Role of the Subesophageal Zone in Sensorimotor Control of Orientation in *Drosophila* Larva

Graphical Abstract

Highlights

- A loss-of-function screen reveals a new set of neurons controlling larval chemotaxis
- These neurons form a premotor center located in the subesophageal zone (SEZ)
- The SEZ contributes to action selection in response to multiple sensory modalities
- The neurons identified in the SEZ are sufficient to initiate reorientation maneuvers

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In Brief

*Drosophila* larvae chemotax by alternating runs with directed turns. To map the sensorimotor pathway directing this process, Tastekin et al. combine a genetic screen with high-resolution behavioral analysis and acute functional perturbations. They find that the subesophageal zone plays a critical role in the sensory control of orientation behaviors.
Role of the Subesophageal Zone in Sensorimotor Control of Orientation in *Drosophila* Larva

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SUMMARY

Chemotaxis is a powerful paradigm to investigate how nervous systems represent and integrate changes in sensory signals to direct navigational decisions. In the *Drosophila melanogaster* larva, chemotaxis mainly consists of an alternation of distinct behavioral modes: runs and directed turns. During locomotion, turns are triggered by the integration of temporal changes in the intensity of the stimulus. Upon completion of a turning maneuver, the direction of motion is typically realigned toward the odor gradient. While the anatomy of the peripheral olfactory circuits and the locomotor system of the larva are reasonably well documented, the neural circuits connecting the sensory neurons to the motor neurons remain unknown. We combined a loss-of-function behavioral screen with optogenetics-based clonal gain-of-function manipulations to identify neurons that are necessary and sufficient for the initiation of reorientation maneuvers in odor gradients. Our results indicate that a small subset of neurons residing in the subesophageal zone controls the rate of transition from runs to turns—a premotor function compatible with previous observations made in other invertebrates. After having shown that this function pertains to the processing of inputs from different sensory modalities (olfaction, vision, thermosensation), we conclude that the subesophageal zone operates as a general premotor center that regulates the selection of different behavioral programs based on the integration of sensory stimuli. The present analysis paves the way for a systematic investigation of the neural computations underlying action selection in a miniature brain amenable to genetic manipulations.

INTRODUCTION

When exposed to an attractive odor, *Drosophila* larvae quickly locate the spatial position of its source [1]. To navigate in odor gradients, the larva relies on active sensing by directing motion based on the spatiotemporal comparison of changes in the olfactory stimulus. Upon sensory integration, the perceived time course of the odor concentration controls when and where the animal turns [2, 3](Figure 1A). Runs are elongated when the concentration increases in time (up-gradient motion), and turns are facilitated when the concentration decreases in time (down-gradient motion). Prior to the initiation of a turn, the larva scans the local odor gradient through lateral head casts. The majority of turns are implemented toward the direction of higher odor concentrations, resulting in gradient ascent.

Odors are represented in the activation pattern of 21 peripheral olfactory sensory neurons (OSNs), each expressing one (or occasionally two) distinct odorant receptor(s) besides the ubiquitous co-receptor Orco [4–6]. The OSN activity is then transformed by the local interneurons of the larval antennal lobe into a pattern of activity of the projection neurons (PNs) [7, 8], which, in turn, excites Kenyon cells in the mushroom bodies (MBs) and downstream partners in the lateral horn (LH) [9, 10]. In contrast with our understanding of the peripheral encoding of odors and the behavioral principles underlying larval chemotaxis, little is known about the circuits that convert olfactory stimuli into sequences of behavior.

Recent efforts to map the sensorimotor pathway controlling innate chemotaxis have identified a class of *odd*-expressing neurons in the larval brain [11]. These neurons—called Odd neurons—are thought to adjust the sensitivity of the olfactory system to changes in odor concentrations detected during chemotaxis. Constitutive impairment of the function of the Odd neurons reduces the efficiency of gradient ascent. Odd neurons receive inputs from PNs and Kenyon cells, but their downstream targets remain unknown. More generally, how the extrinsic neurons of the MBs and the LH connect to the motor system in the ventral nerve cord (VNC) remains elusive. To identify new circuit elements bridging the LH and MBs to the VNC, we followed an unbiased loss-of-function screen on a large collection of GAL4 enhancer trap lines. This screen pinpointed the existence of a group of neurons in the subesophageal zone with a function necessary and sufficient to transform time-varying sensory signals into an organized sequence of motor responses.
RESULTS

