

Specific Kinematics and Motor-Related Neurons for Aversive Chemotaxis in *Drosophila*

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Summary

Background: Chemotaxis, the ability to direct movements according to chemical cues in the environment, is important for the survival of most organisms. The vinegar fly, *Drosophila melanogaster*, displays robust olfactory aversion and attraction, but how these behaviors are executed via changes in locomotion remains poorly understood. In particular, it is not clear whether aversion and attraction bidirectionally modulate a shared circuit or recruit distinct circuits for execution.

Results: Using a quantitative behavioral assay, we determined that both aversive and attractive odorants modulate the initiation and direction of turns but display distinct kinematics. Using genetic tools to perturb these behaviors, we identified specific populations of neurons required for aversion, but not for attraction. Inactivation of these populations of cells affected the completion of aversive turns, but not their initiation. Optogenetic activation of the same populations of cells triggered a locomotion pattern resembling aversive turns. Perturbations in both the ellipsoid body and the ventral nerve cord, two regions involved in motor control, resulted in defects in aversion.

Conclusions: Aversive chemotaxis in vinegar flies triggers ethologically appropriate kinematics distinct from those of attractive chemotaxis and requires specific motor-related neurons.

Introduction

All motile organisms are guided by environmental chemical cues. *Escherichia coli* aggregates at an attractant source by coupling the frequency of random changes in orientation to changes in attractant concentration over time [1]. *Caenorhabditis elegans* uses an analogous mechanism but biases the reorientation events according to the direction of the gradient [2]. Still more sophisticated mechanisms, involving active sampling, specific orienting movements, and decision making, underlie chemotactic strategies in *Drosophila* and mammals [3–6]. These strategies rely on complex neural processing, but the circuits for their execution remain unknown.

Adult *Drosophila* represents an outstanding model for investigating the neural control of chemotaxis. Flies are similar to

vertebrates in the organization of their olfactory circuits [7]; their bipartite central nervous systems, comprising a brain and a ventral nerve cord (VNC)/spinal cord; and their complex repertoire of movements, made possible by articulated appendages. As a result, olfactory-motor transformations in flies are likely distinct from those in simpler organisms. Flies seek odorants indicative of food [8–11] and avoid many volatile chemicals, especially at high concentrations [10–13]. Their olfactory behavior has been used extensively to study olfactory coding [3, 8–10] and neural plasticity [14]. However, beyond the circuits for sensing and processing olfactory information, the neurons that execute these behaviors are largely unknown. The genetic tools in *Drosophila* enable access to specific neural components [15]. Furthermore, recent advances in the tracking of freely walking flies make it possible to screen for behavioral defects and allow dissection of the temporal details of chemotactic behavior [16, 17].

Chemotactic behaviors can be elicited by dedicated olfactory receptor neurons [12, 13]. Moreover, distinct input channels were reported to mediate aversion and attraction to vinegar at different concentrations [8]. To what extent is this sensory separation preserved as information flows through the nervous system? One possibility is that attraction and aversion converge onto one circuit that is bidirectionally regulated; alternatively, these opposing tasks may each recruit distinct circuits for execution. Here we combine quantitative analysis of behavior with genetic perturbations to compare aversive and attractive chemotaxes. Our results demonstrate that these tasks are characterized by distinct kinematics and that aversion engages specific neuronal populations for execution.

Results

Aversion and Attraction Both Enrich Turns, but the Kinematics Differ

To observe adult fly chemotaxis with high temporal resolution and throughput, we simultaneously tested 15–20 flies in a four-quadrant arena [8], recording the trajectories of each individual [16]. A typical experiment began with 2.5 min of air delivery to all four quadrants (“control period”) and continued for 5 min more after an odorant entered one quadrant (“odorant period”). As previously reported, control flies were robustly repelled by 10% acetic acid (Figure 1A), as well as a number of other compounds at high concentration (see Figure S1 available online; data not shown), and were attracted to apple cider vinegar (Figure 1B).

A variety of changes in the movements of individual flies might account for the changes in their spatial distribution during chemotaxis. Visual inspection suggested that flies approaching the aversive quadrant from an air quadrant turned back near the quadrant boundary, a behavior that could directly contribute to aversion (Figures 1D and S1; Movie S1). Conversely, turns toward an attractive odorant source were also observed, although at a lower frequency (Figure 1E; Movie S2). To describe turns under both aversive and attractive conditions, we approximated the contour lines of odorant concentrations by concentric circles centered on the bottom

