiple comparison test; no statistical significance observed.
Extended Data Fig. 4 | See next page for caption.
Extended Data Fig. 4 | Rapidly fluctuating network correlations between DANs and different behavioral variables. (a) Representative traces from two flies showing the net motion of each animal (top), overlay of γ DAN activity (colored) and either forward velocity (middle rows, black) or turning velocity (bottom rows, black) during a period of continuous locomotion (epoch shown by gray dashed box in top trace). DAN activity is normalized to minimum and maximum values during the selected bout of walking. (b) Average activity of γ DANs aligned to increases in forward velocity during bouts of continuous movement. N = 9,772 movements in 74 flies. (c) Average activity of γ DANs aligned to increases in turning velocity during bouts of continuous movement. N = 11,667 movements in 74 flies. (d) Left: overlay of DAN activity in different compartments during epochs designated in (a). Top: same epoch as left panel of (a). Bottom: same epoch as right panel of (a). Middle: running cross-correlation between pairs of γ DANs for the traces at left. Right: histograms of running correlation. (e) Histogram of running cross-correlation between pairs of γ DANs for all flies. Shuffled controls (random 1-20 sec temporal shift) in black. N = 74 animals, 178 5-minute trials. (f) Partial correlations between γ DANs to control for potential relationships that arise from common behavioral signals. N = 74 animals, 178 5-minute trials. ANOVA followed by Tukey’s multiple comparison test. Data labeled with different letters are significantly different from each other (p < 0.00001). (g) Proportion of the variance (R²) in net motion (left), forward velocity (middle), and |angular velocity| (right) explained by individual and all DANs. N = 66 animals, 119 5-minute trials. ANOVA followed by Tukey’s multiple comparison test. Data labeled with different letters are significantly different from each other (p < 0.0005). (h) No significant relationships are apparent between intercompartmental correlations and behavioral parameters. All Pearson correlation coefficients are either weak (|r|<0.1) or not significant, see Supplementary Table 2.
Extended Data Fig. 5 | See next page for caption.
Extended Data Fig. 5 | DAN-motor correlations vary across conditions. (a) Same analysis as in Fig. 3f but offset by 15 sec such that animals were walking only in clean air. N = 26 flies, 143 epochs. (b) Same analysis as in Fig. 3c but offset by 15 sec such that animals were walking in clean air. Fisher r-to-z transformation indicates no significant differences in correlation coefficients between upwind displacement and Δ|heading| in and out of odor (z = -1.32). N = 26 flies, 143 odor presentations. (c) Average γ DAN ΔF/Fo shows no correlations with an animal’s net displacement (left) or total scalar distance traveled (right) during odor presentations. Displacement was normalized (divided by) an individual’s average walking speed. Pearson correlation coefficient (r) indicated where relationship is statistically significant (p < 0.055, Bonferroni correction). N = 26 flies, 143 epochs. (d) ΔF/Fo of DANs in the γ2 vs γ4 compartments during odor presentation. Pearson coefficient (r) indicated where relationship is significant (p < 0.0001, see Supplementary Table 2). N = 26 flies, 143 odor presentations. (e) Same analysis as in Fig. 4d but offset by 15 sec such that flies were walking in clean air. N = 22 flies, 52 odor presentations. (f) Same analysis as in Fig. 4b but offset by 15 sec such that animals were walking in clean air. N = 22 flies, 52 odor presentations. (g) Filters predicting DAN activity from |heading| (top) or forward velocity (bottom) as animals walked in clean air, under low (lighter) or high (darker) airflow conditions. ± 95% confidence interval obscured by thickness of the data line. (h) Average γ DAN ΔF/Fo plotted as a function of upwind displacement (left), average Δ|heading| (middle), and average Δforward velocity (right) during odor presentation from Fig. 3f however here data from the low airflow context was subsampled such that the variance of the Δ|heading| was statistically equal to that of the high airflow context. Top: histogram showing distribution of behavioral variables. Pearson coefficient (r) indicates where relationship between subsampled variables is significant (p < 0.05 with Bonferroni, see Supplementary Table 2). Nlow airflow = 135 odor presentations. (i) Same as (h) with data from the high airflow context subsampled such that the variance of the Δforward velocity was statistically equal to that of the low airflow context. Nhigh airflow = 50 odor presentations.
Extended Data Fig. 6 | Analysis of dynamic DAN-motor correlations. (a) Average predicted γ2 odor responses generated by applying high airflow filters to low airflow behavioral data, plotted as a function of upwind displacement (left), average |heading| (middle), and average forward velocity (right) during odor presentation under low airflow conditions. Best fit line and Pearson coefficient (r) indicated where relationship is significant (p < 0.0001, Bonferroni correction, see Supplementary Table 2). N = 26 flies, 143 odor presentations. (b) As in (a) but predicted DAN odor responses generated from applying low airflow filters to high airflow behavioral data plotted against behavior under high airflow conditions. Best fit line and Pearson coefficient (r) indicated where relationship is significant (p < 0.0001, Bonferroni correction). N = 22 flies, 52 odor presentations. (c,d) Same as (a,b) except for γ3 DAN odor responses. N = 26 flies, 143 odor presentations (c), N = 22 flies, 52 odor presentations (d). (e) Average predicted DAN odor responses generated by applying filters derived as animals walk in clean airflow to the behavioral data plotted as a function of upwind displacement (left), average |heading| (middle), and average forward velocity (right) as animals walked in clean air, under low airflow. Best fit line and Pearson coefficient (r) indicated where relationship is significant (p < 0.01 with Bonferroni correction, see Supplementary Table 2). N = 26 flies, 143 odor presentations. (f) Same as (e) except under high airflow. N = 22 flies, 52 odor presentations.