We conducted a genetic screen of over 1,100 P[GAL4] enhancer trap lines (NP-GAL4) provided by the Drosophila Genetic Resource Center (DGRC) [12]. Using the GAL4/UAS expression system, each GAL4 line drove the expression of the tetanus toxin light chain (TNT, insertion “E”) to inhibit synaptic transmission in a defined subset of neurons [13]. By means of this strategy, we silenced subsets of neurons in different regions of the CNS and subsequently examined the existence of loss-of-function defects in the timing and the direction of turns. Accordingly, we devised a high-throughput assay consisting of an agarose-coated Petri dish with a single odor source (1-hexanol, Figure 1B, bottom panel). We quantified the ability of a group of 15 larvae to accumulate at the peak of the odor gradient by monitoring the average distance of individual animals (<d>) with respect to the odor source (Figure 1C). In a dish devoid of any odor source, we assessed the existence of locomotor defects by measuring the dispersal of larvae from the start position (Figure 1B, top panel). As outlined in Figure 1D, the results of the two assays were combined to classify the loss-of-function phenotypes into four groups: normal locomotion and chemotaxis (49%), locomotor defects only (10%), locomotor and chemotactic defects (19%), and chemotactic defects only (22%).

We examined the expression pattern of the lines displaying only a chemotactic defect and selected those showing a...
relatively sparse neural pattern that excluded peripheral and secondary olfactory neurons. The chemotactic ability of each line crossed to TNT was assayed in a high-resolution chemotaxis assay [2, 14, 15] by quantifying the percentage of time individual larvae spent in the vicinity of the source (Figure 1E; Figure S1; Table S1). We observed that control larvae (w−/− and w−/− × UAS-TNTE) accumulated at the source, whereas anosmic larvae lacking the odorant co-receptor Orco did not stay in the vicinity of the source. Using the Orco−/− null mutant and wild-type (w−/−) as references, the loss-of-function phenotypes of the selected NP-GAL4 lines ranged from a complete impairment in chemotaxis to a mild impairment (Figure 1E). The GAL4 lines associated with a strong chemotactic defect displayed a broad expression pattern encompassing hundreds of neurons in both the central brain and the VNC (e.g., NP2351 in Figure 1F, left panel). Conversely, GAL4 lines with a weak chemotactic phenotype typically showed narrow expression patterns restricted to less than a dozen neurons (e.g., NP1613 in Figure 1F, right panel). We concentrated on the line NP4820-GAL4 as it combined a pronounced phenotype with a relatively sparse neural coverage that could be reduced through intersectional strategies [13] (Figure 1F, middle panel).

Participation of the NP4820-Labeled Neurons in the Control of Run-to-Turn Transitions

The NP4820-GAL4 line covers a heterogeneous set of neurons in the brain lobes (BLs), the subesophageal zone (SEZ), and the VNC (Figure 2A). We adopted a statistical approach to register single cells in individual brain hemispheres and compared them across preparations (Table S2). Within the BL, two groups of neurons consistently showed strong labeling: anterior dorsolateral (ADL) and Kenyon cells (KCs). The labeled neurons included neither the OSNs nor the interneurons and PNs of the antennal lobe. To characterize the nature of the sensorimotor defects underlying the loss-of-function phenotype of NP4820-TNTE (NP4820×UAS-TNTE), we tested the ability of single larva to chemotax toward a single source of 1-hexanol—an odorant eliciting activity in at least 8 out of the 21 larval OSNs (broad activation pattern) [3]. Positive controls matching the loss-of-function experiments (w−/− × UAS-TNTE) displayed zigzaging movements under the source (Figure 2B, left panel). Upon overshooting the position of the source, most runs were quickly terminated by a turn toward the source [2] (Figure 2B, left panel; Figure S1B, bottom panel). The topology of the trajectory of NP4820-TNTE was distinct from the controls: runs were generally much longer than those of positive controls, leading to a wider spatial dispersal of larvae.
In addition to 1-hexanol, we also tested chemotaxis in response to a second odor, ethyl butyrate, which activates only three out of the 21 OSNs (narrow activation pattern) [5]. We observed no dependence between the broadness of the peripheral activation pattern of the OSNs and the strength of the phenotype. We found that NP4820>TNTE larvae spent significantly less time under the odor source than the controls. In addition, NP4820>TNTE larvae showed a significant reduction in turn frequency (Figure 2C) and a slightly lower speed than the controls (Figure S1C). Although the time spent under the odor zone is dependent on the locomotion dynamics, the small differences observed in average speeds could not account for the significant decrease in the time spent under the odor zone. The tendency of larvae to maintain their ongoing direction of motion was measured through the persistence length of their trajectories (see Experimental Procedures). Using this metrics, we concluded that the trajectories of NP4820>TNTE were straighter than those of the control genotypes. In spite of the lengthened reaction time induced by the loss-of-function manipulation, larvae were still capable of reorienting toward the source, which demonstrates the ability of NP4820>TNTE larvae to perceive the odor gradient (Figure 2B).