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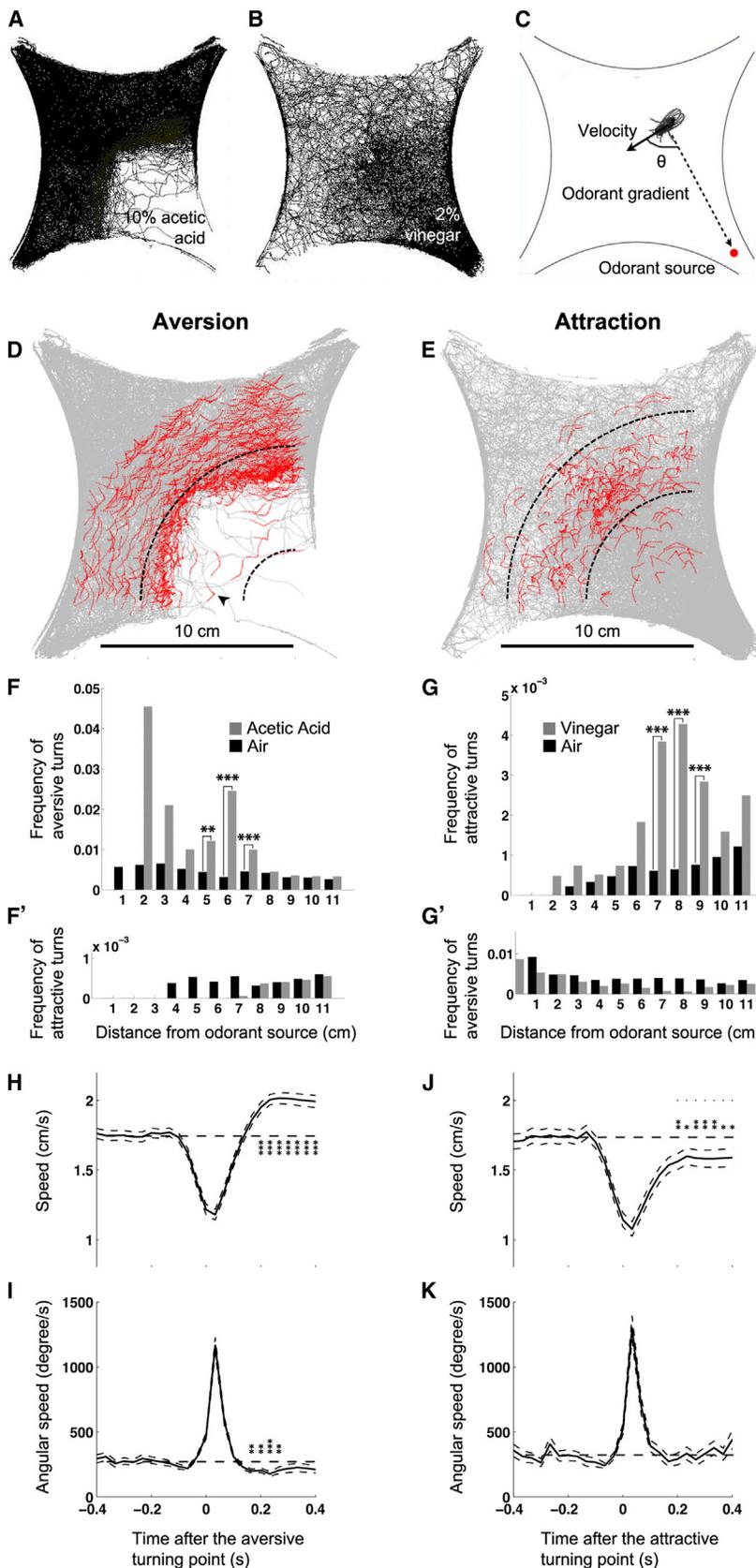


Figure 1. Aversive and Attractive Chemotaxes Enrich Turns but Differ in Their Kinematics

(A and B) Trajectories of *UAS-shi^{ts1}/+* flies with 10% acetic acid (A) or 2% vinegar (B) in the bottom right quadrant. Each black dot represents the appearance of one fly in one frame, and data were pooled over all periods, as specified in Figure S3.

(C) Definition of the orientation angle θ . Red dot represents the odorant source. Dashed arrow represents the approximated direction of the odorant gradient. Solid arrow represents the direction of the velocity.

(D and E) Turn segments (red) flanking the aversive turning points in response to 10% acetic acid (D) or attractive turning points in response to 2% vinegar (E). Arrowhead in (D) represents an exemplary turning point. Gray dots are the same as black dots in Figures 1A and 1B. The dashed lines specify the borders (2.5–7.5 cm in D and 5.5–9.5 cm in E) of areas for data collection in Figures 1H–1K.

(F–G) The relation between the distance from the odorant source and the frequency of aversive (F and G') or attractive (F' and G) turns in chemotactic aversion (F and F') and attraction (G and G'). Compared to the air control, the aversive odorant (acetic acid) condition enriches aversive turning points (F), whereas the attractive odorant (vinegar) condition enriches attractive turning points (G). For each bin, the number of aversive turns against the total number of tracked fly positions between the control and the odorant periods were compared with a chi-square test, and only significantly different bins were indicated.

(H–K) The temporal profiles of speed (H and J) and angular speed (I and K) around the aversive (H and I) or attractive (J and K) turning points. Solid traces represent the means of all individual data points over time; dashed traces represent mean \pm SEM. The comparisons were made between mean speed or angular speed before turning (–0.4 s to –0.2 s) and at every time point after turning (0.17 s to 0.4 s), with the turning point as time 0. Only significantly different time points were indicated. (Wilcoxon test; $n = 387$ for aversion, $n = 203$ for attraction).

Throughout the paper: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

right corner of the arena (“odorant source,” red dot in Figure 1C), based on direct visualization of airflow in the arena (Movie S3). By definition, the odorant gradient at any position

attractive turns were not enriched in aversion (Figures 1F' and S1), and aversive turns were not enriched in attraction (Figure 1G').

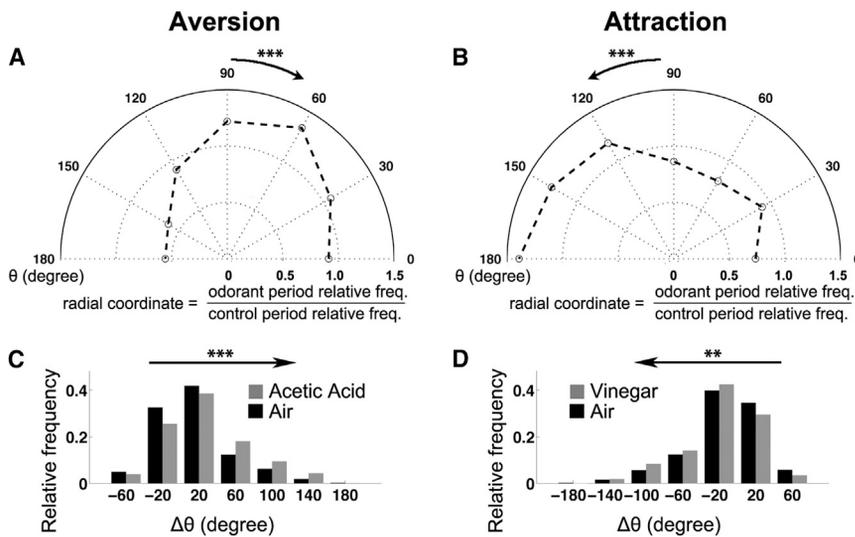


Figure 2. Odorant Gradients Modulate Turn Initiation and Direction

(A and B) Distribution of velocity orientations in the border area (see Figures 1D and 1E) upon turn initiation in aversion (A; $n = 2,954$ for the control period, $n = 2,276$ for the odorant period) or attraction (B; $n = 4,642$ for the control period, $n = 4,238$ for the odorant period) in the polar coordinate system. Radial coordinate represents the ratio of the relative frequency during the odorant period and the relative frequency during the control period. Angular coordinate represents θ (defined in Figure 1C; in degrees) upon turn initiation. The velocity orientation upon turn initiation is biased toward the odorant source in aversion (arrow pointing to the right in A) and away from the odorant source in attraction (arrow pointing to the left in B) compared to their control periods, respectively.