Extended Data Fig. 7 | See next page for caption.
Extended Data Fig. 7 | Cross-correlation analysis between DAN activity and behavior during odor pursuit. (a) Organization of cross-correlation matrix comparing DAN activity to past, present, and future behavior, in and out of odor. (b) Auto-correlation of forward velocity (left) and |heading| (right) during the 10 sec prior to odor and the 10 sec of odor presentation. Colored points indicate statistically significant correlations (Pearson correlation coefficient, \( p < 0.05 \), no Bonferroni correction). \( N = 26 \) flies, 143 odor presentations. Note the correlation between an animal’s current and past or future forward velocity extend throughout the trial, while the correlation between an animal’s current and past or future heading lasts < 3 sec. (c,d) Cross-correlation matrix between forward velocity (left) or |heading| (right) and \( \gamma \) DAN activity during the 10 sec prior to odor onset and the 10 sec during odor presentation under low (c) and high (d) airflow conditions. Only relationships that are statistically significant by Pearson cross correlation (\( p < 0.05 \), no Bonferroni correction, see Supplementary Table 2) are shown in color indicated by green-magenta scale. \( N = 26 \) flies, 143 odor presentations (c), \( N = 22 \) flies, 52 odor presentations (d). (e,f) Same analysis as in (c,d) but over a 20-sec period during which only clean air is presented to the animal. Colored points indicate statistically significant correlations (\( p < 0.05 \), no Bonferroni correction). \( N = 26 \) flies, 143 odor presentations (e), \( N = 22 \) flies, 52 clean air epochs (f).
Extended Data Fig. 8 | Correlations between DAN activity and current and future behavior emerge during odor tracking. (a) Representative trial showing fictive 2D trajectory, forward velocity, |heading|, and $\gamma$ DAN activity in which the fly reorients and tracks upwind in response to apple cider vinegar in the low airflow context. Black trajectories indicate clean air, orange indicates time of odor presentation. Shaded areas represent epochs used in nested linear model (b). (b) A nested linear model predicting $\gamma$ DAN activity during the initial phase of odor presentation under low airflow conditions ($t = 1-4$ sec after odor onset) based on an animal’s average heading 10 sec prior to odor onset ($h_o$), initial $\Delta$forward velocity ($t = 1-4$ sec, $\Delta V_{1-4}$), initial $\Delta$|heading| ($t = 1-4$ sec, $\Delta h_{1-4}$), and future $\Delta$|heading| ($t = 7-10$ sec, $\Delta h_{7-10}$, a time window when behavioral autocorrelations are no longer relevant). Fraction of DAN variance explained as a function of which predictors were included in the model, for odor presentation (colored lines) and same temporal epochs offset 10 sec prior to the odor presentation (black) when the fly walked in clean air. F-test, $p < 0.05$ (*), $p < 0.01$ (**) with colored asterisk depicting significant differences in odor and black asterisk depicting significant differences in clean air. $N = 26$ flies, 143 odor presentations. (c) Same as (b) except under high flow conditions. $N = 22$ flies, 52 odor presentations.
Extended Data Fig. 9 | DAN-movement relationships during odor tracking in low airflow conditions are comparable in starved and fed animals. (a) Linear filters predicting DAN activity using forward velocity (Vf, left) or |heading| (|h|, right) in fed (colored lines) and starved (black dashed lines) flies during odor tracking over a 4 second window. N = 10 flies, 49-53 odor presentations. (b) Average predicted DAN activity generated by applying linear filters from fed animals plotted as a function of upwind displacement (left), average |heading| (middle), and average forward velocity (right) during odor in fed individuals. Best fit line and Pearson correlation coefficient (r) indicated where relationship is statistically significant (p < 0.0001 with Bonferroni correction, see Supplementary Table 2). (c,d) Average predicted γ2 DAN odor responses generated by applying filters derived from fed (c) or starved (d) animals to behavioral data from starved (c) or fed (d) animals, plotted as a function of average |heading| (left) or average forward velocity (right) during odor presentation. Best fit line and Pearson correlation coefficient (r) indicated where relationship is statistically significant (p < 0.0005 with Bonferroni correction, see Supplementary Table 2). N = 10 flies, 49 (fed) and 53 (starved) odor presentations. (e,f) Same as (c,d) but for γ3 DANs. (g,h) Same as (c,d) but for γ4 DANs.
Extended Data Fig. 10 | Optogenetic inhibition or excitation of PAM DANs bidirectionally influences upwind tracking behavior. (a) Average upwind velocity during odor presentations preceding optogenetic inhibition (–) and during odor presentations paired with optogenetic inhibition (+) for the indicated genotypes in starved animals. PAM DANs (mB042B driver) > GtACR1 (N = 63, top left), PAM DANs (mB196B driver) > GtACR1 (N = 49, top middle), PAM DANs mB042B-Gal4 parental controls (N = 33, top right), PPL DANs (mB504B driver) > GtACR1 (N = 30, bottom left), γ4 DANs (mB312B driver) > GtACR1 (N = 54, bottom middle), UAS-GtACR1 parental controls (N = 48, bottom right). Paired two-sided t-test with Bonferroni correction, p < 10^{-5} (**), see Supplementary Table 2. (b) Top: average upwind speed in odor presentations preceding optogenetic activation (–) and in odor presentations paired with optogenetic activation (+) in fed PAM DANs (mB042B driver) > CsChrimson flies (left) and UAS-CsChrimson parental controls (right). N = 60 paired cohorts of PAM > CsChrimson and parental control animals assayed together during a single experiment. Bottom: average upwind speed of fed animals in clean air preceding optogenetic activation (–) and with optogenetic activation (+) for fed PAM DANs (mB042B driver) > CsChrimson flies (left) and UAS-Chrimson parental controls (right). N = 44 paired cohorts of PAM > CsChrimson and parental control animals assayed together during a single experiment. Paired two-sided t-test with Bonferroni correction, p < 10^{-5} (**), see Supplementary Table 2.
Reporting Summary