A more detailed inspection of the sequences of postures adopted by NP4820>TNTE larvae and controls showed that the loss of function alters the stereotypic nature of transitions between runs, head casts, and turns (Figure 3A, left panel). After an elongated run, NP4820>TNTE larvae typically stopped and moved backward for several seconds before they initiated a turn—a behavioral routine that was not observed in wild-type larvae (Figure 3A, right panel). Next, we investigated the ability of loss-of-function larvae to implement their turns during down-gradient runs and to turn toward the odor gradient. This ability of NP4820>TNTE larvae to perceive the odor but fail to integrate concentration...
changes as effectively as wild-type. Finally, we examined the stereotypy in reorientation maneuvers. Prior to the execution of a turn, the head casting dynamics was reduced in NP4820>TNTE compared to wild-type controls (Figure 3C). This change was coupled with a decrease in the strength of the olfactory signal preceding a turn (data not shown). We conclude that NP4820-labeled neurons are involved in the timing of transitions from runs to turns and that they are necessary to organize turning maneuvers.

**Refined Mapping of the Loss-of-Function Phenotype onto the Subesophageal Zone**

To exclude that the loss-of-function phenotype stemmed from a defect in the motor circuits of the VNC, we applied an intersectional strategy [13] to specifically silence neural activity in the brain lobes and the subesophageal zone (Figure 4A). First, the NP4820>TNTE transgenes were combined with teashirt-GAL80 (tsh-GAL80) to prevent expression of TNT in the thoracic and abdominal ganglia of the VNC [16] (Figure 4A, green box, and Figure 4C). The chemotactic defects were conserved even upon repression of TNT expression exclusively in the VNC (Figures 4B–4D). NP4820,tsh-GAL80;UAS-mCD8::GFP larvae displayed significant variability in cellular coverage across individual VNCs. Very few cells were unaffected by the expression of GAL80 in the VNC, and none of them were consistently found in all preparations. The contribution of residual cells from the VNC could be ruled out based on two additional lines of evidence: (1) tsh-GAL80 robustly suppressed expression of GAL4 in the VNC of second-instar larvae (Figure S2A). The absence of TNT expression in the VNC did not abolish the loss-of-function phenotype at this stage (Figure S2B). (2) As incomplete suppression of GAL80 expression in NP4820,tsh-GAL80;UASTNTE might lead to minimal expression of TNT, we used a flippase-based strategy to restrict expression of TNT to the neurons found in the VNC (Figure S2C). Silencing any residual neurons in the VNC did not induce a loss-of-function phenotype (Figure S2D). These results bar the possibility that the loss-of-function phenotype is due to a locomotor impairment originating from the interneurons and/or a motor circuit comprised in the VNC [17].

To further dissect the contribution of the neurons labeled by NP4820, we combined NP4820>TNTE with Cha3.3-GAL80 [18] (Figures 4B–4D). The addition of Cha3.3-GAL80 reproducibly suppressed the expression of GAL4 in two regions of the brain, the subesophageal zone (cellular coverage reduced from 16.1 ± 3.3 to 3.3 ± 1.6 neurons) and the ADL group (coverage reduced from 8.9 ± 1.9 to 0.5 ± 1.2 neurons, Figures S3A and S3B), but it did not affect the labeling of KCs (Figures S3A and S3B). In contrast with the tsh-GAL80 manipulations, larvae with restored function in Cha3.3-positive neurons did not show a strong chemotactic impairment (Figure 4D). To test the contribution of neurons of the ADL group (Figure 2A; red box in Figure 4A), we took advantage of a GAL4 driver line that specifically covers this population of neurons: R29F12 [19] (Figure 4Cv). Upon silencing of the neurons labeled by R29F12, we observed no chemotactic defect (Figure 4D), which strongly suggests that the ADL group is not responsible for the loss-of-function phenotype induced by NP4820. Next, we considered the contribution of the MBs by functionally restoring embryonic-born and larval-born KCs with the MB247-GAL80 construct [20]. Expression of TNT in all NP4820-positive cells, except for the KCs covered by MB247-GAL80, did not abolish the loss-of-function phenotype (Figures S3C and S3D). Finally, to rule out the possibility that the loss-of-function phenotype was due to a developmental defect, we conducted a temporal control of the expression of TNT with a temperature-sensitive isoform of GAL80 (GAL80(TS)) [21] (Figure S3E). In summary, we used a combination of negative intersectional manipulations described in Figure 4A to map the neurons underlying the loss-of-function phenotype of NP4820 onto approximately 12 cells located in the subesophageal zone.