(C) Distribution of $\Delta\theta$ after turning, for events initiated when the flies were moving toward the odorant source (red dot in Figure 1C) during the control ($n = 1,368$) and aversive ($n = 1,322$)

odorant period. The arrow pointing to the right indicates that the whole distribution during the aversive period is biased to align the flies against the odorant gradient ($>0^\circ$).

(D) Distribution of $\Delta\theta$ after turning, for events initiated when the flies were moving away from the odorant source (red dot in Figure 1C) during the control ($n = 1,887$) and attractive ($n = 2,081$) odorant period. The arrow pointing to the left indicates that the whole distribution during the attractive period is biased to align the flies with the odorant gradient ($>0^\circ$).

All statistical significance in this figure was assessed by Wilcoxon test. The comparisons were made between the entire distributions of θ in the control period and the odorant period in (A) and (B) and the entire distributions of $\Delta\theta$ in the control period and the odorant period in (C) and (D). The genotype is *UAS-shi^{TS1}/+*, which also serves as a control for Figure 4.

To determine whether these turns represented quantitatively similar changes in behavior, we examined the fine structure of turning movements, based on the region where turns were enriched (between the dashed curves in Figures 1D, 1E, and S1). We calculated the average walking speed and angular speed of aversive and attractive turns aligned by the “turning points” (as defined above). As expected, turns were associated with a reduction in speed (Figures 1H, 1J, and S1) and a peak in angular speed (Figures 1I, 1K, and S1), the magnitudes and durations of which were almost identical between aversion and attraction. However, after aversive turns, speed increased significantly (Figures 1H and S1), and angular speed decreased significantly (Figures 1I and S1) compared to the baseline values before turns. Thus, after aversive turns, flies moved more quickly and followed straighter paths. Neither of these modulations was observed in attractive turns, and the speed after a turn even decreased (Figures 1J and 1K). The difference between aversive and attractive turning kinematics was not simply due to the asymmetric geometry in our definition of turning points (see Experimental Procedures), because aversive turns during the control period lacked the speed increase and angular speed decrease observed in aversive turns during the odorant period (Figure S1, compare to Figures 1H and 1I). Intriguingly, these odorant-free turns were qualitatively similar to attractive turns, implying that attraction, unlike aversion, might not induce specific types of turns. Finally, we note that, despite our selection of a region of interest, none of these conclusions were sensitive to the precise borders chosen, because those chosen included 45% of aversive turns (red segments in Figure 1D) and 64% of attractive turns (red segments in Figure 1E). As a result, adding or removing a few turn segments by shifting the borders did not significantly alter the ensemble statistics. Thus, both aversion and attraction increase the frequency of turning events as flies transition into or out of the odorant quadrant, respectively, but these turns are

quantitatively different, suggesting different underlying executive programs.

Odorants Modulate Turn Initiation and Direction

We envisioned two mechanisms that could contribute to the enrichment of aversive turns in aversion (or attractive turns in attraction). First, a fly could modulate the frequency of turn initiation depending on whether it is moving up or down an odorant gradient. Second, when a fly was turning, it could utilize the spatial odorant gradient to determine its turn direction. We tested whether either or both of these two mechanisms were used.

To test for modulation of the frequency of turn initiation, we identified all turns (see Experimental Procedures) and classified them by the direction of fly movement prior to turn initiation. To determine the direction of movement, we calculated the angle θ between each fly’s velocity and the odorant gradient ($0^\circ \leq \theta \leq 180^\circ$, Figure 1C). When $\theta = 0^\circ$, the fly was walking exactly toward the odorant source; at $\theta = 180^\circ$, the fly was moving exactly in the opposite direction. We found that, in the aversion assay, flies were more likely to be moving toward the odorant source (up the gradient) right before turns began ($\theta < 90^\circ$, Figures 2A and S2), while in the attraction assay, flies were more likely to be moving away from the source ($\theta > 90^\circ$, Figure 2B). Odorant concentration changes thus modulate the frequency of turn initiation in both attractive and aversive contexts.

We next tested whether the direction of induced turns were influenced by the direction of the odorant gradient. We generated the metric $\Delta\theta$ by subtracting the absolute value of θ at turn initiation from that at turn termination. A positive or negative $\Delta\theta$ means that the turn shifts the walking direction away from or toward the odorant source, respectively. For turns initiated when the flies were moving toward the aversive odorant source, we compared the $\Delta\theta$ distribution to that observed in

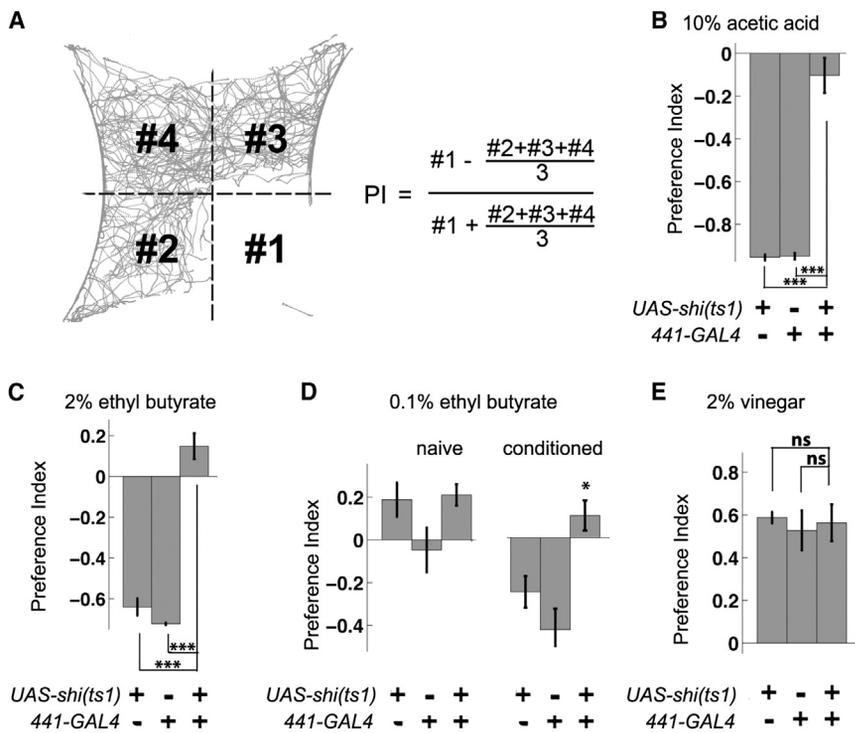


Figure 3. *441 > shi^{ts1}* Affects Aversive, but Not Attractive, Chemotaxis

(A) Definition of the preference index (PI). Each number represents all positions flies visit in a particular quadrant counted over a defined period of time. For aversion, the PI was calculated 2.5–5 min after the odorant onset, when the index reached a steady state (Figure S3). For attraction, the PI was calculated 1–3 min after the odorant onset to reduce the impact of habituation (Figure S3).