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

Statistics

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

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

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection
Two-photon imaging data were collected using PrairieView 5.4 (Bruker). FicTrac (https://rjdmoore.net/fictrac/) was used to track and quantify parameters of locomotion. Custom built Arduino-based hardware was programmed with C and Python for closed loop and behavioral experiments.

Data analysis
MATLAB (R2018a), Python (3.4.7), ImageJ (ver: 2.0.0-rc-68/1.52g), neurPrint, navis, Neuronbridge, NoRMCorre were used for data analysis and statistical tests. Code for all analyses is available at a publicly accessible repository.

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

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:
- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

All behavioral and neural data at a publicly accessible repository.
Field-specific reporting

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

Life sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Life sciences study design

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

Sample size

No statistical tests were used to determine sample size. Preliminary experiments were used to assess variance and determine adequate sample sizes in advance of conducting the experiment. The N=10-20 flies used for experiments are comparable to the other functional and behavioral studies in Drosophila (e.g. Bracker, et al., 2013; Lin et al., 2014; Lewis, et al., 2015; Ichinose, et al., 2015; Owald, et al., 2015; Chang, et al., 2016).

Data exclusions

We did not exclude flies or data from any analysis, unless flies appeared unhealthy at the time of the experiment, did not walk smoothly on the ball due to issues with tethering, or failed to respond once to odor, suggesting that their antenna may have been damaged. We excluded trials in which FicTrac software failed to continuously track the fly’s movements, as in these instances the animal was not maintained in the closed-loop virtual reality configuration.

Replication

The main findings of the paper were confirmed by multiple complementary experiments. All repeats in our experiments were biological replicates (i.e. different groups of flies in behavioral experiments and different fly brains in imaging experiments) and were performed over the course of multiple days to assure reproducibility. The specific number of replicates for each experiment is detailed in the legend of each figure. Briefly, odor responses under low airflow conditions were performed in N=26 flies. Odor responses under high airflow conditions were performed in N=22 flies. Odor responses under low airflow conditions in starved/fed animals were performed in N=10 flies. Optogenetic experiments were performed in N>30 7-fly cohorts.

Randomization

Flies were group housed separated by genotype, and individual flies were selected randomly for functional or behavioral experiments.

Blinding

The experimenter was not blind to fly genotypes during data collection as this was not relevant to most imaging experiments and was not logistically possible. Real-time interpretation of data, however, was impossible and there were no subjective observational experiments where blinding would be necessary.

Reporting for specific materials, systems and methods

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

Materials & experimental systems

| n/a | Involved in the study |
|-----|----------------------|
| X   | Antibodies           |
| X   | Eukaryotic cell lines|
| X   | Palaeontology and archaeology|
| X   | Animals and other organisms|
| X   | Human research participants|
| X   | Clinical data        |
| X   | Dual use research of concern|

Methods

| n/a | Involved in the study |
|-----|----------------------|
| X   | ChIP-seq             |
| X   | Flow cytometry       |
| X   | MRI-based neuroimaging|

Animals and other organisms

Policy information about studies involving animals: ARRIVE guidelines recommended for reporting animal research

Laboratory animals

We used 1-7 day old female Drosophila melanogaster strains. All relevant genotypes with citations, strains, and sources are described in the Methods section.

Wild animals

This study did not involve wild animals.

Field-collected samples

This study did not involve samples collected in the field.

Ethics oversight

No ethical approval was required because we only used insects in this study.

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