**Generalization of the Chemotactic Phenotype to Other Sensory Behaviors**

We asked whether the NP4820-labeled neurons had a function pertaining to other types of sensory-driven behaviors. To address this question, we examined the orientation behavior of NP4820>TNTE larvae during phototaxis and thermotaxis [22–24]. For most of its development, the Drosophila melanogaster larva displays strong light avoidance. We elicited photophobic responses in a controlled light well—a landscape where the absence of light at the center of the arena represented a zone of “comfort” and the edges produced avoidance responses (Figure 5A).

Single larvae were introduced at the center of the light well. For the positive controls (w/+/ x UAS-TNTE), foraging away from the dark center quickly led to aversive responses that re-oriented the larva toward the center (Figure 5A, left panel). Consistent with the chemotactic phenotype, the response of NP4820>TNTE to an increase in light intensity was delayed (Figure 5A, right panel). The amount of time spent by NP4820>TNTE larvae in the region of low light intensities was significantly reduced compared to controls, as was the frequency of turning (Figure 5B). Besides lengthening their runs, NP4820 loss-of-function larvae persisted in a given direction of motion longer than controls (Figure 5B). Next, we tested the ability of NP4820>TNTE larvae to orient in a temperature gradient. In a stable linear gradient ranging from 20°C to 36°C, we monitored individual larvae introduced at the gradient’s center. Wild-type larvae showed a strong preference for the region with temperatures ranging from 23°C to 25°C (Figures 5C and 5D). In contrast, NP4820>TNTE larvae turned less frequently and dispersed more widely, which decreased their ability to locate the region of preferred temperatures. We then asked whether the loss-of-function phenotype observed for phototaxis and thermotaxis originated from the functional impairment of the subesophageal zone. By using Cha3.3-GAL80, we rescued the subesophageal zone and ADL function in NP4820-TNTE larvae and restored orientation responses to light and temperature (Figure S4). In addition, silencing the ADL group labeled by the R29F12 driver line did not produce a defect in phototaxis and thermotaxis (Figure S4). Together, these results indicate that the subesophageal zone hosts a group of neurons that are necessary for organizing transitions between orientation responses elicited by multimodal sensory stimuli.

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A close-up view of the SEZ region is shown for NP4820,tsh-GAL80>UAS-mCD8::GFP (ii) to be compared with NP4820,Cha3.3-GAL80>UAS-mCD8::GFP (iii). (iv and v) ADL neurons in NP4820 (iv) are also covered by R29F12 (v). The expression of GFP in ADL neurons is also abolished by Cha3.3-GAL80 (see also Figure S3). Green: anti-GFP, magenta: anti-nc82 combined with anti-synapsin.

(D) Quantification of chemotactic behavior of R29F12>UAS-TNTE and NP4820>TNTE upon GAL80 intersectional repression. Percentage of time spent in the odor zone (left, one-way ANOVA followed by post hoc t test with Bonferroni correction at p < 0.05). Error bars denote SEM. Turn frequency (middle, one-way ANOVA followed by post hoc t test with Bonferroni correction at p < 0.05). Error bars, SEM. Persistence length (right, Kruskal-Wallis followed by Wilcoxon test with Bonferroni correction at p < 0.05). In the box plots, the median is indicated by the black horizontal line. Box boundaries represent first and third quartiles; whiskers are 1.5 interquartile range; outliers are indicated by dots. n = 14–21 trajectories.

A Subset of Neurons Included in the Subesophageal Zone Is Sufficient to Initiate Reorientation Maneuvers

To test whether the NP4820-positive neurons in the subesophageal zone are sufficient to elicit transitions between runs and turns, we exploited optogenetics to implement acute gain-of-function perturbations with light. By using CsChrimson, an optical actuator with excitation wavelength shifted to the deep red range to which adult flies and larvae are largely insensitive [26].
we found that brief red light flashes evoked reliable head casts in NP4820-UAS-CsChrimson::mVenus larvae. This behavior was quantified by measuring the instantaneous change in head angle (absolute head angular speed). An increase in head angular speed was reliably triggered by each light flash. In contrast, wild-type controls (*w*/*Cyo/*Cyo UAS-CsChrimson::mVenus) showed very infrequent bouts of head casts that were not correlated with the temporal succession of the light flashes (Figure 6A). We adopted a receiver operating characteristic (ROC) approach [27] to discriminate negative (wild-type) controls from the gain-of-function phenotype. To this end, we devised a stimulation protocol in which larvae were exposed to eight consecutive 6-s red-light flashes. Successive flashes were separated by 30 s. A larva was flagged as responding to the light flashes when the instantaneous head angular speed increased above a threshold determined by the ROC analysis.
Figure 6. Sufficiency of Subesophageal-Zone Neurons to Trigger Head Casts and Turns