(B) Strong aversion to 10% acetic acid in control animals is almost completely abolished in *441 > shi^{ts1}* flies.

(C) *441 > shi^{ts1}* abolishes aversion to 2% ethyl butyrate.

(D) *441 > shi^{ts1}* abolishes learned aversion. To associate the naturally attractive or neutral odorant (0.1% ethyl butyrate) with a negative valence, we repetitively delivered it to the flies coupled with electric shocks right before testing in the arena.

(E) *441 > shi^{ts1}* does not affect the PI to 2% vinegar. Columns are for mean PIs of multiple independent runs; error bars represent SEM ($n \geq 4$). (B), (C), and (E): t test was performed with Holm-Bonferroni post hoc correction; (D): two-way ANOVA tests the significance of the interaction between genotype and conditioning.

the control period of the same experiment. Given that we selected the turns whose initial θ was smaller than 90° , the $\Delta\theta$ distribution was inherently biased to be above 0° . However, the frequency of positive $\Delta\theta$ was further increased under the aversive condition compared with controls, demonstrating that the local odorant gradient indeed modulated the flies' turn direction (Figures 2C and S2). An attractive odorant gradient modulated turn direction in a similar fashion, enriching negative $\Delta\theta$ in turns triggered while moving down the gradient (Figure 2D). Thus, odorant gradients modulate the direction of turning in both aversion and attraction.

In summary, turns evoked during chemotaxis involve both mechanisms proposed at the beginning of this section. When a fly walks toward an aversive odorant (or away from an attractive odorant), it is more likely to start turning, and the turn direction is biased away from the aversive odorant (or toward the attractive odorant).

A Screen for Neurons Necessary for Aversion

Aversion and attraction could reflect different dynamical states of the same motor control circuit or could engage distinct circuits for execution. To distinguish between these possibilities, we manipulated the underlying circuits. Under our experimental conditions, aversion was robust while attraction was sensitive to genetic background. We therefore screened for neurons necessary for aversion to 10% acetic acid. We chose 103 *GAL4* lines from an integrase swappable in vivo targeting element enhancer trap collection [18], each of which allows transgene expression in specific neuronal populations. These lines were selected for relatively sparse expression and against expression in olfactory receptor neurons or projection neurons. To impair synaptic output from targeted neurons, we expressed *UAS-shibire^{ts1}* (*UAS-shi^{ts1}*), which acutely abolishes synaptic transmission at the restrictive temperature used in our assays [19].

The metrics described above were data intensive and might not capture every aspect of chemotaxis, making them impractical for a screen. We therefore defined a preference index (PI) as a comprehensive chemotaxis indicator (Figure 3A). A PI of 1 means that the flies always remain within the odorant quadrant, a PI of 0 means that they spend as much time in the odorant quadrant as in the air quadrants, and a PI of -1 means that they never enter the odorant quadrant. We identified four lines that strongly impaired aversion (Figure S3) and characterized in detail two of these, *441-GAL4* and *918-GAL4*.

441-Targeted Silencing Affects Aversion, but Not Attraction

Expression of *UAS-shi^{ts1}* under the control of *441-GAL4* (*441 > shi^{ts1}*) almost completely abolished aversion to 10% acetic acid, as measured with the PI (Figure 3B). This line therefore allowed us to address whether the circuit elements necessary for acetic acid aversion are shared by other chemotactic behaviors. We found that *441 > shi^{ts1}* flies also abolished aversive responses to both 2% ethyl butyrate (Figure 3C) and 0.1% E2-hexenal (Figure S3). Flies can also be conditioned to avoid naturally attractive or neutral odorants through their association with electric shocks. Despite its distinct origin from innate aversion, learned aversion to 0.1% ethyl butyrate was also abolished by *441 > shi^{ts1}* expression (Figure 3D). *441+* cells are thus broadly required for olfactory aversion. Interestingly, the attractive response to vinegar odor as measured by PI was completely unaffected (Figure 3E). These results implied a separation of neural circuits for olfactory aversion and attraction.

441-GAL4+ Cells Regulate the Completion of Aversive Turning

We next examined the possible behavioral mechanisms underlying the abolition of aversion in *441 > shi^{ts1}* flies using

our kinematic metrics. Unlike control flies, which increased aversive turns in response to 10% acetic acid (Figures 1F and 4B), $441 > shi^{ts1}$ flies did not (Figure 4A). To understand how $441 > shi^{ts1}$ disrupts aversive turns, we analyzed turn initiation and direction. Intriguingly, while $441 > shi^{ts1}$ flies were still biased to initiate turning when moving toward the aversive odorant source, just as in controls (Figure 4C, compare with Figures 2A and 4D), they lost all directional modulation of $\Delta\theta$ (Figure 4E, compare to Figures 2C and 4F). $441 > shi^{ts1}$ flies thus still sensed the aversive odorant and initiated turns but failed to complete the late stages of the turning pattern necessary to appropriately reorient themselves.