(A) Gain-of-function manipulations: NP4820-positive neurons elicit head casting when artificially activated by means of optogenetics. Time course of the absolute value of the angular speed of the head ($|\text{d}q/\text{d}t|$) for a representative larva. Top: angular speed of the head for $w^{1-}/C_0$ larvae. In a sequence of eight flashes of 6 s, the head angular speed exceeds the threshold (100 $^\circ/\text{s}$, dashed horizontal line) only twice as denoted by the black marks. Bottom: angular speed of the head for NP4820>UAS-CsChrimson::mVenus larvae. The angular speed of the head exceeds the threshold for eight out of the eight consecutive flashes. Red shadings in the background indicate the light flashes. Horizontal dashed line indicates the threshold and the black marks denote the instances where the angular speed exceeds the threshold.

(B) Optogenetic clonal analysis of the gain-of-function phenotype in NP4820 larvae. Clones were generated by using the “flip-out technique” (see Experimental Procedures and Figure S5). Expression pattern associated with a representative negative clone (top-left) and a representative positive clone (top-right). White arrows indicate the subesophageal-zone cells and yellow arrow indicates their projections through the VNC. Small white arrowheads indicate immature larval-born neurons. Green, anti-GFP targeting mVenus; magenta, anti-nc82 combined with anti-synapsin. Bottom panels: temporal series of the head angular speed for ten representative negative clones (left) and ten positive clones (right). In this schematic representation, we use a black mark to denote instances where the angular speed of the head exceeds the threshold (100 $^\circ/\text{s}$) during a flash. Red shadings indicate consecutive 6-s flashes, as described in (A).

(C) Schematic representation of the NP4840-labeled subesophageal-zone (SEZ) neurons found in the positive clones together with their projections. Schematic depiction of the lateral (left) and dorsal views (right) of the larval brain. Afferent OSN axons projecting onto the larval antennal lobe are shown in blue. SEZ neurons and their projections are shown in green: neuron #1 (dark green) was observed in one brain; neuron #2 (green) projects to the VNC, and it was observed in three brains; neuron #3 (light green) was observed in one brain. No SEZ neurons were observed in the negative clones (Figure S5C). Symbols of different anatomical

(legend continued on next page)
when the time course of its head angular speed was sufficiently high for minimum six out of the eight flashes (Figure 6A). We defined a threshold value on the head angular speed that maximized the rate of true-positives while minimizing the rate of false-positives (value: 100°/s, see also Figure S5A).

Next, we took advantage of a stochastic flippase-induced recombination technique to create mosaic larvae with CsChrimson expression restricted to random subsets of NP4820-positive neurons (see Experimental Procedures and Figure S5B). Individual clones were tested in a series of eight consecutive light flashes before isolation of their brains. The expression pattern of each clone was assessed by a direct staining against the mVenus tag fused to CsChrimson. We tested a total of 70 clones out of which we identified ten positive hits (Figure 6B, bottom panels), a number well above the expected number of false-positives per neuronal group (3.5 out of 70, Figure S5A). The expression pattern of a positive (light-responsive) and a negative (light-indifferent) clone is illustrated in Figure 6B (top panels) with the corresponding behavioral time courses shown in Figure S5B.

The neurons appearing more frequently than the expected rate of false-positives formed five groups (Figures S5C and SSD). One group was observed in the posterior dorsolateral (PDL) region with no contralateral projection; this group corresponded to unspcific secondary lineage neurons with immature neurites. The same observation was made for neurons covering the anterior dorsolateral commissural (ADLC) fiber. The immature aspect of various neurons constituting secondary lineages (SLs) precluded their functional involvement to the phenotype. As the intersectional manipulations listed in Figure 4A permitted us to exclude the contribution of a third group of neurons included in the VNC, we were left with three neurons in each hemi-segment of the subesophageal zone (Figures 6C and S5D). None of the subesophageal-zone neurons were found in negative clones. These observations suggest that the gain-of-function phenotype originates from the subesophageal-zone neurons observed in positive clones. We complemented this functional dissection with thermogenetic perturbations by means of the temperature-gated ion-channel dTrpA1 [28] (Figures S5E and S5F).

Together, the results of the optogenetic and thermogenetic gain-of-function analysis indicate that acute activation of the NP4820-labeled neurons in the subesophageal zone was sufficient to initiate reorientation maneuvers.