If 441 - $GAL4$ labeled circuit components necessary for the execution of an aversion-specific motor program, artificial activation of these cells might trigger elements of this program. To test this, we expressed Channelrhodopsin2 with 441 - $GAL4$ ($441 > ChR2$), allowing $441+$ neurons to be activated by light [20], while incorporating a *norpA* mutation to block phototransduction. In these blind flies, angular speed peaked during blue light illumination (mimicking turning) and then dropped below baseline levels (mimicking turn suppression; Figure 4G, compare to Figure 4H). Moreover, this turn suppression coincided with a speed increase (Figure 4I, compare to Figure 4J). These kinematic changes are strikingly similar to those accompanying natural aversive turns (Figures 1H and 1I). Both resting and walking $441 > ChR2$ flies transitioned into fast walking after illumination (data not shown), indicating that the artificially induced locomotion overrode the flies' endogenous locomotion status. We note, however, that the light-induced pattern of neuronal activity was unlikely to completely mimic the natural pattern due to its synchrony and uniformity, which might account for the lack of an initial speed decrease during illumination. Alternatively, since $441+$ circuits are not necessary for turn initiation (Figures 4C and 4D), it is possible that the speed decrease at the beginning of turns is controlled by neurons upstream of those labeled by 441 - $GAL4$. Thus, 441 - $GAL4$ targets circuit components that are not only necessary for completion of aversive turns but also sufficient to induce aspects of kinematics characteristic of aversive turns.

The spatial distribution of $441 > shi^{ts1}$ flies in attraction appeared normal (Figure S4; also compare to Figure 1B), although the attractive turns were no longer enriched (Figure S4; also compare to Figure 1G) and the bias of turn initiation was changed (Figure S4; also compare to Figure 2B). Thus, aversion and attraction display both different turn kinematics and distinct dependence on turning. We infer that odorants may generate attraction through another mechanism.

441-GAL4 Targets Motor-Related Neural Structures

We sought to identify the $441+$ cells responsible for aversion using intersectional strategies. 441 - $GAL4$ labels glia and neurons in both the brain and the VNC (Figures 5A and 5A'). We first tested whether neuronal expression of shi^{ts1} was sufficient to cause the phenotype with a split $GAL4$ strategy [21]. We replaced the $GAL4$ in 441 - $GAL4$ with the $VP16$ activation domain (AD) and introduced a second transgene expressing the $GAL4$ DNA-binding domain (DBD) with a pan-neuronal promoter, *elav*. A functional $GAL4$ was therefore only reconstituted in $441+elav+$ cells when we combined 441 - $VP16AD$ and *elav*- $GAL4DBD$ to express shi^{ts1} . This manipulation still abolished aversion (Figure S5), indicating that neuronal expression of shi^{ts1} in $441+$ cells is responsible for the aversion deficit. To

assess the contributions of 441 - $GAL4$ expressions in the brain versus the VNC, we introduced a VNC-specific suppressor of $GAL4$, *tsh-GAL80* [22]. *tsh-GAL80* greatly reduced 441 - $GAL4$ expressions in both neurons and glia in the VNC, but not in the brain (Figures 5B and 5B'). In behavioral experiments, adding *tsh-GAL80* to $441 > shi^{ts1}$ flies restored the PI to about half of the PI observed in controls (Figure 5C), indicating that $441+tsh+$ neurons were necessary for aversion. These data also suggest either that *tsh-GAL80* was not strong enough to completely block 441 - $GAL4$ or that additional $441+tsh-$ neurons contribute to aversion.

In the brain, 441 - $GAL4$ has prominent expression in the ellipsoid body (EB, arrowhead in Figure 5A), which was reported to be necessary for olfactory aversion in a different assay [23]. To examine the role of these neurons, we generated two EB - $GAL80$ s using enhancer fragments from a large collection of enhancer- $GAL4$ fusions [24, 25] (Figure S5; data not shown). $R13C06$ - $GAL80$ effectively blocked 441 - $GAL4$ in the EB (Figure 5D, open arrowhead) and partially restored aversion in $441 > shi^{ts1}$ flies (Figure 5C). Similar results were obtained using an independent EB - $GAL80$ driven by a second enhancer, $R11F03$ (Figure 5C). These data indicate that inactivation of EB neurons also contributed to loss of aversion when all $441+$ neurons were inactivated. However, EB - $GAL4$ s with the same two enhancers did not affect aversion when expressing shi^{ts1} (Figure S5), suggesting that $441+$ EB neurons mediate aversive chemotaxis redundantly with other $441+$ neurons. Finally, $R13C06$ - $GAL80$ and *tsh-GAL80* together did not restore the PI beyond the level of single $GAL80$ (Figure 5C). This could be due to incomplete suppression of 441 - $GAL4$ in the EB or *tsh+* neurons by $GAL80$; alternatively, $441 > shi^{ts1}$ may disrupt a third aversion-related circuit not accounted for by either $GAL80$ line.

Given the implied importance of $441+$ VNC neurons, we directly tested their sufficiency to induce locomotion in decapitated $441 > ChR2$ flies [26]. Headless $441 > ChR2$ flies immediately started to swing their legs upon illumination (Figure S5; Movie S4), a behavioral change that was never observed in controls lacking 441 - $GAL4$ (Figure S5; Movie S5) or with a $GAL4$ labeling many motor neurons (data not shown). Doubling the intensity of light stimulation caused nearly half of the headless flies to transition from leg swinging to nondirectional displacements, jumps, or forward walking (Figure S5; Movie S6). Although these headless flies did not maintain steady walking, perhaps due to the absence of brain-mediated coordination, this result demonstrated that activation of $441+$ VNC neurons generated new locomotor patterns, even in sluggish, headless flies, reminiscent of the transition from resting to fast walking in intact flies.

We also attempted to narrow down the most likely VNC neurons involved in aversion using morphological analysis. $441 > mCD8$ - GFP only weakly labeled neuronal processes, and detailed studies of these neurons were confounded by glial labeling. We therefore expressed a strong dendritic marker (*DenMark*) with 441 - $GAL4$ and removed glia expression using *repo-GAL80*. To facilitate comparison between individual VNCs, we took multiple image stacks under the same conditions both with and without *tsh-GAL80*, registered them to a standard VNC [27], and averaged the expression intensity of *DenMark* within each genotype (Figures 5E₁-5F₂' and S5). *tsh-GAL80* suppressed $441 > DenMark$ expression in the abdominal ganglia (dashed boxes in Figures 5E₁-5E₂'; compare to Figures 5F₁-5F₂'), a region of the VNC that does not control locomotion. Outside the abdominal ganglia,

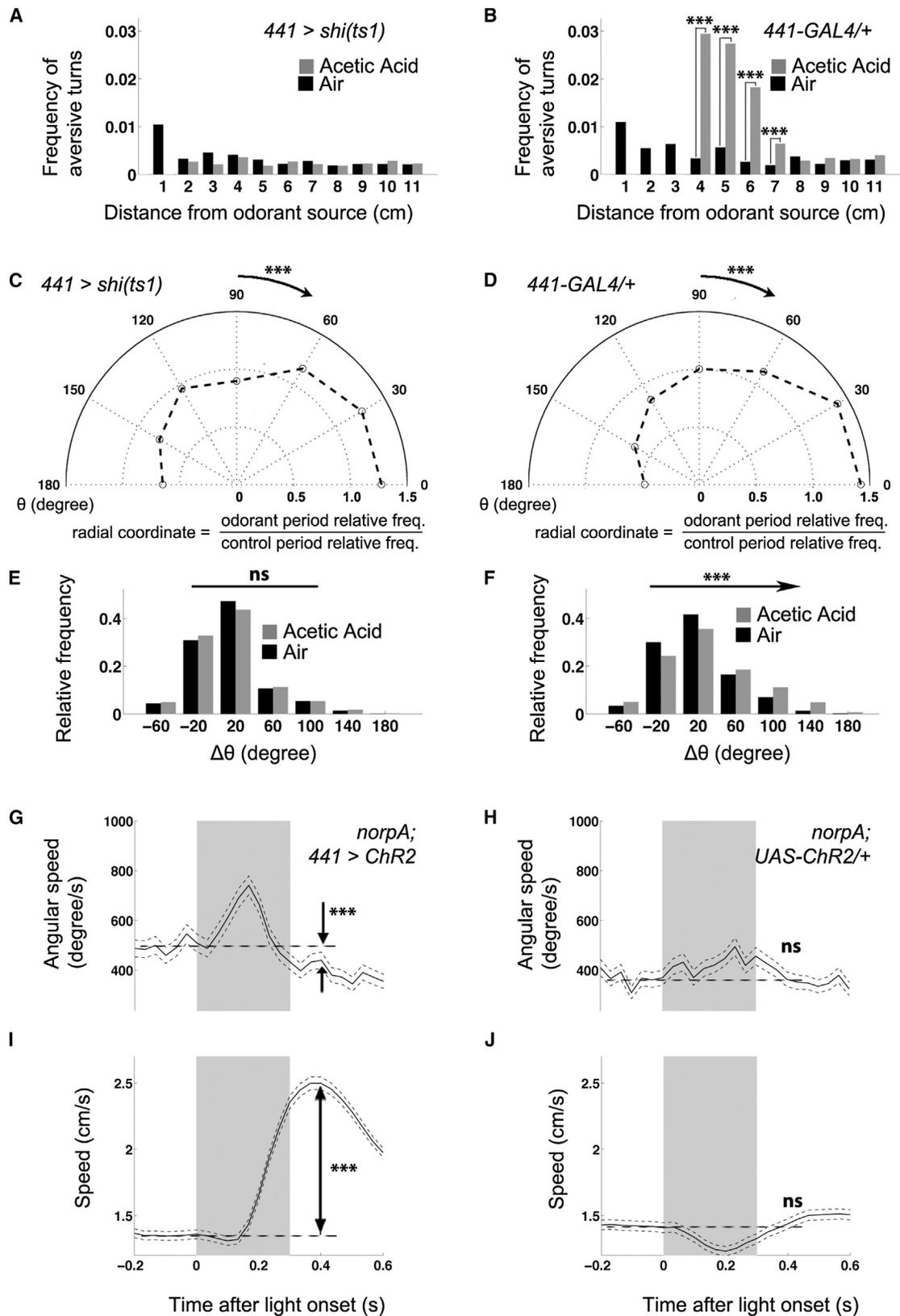


Figure 4. *441-GAL4* Defines Neurons for the Completion of Aversive Turns

(A and B) The enrichment of aversive turns in response to 10% acetic acid is abolished in *441 > shi^{ts1}* (A) compared to *441-GAL4* (B) and *UAS-shi^{ts1}/+* (Figure 1F) controls.

(C and D) The modulation of turn initiation, as measured by the distribution of θ upon turn initiation in the border area, still persists in *441 > shi^{ts1}* (C; n = 2,765 for the control period, n = 3,341 for the odorant period) compared to *441-GAL4/+* (D; n = 2,552 for the control period, n = 1,897 for the odorant period) and *UAS-shi^{ts1}/+* (Figure 2A) controls.

(legend continued on next page)

441 > *DenMark* expression was only suppressed by *tsh-GAL80* in neurites extending from ventromedial cell bodies (arrows in Figures 5E₁, 5E₂, and S5; compare to the dashed arrows in Figures 5F₁ and 5F₂) to beneath the dorsal surface of the VNC (arrows in Figures 5E₁' and 5E₂'; compare to the dashed arrows in Figures 5F₁' and 5F₂') and those along the midline within the VNC (arrowheads in Figures 5E₁', 5E₂', and S5; compare to the open arrowhead in Figures 5F₁' and 5F₂').

In summary, we identified two circuit element candidates, the EB and small subsets of VNC neurons, that are causally linked to aversive chemotaxis and are likely involved in motor control (see Discussion).

918-GAL4+ Neurons Are Also Required for Aversion and Overlap with 441+ VNC Neurons

The second line identified in our genetic screen (Figure S3), 918-GAL4, strongly supported the importance of the VNC neural elements defined by 441-GAL4 for aversion. 918 > *shi^{ts1}*, like 441 > *shi^{ts1}*, attenuated aversion, but not attraction (Figures 6A–6C), a deficit that was fully rescued by *tsh-GAL80* (Figure 6A). 918-GAL4 appeared to label the 441+ ventromedial neurons as well, based on the stereotypic locations of their cell bodies (arrows in Figure 6D; compare to those in Figures 5E₁–5E₁'). Labeling of these ventromedial neurons was also suppressed by *tsh-GAL80* (Figure 6E, dashed arrows). In the brain, *tsh-GAL80* only suppressed 918 expression in some local interneurons in the antennal lobes (Figure S6).