To fully ascertain the role of the subesophageal-zone neurons in the control of run-to-turn transitions, we used another GAL4 line (R23F01) with a narrow expression pattern similar in coverage to the NP4820-positive cells located in the subesophageal zone (Figure 6D). Line R23F01 consistently labeled four to six neurons in subesophageal zone together with three to 12 neurons with weaker expression level in the same region (Table S2). In addition, R23F01 showed stochastic labeling of one to six neurons in VNC, and it occasionally included a small number of KGs in each hemisphere (Figure S6B). Functional impairment of the R23F01-labeled cells by TNT led to a loss-of-function phenotype similar to NP4820: when tested in an odor gradient R23F01>TNTE displayed a reduction in turn frequency associated with a decreased ability to track the odor source (Figures S6A–S6C). Optogenetics-based gain-of-function perturbations induced by expressing CsChrimson in the R23F01-positive cells were sufficient to induce robust head casts (Figure 6D). Coupling the R23F01-GAL4 driver with tsh-GAL80 removed any expression in the VNC, which preserves the gain-of-function phenotype (Figures S6D and S6E).

**DISCUSSION**

In insects as in vertebrates, our understanding of the sensorimotor processes governing chemotaxis has been hampered by the absence of a connectivity diagram to guide circuit-functional hypotheses [29]. While the anatomy and function of neurons forming the first layers of the fly olfactory system are reasonably well characterized, little is known about the circuits and computational principles transforming olfactory inputs into orientation behaviors. At the motor end of the pathway, we have witnessed the emergence of models explaining how behavioral routines come about in terms of dynamical patterns of activity within the VNC network of the larva [30, 31], and how these patterns of activity produce stereotypical sequences of muscle contraction [32, 33]. In the absence of a connectivity diagram, we carried out a forward screen to identify new sets of neurons responsible for the control of specific sensorimotor tasks.

The sensorimotor pathway connecting the peripheral olfactory neurons to the motor system must achieve at least four tasks: (1) it encodes features of the stimulus that are relevant to the behavior; (2) it integrates dynamical sensory inputs with other sensory and contextual information, including internal states; (3) it converts this information into decisions directing the switch between distinct behavioral programs; (4) it implements the execution of specific motor programs. The objective of the present study was to identify circuit elements contributing to the modulation of action selection by sensory inputs. To this end, we conducted a loss-of-function behavioral screen to determine the necessity of specific groups of neurons to regulate transitions between running and turning.

We found that the subesophageal zone hosts a small group of neurons contributing to the control of larval chemotaxis. Inhibiting these neurons did not completely impede orientation in odor gradients, but it compromised the timing and coordination of reorientation maneuvers (Figures 2 and 3). Acute activation of these neurons through optogenetics and thermogenetics was...
sufficient to initiate reorientation maneuvers (Figures 6 and S5). The reduction in chemotactic performance is due to a partial loss of function of the subesophageal zone is reminiscent of the phenotypic impairment of odd-expressing neurons in the larval brain [11], except that head casting is enhanced upon silencing of the Odd neurons, whereas it is reduced upon silencing of the subesophageal-zone neurons. With regard to the subesophageal-zone neurons, we generalized the loss-of-function phenotype to three different modalities—odor, light, and temperature (Figures 2 and S5). This finding rules out that the NP4820-positive neurons are solely involved in olfactory processing, hinting at a higher-order function. In addition, it indicates that the subesophageal zone combines information arising from different sensory modalities according to their respective valences—positive for chemotaxis, negative for phototaxis and thermotaxis in warm environments. For chemotaxis to produce gradient ascent and phototaxis to produce gradient descent [2, 3, 22], the detection of increases in stimulus intensity must have an opposite modulatory effect on the behavioral output: positive gradients should suppress turning for attractive odors whereas it should facilitate turning for light. Although we still ignore how this sign reversal is implemented, it is expected to occur at a site upstream of the subesophageal zone, possibly in the first-order sensory neurons or directly downstream from them [34].

The subesophageal zone forms an anatomical bridge between the brain lobes and the ventral nerve cord. It is known to contribute to the representation of gustatory inputs in the larva [35]. Besides this function for taste, we hypothesize that the subesophageal zone acts as a multimodal hub where sensory information descending from the brain is combined and transformed before the release of motor commands. Using negative intersections (Figure 4A), we dissociated the loss-of-function phenotype from the locomotor system contained within the VNC [17]. This observation makes a pure motor function of the subesophageal zone very unlikely. Although we cannot exclude a direct modulatory effect of the subesophageal-zone neurons on the anterior lobe and the optic neuropile [8, 10, 36], such a feedforward mechanism would not explain the induction of head casts upon acute gains of function (Figures 6 and S5). Instead, we favor a model in which the subesophageal zone is included in the sensorimotor pathway downstream of the mushroom bodies and lateral horn. This model is compatible with the function of the subesophageal zone as a premotor center modulating the maintenance of locomotion during exploratory behavior in invertebrates [37, 38]. Parasitic wasps inject a venom cocktail into the nervous system of cockroaches to modulate the motor behavior of their prey. A main target of the wasp’s venom is the cockroach’s subesophageal zone. Modulation of activity in the subesophageal zone alters the cockroach’s spontaneous and sensory-evoked walking behavior and reduces the sensitivity of the animal to various stimuli. In adult flies, subesophageal-zone neurons have been shown to modulate innate feeding behavior driven by gustatory inputs based on internal state [39]. In light of previous studies and the present work, we speculate that the subesophageal zone of the Drosophila larva plays an active role in directing the selection of distinct motor programs by combining contextual information from the environment.

In summary, our results argue that the subesophageal zone hosts a group of neurons acting as a watershed that uses multimodal sensory signals produced by the larva’s own motion to modulate the probability of transitions between elementary behavioral routines (straight runs and reorientation maneuvers). Thirty years after the first analysis of larval foraging behavior as a handle on the transition between elementary motor programs [40], our work contributes to ongoing efforts to map the neural correlates of action selection in the Drosophila larva. In future work, an anatomical reconstruction of the subesophageal zone at the resolution of single synapses [41] will help us clarify the nature of the computations achieved by the subesophageal zone to initiate and maintain the motor programs underlying foraging and orientation behaviors in response to sensory stimuli. By combining new collections of GAL4 drivers [19, 42] with functional analysis and behavioral quantification [43], we are now in a position to undertake a systematic reconstruction of the sensorimotor pathway and computational logic underpinning larval chemotaxis.

**EXPERIMENTAL PROCEDURES**

**Fly Stocks**

For the primary screen, stocks were raised on standard cornmeal medium at 18°C on 12 hr-day:12 hr-night cycle; they were shifted to room temperature at least 3 hr before conducting the behavioral tests. We introduced bromophenol blue to the food to stain the digestive tract of larvae in order to enhance the contrast between individuals and the agarose substrate in the background. This method greatly improved the resolution when tracking groups of larvae. Other than the flies used in the primary screen (Figure 1D), stocks were raised at 23°C in non-colored medium. As a “wild-type” control, w1118 strain (denoted as w+/+) or a heterozygous cross between w+/+ and the respective parental lines were used. For the primary screen, we used a selection of 1118 GAL4 insertion lines on the second or the third chromosome. The lines originated from the NP collection available from the Drosophila Genetic Resource Center in Kyoto [13]. The following stocks were used: w;UAS-TNTE++; w;Orco-GAL4;UAS-mCD8::GFP (gift from L. Vosshall), P[GawB][1092]80-GAL4 [44], w;Orco202 (denoted as Orco−) [6], w;ub-GAL80[ ] [21], w;UAS-dTomA;+ (gift from P. Garrity), w;sh-LexA;GAL80++; (gift from the Simpson lab), w;Uas-;Cha3.3-GAL80 [18], elav-GAL4;+; (stock#: 458, Bloomington Stock Center), w[1118];P[y+;+7.7] w[mC]=20UXAS-IUV-CsChrimson::mVenus;attP2 (stock#: 55136, Bloomington Stock Center), w[1118];P[y+;+7.7] w[mC]=R29F12-GAL4;attP2 (stock#: 49495, Bloomington Stock Center), w[1118];P[y+;+7.7] w[mC]=GM2R3F01-GAL4;attP2 (stock#: 47335, Bloomington Stock Center), w;MB247-GAL80++; (gift from H. Tanimoto), w;+;ub-GAL80> and hs-flp/sp:cyo/MKRS7M6 (gift from K. Scott), w;FJRC79-8XLexAop2-FPl; attp40; and P[FJRC39-10XUSAS-FRT-STOP-FRT-E86etLC at attP2 (gift from B. Peiffer and G.M. Rubin), sh-LexA on the second chromosome (gift from J.-M. Knapp and J.H. Simpson), w;+;57C10-LexAop65 at attP2 [45].