To test whether 918-GAL4 and 441-GAL4 label the same VNC neurons and, if so, whether these neurons play essential roles in aversion, we replaced the GAL4 in 441-GAL4 with GAL80 at exactly the same genomic locus [18]. Indeed, 441-GAL80 rescued the aversion deficit in 918 > *shi^{ts1}* flies (Figure 6A). We used *UAS-mCD8-GFP* to visualize the pattern of 918-GAL4 after 441-GAL80 suppression but did not find any apparent effect (data not shown). We reasoned that the intersectional effect might be sufficient to alter behavior but insufficient to cause a change of expression visualized using a stable marker like *mCD8-GFP*. To sensitize our expression analysis, we utilized *UAS-CL1-GFP*, an unstable GFP with a degradation tag. The ventromedial cell bodies were labeled in 918 > *CL1-GFP* VNCs (Figure 6F, arrows), and the reporter expression was markedly reduced by 441-GAL80 (Figure 6G, dashed arrows; Figure S6). The intersection between 441 and 918 thus confirmed their overlapping expression patterns and indicated that the neurons they label in common are necessary for aversion.

The ventromedial cell bodies and dorsal projection patterns are also characteristic of tyraminerpic/octopaminergic neurons, which express tyrosine decarboxylase 2 (*dTdc2*) and can be targeted by *dTdc2-GAL4* [28] (Figure S6). We therefore combined 441-GAL4 and *dTdc2-GAL4* in the same fly and found that two GAL4s in combination labeled as many neurons in the posterior cluster as *dTdc2-GAL4* alone and slightly

more than 441-GAL4 alone, confirming that the 441+*tsh*+ ventromedial neurons are also *dTdc2*+ (Figure S6). *dTdc2* > *shi^{ts1}* did not affect aversion (Figure S6), which could either be caused by redundancy similar to the case of *EB* > *shi^{ts1}* or imply the involvement of other 441+918+ VNC neurons in aversion.

Discussion

To our knowledge, this is the first direct demonstration that odorants modulate turn initiation and direction in freely walking insects. Moreover, aversive and attractive turns involve distinct kinematics. Intuitively, our quantitative analyses reveal that flies speed up and follow straighter trajectories after turning away from a noxious smell, which should shorten their exposure to potential harm. Such a strategy is not employed for attraction. Chemotaxis has been studied in tethered adult flies, paradigms in which mimicking the olfactory inputs a freely moving fly would encounter proved challenging. For example, in a “fly-on-the-ball” paradigm, aversion was not triggered, even using a strong repellent [4, 29]. In another study, flying flies responded symmetrically to aversive and attractive odors [30]. Our more naturalistic approach provided new insights into the relationship between aversive and attractive chemotaxes.

In bacteria [1], aversion and attraction are achieved through bidirectional modulation of the same mechanism. Similarly, in *C. elegans*, aversion and attraction are thought to utilize a push-pull mechanism on one set of antagonizing command neurons [2]. Our genetic inactivation experiments suggest that, in flies, aversion is executed through specific neurons distinct from attraction. We identified two candidate circuit components, the EB and a subset of the VNC neurons, that appear redundantly necessary for aversive chemotaxis (Figure 6H). The EB is part of the central complex, defects in which are associated with uncoordinated walking [31]. In grasshoppers and cockroaches, activating central complex neurons induces specific kinematics [32, 33]. In the VNC, *dTdc2*+ neurons are prominent candidates for mediating aversion, although other 441+918+ neurons might be involved. These neurons are homologous to dorsal or ventral unpaired median neurons in other insects because they are octopaminergic and show similar projection patterns [34]. The activity in these neurons is correlated with specific aspects of locomotion in locusts, crickets, and moths [34]. Given that 441 > *shi^{ts1}* flies display defects in aversive turn completion, but not initiation, we postulate that our genetic manipulation does not interfere with the perception, processing, decision-making, or even initiation steps of aversive chemotaxis but rather the execution of motor programs specifically necessary for this behavior. Our discoveries bridge the extensive investigation of olfactory processing in insects such as honeybees and moths [35] with studies focused on motor control mechanisms in species such as cockroaches and stick insects [36].

(E and F) Distribution of $\Delta\theta$ after turning, for events initiated when 441 > *shi^{ts1}* flies (E) were moving toward the odorant source (red dot in Figure 1C) during the control (n = 1,142) and aversive (n = 1,631) odorant periods. The modulation of direction is lost compared to 441-GAL4/+ (F) and *UAS-shi^{ts1}*/+ (Figure 2C) controls.

(G and H) Angular speed peaks during illumination and then falls below baseline level in 441 > *ChR2* flies (G; n = 1,016 before illumination, n = 989 after illumination; n values are the same for speed), but not in control flies (H; n = 890 before illumination, n = 852 after illumination; n values are the same for speed). (I and J) Speed increases after illumination in 441 > *ChR2* flies (I), but not in control flies (J). Gray bar represents the light-on period.

(A) and (B) show the same comparison as in the corresponding panels in Figures 1F and 1G. (C)–(F) show the same comparison as in the corresponding panels in Figures 2A–2D. (G)–(J) show Wilcoxon tests between the average angular speeds (G and H) or speeds (I and J) before (–0.2 s to –0.1 s) and after (0.4 s) illumination, with the light onset being time 0.

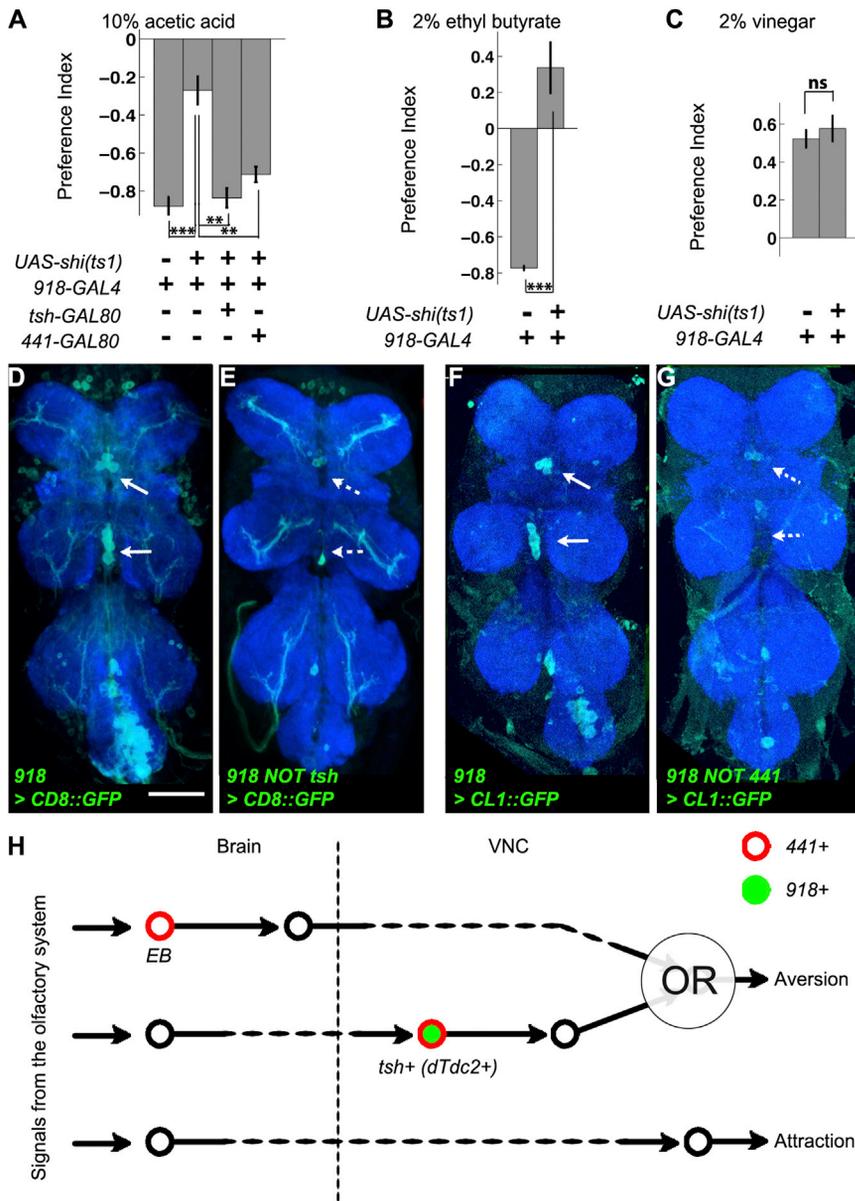


Figure 6. Analyses of *918-GAL4* Support the Role of Specific *441+tsh+* Neurons in Aversion

(A) *918 > shi^{ts1}* attenuates aversion to 10% acetic acid, and this attenuation is suppressed by *tsh-GAL80* or *441-GAL80*.

(B) *918 > shi^{ts1}* abolishes aversion to 2% ethyl butyrate.

(C) *918 > shi^{ts1}* does not affect attraction to 2% vinegar.

Columns in (A–C) are mean PIs of multiple independent runs, and error bars represent SEM ($n \geq 3$); t test was performed with Holm-Bonferroni post hoc correction.

(D) *918-GAL4* expression in the VNC is visualized with a membrane-tagged *GFP*. Arrows point to the ventromedial cell bodies similar to those in Figures 5E₁–5E₁'.

(E) *tsh-GAL80* suppresses VNC expression of *918-GAL4*, including the ventromedial class, as indicated by the dashed arrows.

(F) *918-GAL4* expression is visualized with a destabilized *GFP*. Arrows point to the ventromedial cell bodies.

(G) *441-GAL80* suppressed the expression of *918-GAL4* in the ventromedial class, as indicated by the dashed arrows. Scale bars represent 50 μ m.

(H) A schematic summary of the functionality of neuronal populations underlying aversive chemotaxis inferred from our genetic analysis. Each circle and the line attached to it represent a neuronal population and the direction of information flow; dashed lines represent potential intermediate layers of relay neurons. The “OR” logic gate near the end of the aversive circuit reflects the redundant roles of the pathways mediated by EB and *dTdc2+* VNC neurons.

Due to the asymmetry of the odorant contours, which curve toward the odorant source, a direct comparison between the frequencies of aversive and attractive turns was confounded by the difference in control frequencies (note that Figures 1F and 1G' have a different y axis scale than Figures 1F' and 1G). That is because a turn toward the odorant source does not constitute an attractive turn unless it is more curved than the local odorant contour.

For the temporal profiles of turns, speeds and angular speeds (in the arena-based coordinate system) were collected from 24 flanking frames (0.8 s) in each trajectory, aligned by the turning points, and averaged. For the analysis of turn initiation and direction, we partitioned each trajectory into forward walking and turning segments. Any data point with an angular speed above 400°/s was annotated as turning, and the rest were annotated as forward walking. The threshold was set based on the basal angular speed in Figures 1I and 1K. The angular speed was the absolute value of the orientation difference between two adjacent frames divided by the frame duration (1/30 s). The distribution was skewed such that the mean angular speed was near 400°/s, but the median was only 130°/s. But even for 400°/s, the orientation difference between two adjacent frames was just 400/30 = 13.3°. Thus, the apparently high baseline angular speed reflects the fact that the flies never follow truly straight trajectories. The first frame of one continuous turning segment was defined as its initiation, and the last frame was defined as its termination.

Experimental Procedures

Chemotaxis Paradigm

The four-quadrant behavioral arena was 16.5 cm × 16.5 cm and 1 cm deep [8]. It was placed inside a 33°C box in complete darkness. The airflow was filtered and saturated with water, entered each quadrant at a rate of 40 ml/min, and left through the central hole in the arena floor. One branch of airflow was controlled by solenoid valves through the LabVIEW software (National Instruments), so that for each trial air passed directly into one quadrant for 2.5 min and then was switched to bubble through 5 ml of water (for vinegar and acetic acid) or paraffin oil (for all the other odorants tested) containing an odorant with a specified concentration. The odorant source was replenished for each experiment.

Behavioral Data Analysis

All analyses were performed with MATLAB (MathWorks). For aversion and attraction, all analyses were done with data from the time windows described in Figure S3. The only exception was learned aversion, where the first 2.5 min were analyzed to avoid memory extinction. Except for PI calculation, the data points within 0.5 cm of the arena walls were excluded to avoid the confounding effects of arena edges.

Supplemental Information

Supplemental Information includes Supplemental Experimental Procedures, six figures, and six movies and can be found with this article online at <http://dx.doi.org/10.1016/j.cub.2013.05.008>.

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