**Histology**

The larval CNS and its peripheral olfactory organ (dorsal organ, DO) were dissected and fixed in 4% paraformaldehyde in PBS for 30 min at room temperature. After 3×15 min rinsing in PBS (pH: 7.4) + 0.2% Triton X-100 (TX, Sigma), the tissues were blocked for 30 min with 3% goat serum (Sigma) in PBS-TX. The primary antibody was incubated overnight at 4°C in the blocking solution. After 1×10-min rinse and 2×2-hr rinse (or longer), the samples were incubated overnight with the secondary antibody in PBS-TX kept at 4°C. After 2×10-min rinse in PBS-TX, the CNS and the DOs were mounted in Vectashield mounting medium (Vector Laboratories) on lysine-coated coverslips. Antibodies used were as follows: nc82 s (Developmental Studies Hybridoma Bank) at a dilution of 1:25, 3c11 (Anti SYNORF1, Developmental Studies Hybridoma Bank) at a dilution of 1:25, anti-GFP (product#: A-11120, Invitrogen) at a dilution of 1:500, Texas red goat anti-mouse and fluorescein isothiocyanate (FITC) goat anti-rabbit (Jackson ImmunoResearch) at a dilution of 1:500.
1:500. Imaging was performed with a Leica TCS SPE confocal microscope. Image rendering was performed with Imaris software (Bitplane Scientific Software) and Fiji (imagej.net; http://fiji.sc/). Background correction and adjustments of the brightness and contrast of maximum projections of the confocal stacks were achieved with Fiji.

**Behavioral Assays**

For all behavioral experiments, the arena was coated with 1.5% agarose (Sea-kem-LE, Lonza) prepared with deionized water. Foraging 5- to 6-day-old larvae were dissected from food in a 15% sucrose solution and washed in deionized water before being introduced in the experimental arena. In each trial, approximately 15 animals were tested at the same time. Primary screen assays were as follows: two fresh standard Petri dishes (90-mm diameter, Fisher Scientific) were coated with a 5-mm-thick agarose layer. For each trial, animals were tracked in parallel in both arenas. For the locomotion tests, larvae were introduced in the middle of an odor-free dish where their foraging behavior was monitored for 3 min. For the chemotaxis tests, we used a standard high-throughput assay featuring a small disk of filter paper (5-mm diameter) soaked in an aqueous solution of 1-hexanol 60 mM (CAS: 111-27-3, 98% pure, Sigma-Aldrich). The disk was placed at the center of the dish, and the larvae were introduced approximately 2 cm away from it. Larvae were monitored for 3 min. For each GAL4 line, two independent trials were conducted. The tracking of individual larvae was achieved with a charge-coupled device (CCD) camera (scA1390-17fc, Basler) that acquired images at a frequency of 0.1 Hz. We used a custom-made algorithm in MATLAB (MathWorks) to acquire and analyze images from the camera. The algorithm used the images to compute the position of each animal in real time and returned the time course of the mean distance of the group with respect to the center of the arena, which coincided with the position of the source. As a negative control, we created a reference data set of 20 trajectories for the positive control ($w$ $\rightarrow$ $x$) and standard negative controls for the assessment of both locomotion deficiency (P[Gal4B]109-290-GAL4>UAS-TNTE) and anosmia (Orco-GAL4>UAS-TNTE). After computing the time course of the median position with respect to the arena’s center for all trajectories of the same genotype, we determined the time points of the 3-min experiment for which the positive or negative controls was largest. Following this benchmarking, we defined a cutoff for the p value, which permitted reliable discrimination of impaired behavior without inclusion of false-positives (10 $^{-4}$ for locomotion and 10 $^{-6}$ for chemotaxis). A GAL4 line was associated with a behavioral impairment whenever a significant difference was measured between the median distance of this genotype and the wild-type control for at least three consecutive time points in both trials. For the secondary screen assay, we adopted a machine-vision algorithm previously described in [15] to track single larvae in a small rectangular arena coated with 3% agarose. The arena was set up as a single-odor-source assay [14] to test single larvae at a time. The odor source was placed out of reach of the larva. The two odors tested were 1-hexanol and ethyl butyrate (CAS: 105-54-4, Sigma-Aldrich) dissolved in paraffin oil (Sigma-Aldrich). To create light gradients with inverted Gaussian geometry, we used a blue LED (PLS 0625-030-S, Mitext Systems). Single larvae were placed on a 2.5% agarose slab. We applied total eight light flashes that are 6 s long and separated by 30 s. For thermogenic gain of function, GAL4 driver lines were crossed to UAS-dTrpA1. As a negative control, we used $w$ $\rightarrow$ $x$ $\rightarrow$ UAS-CsChrimson::mVenus. Flies were kept in complete darkness on food supplemented with 0.5 mM all trans-retinal (R2500, Sigma). For optogenetic stimulation we used a red LED with peak emission at 625 nm (PLS 0625-030-S, Mitext Systems). Single larvae were placed on a 2.5% agarose slab. We applied total eight light flashes that are 6 s long and separated by 30 s. For thermogenic gain of function, GAL4 driver lines were crossed to UAS-dTrpA1. As a negative control, we used $w$ $\rightarrow$ $x$ $\rightarrow$ UAS-dTrpA1. As a positive control we used pan-neural elav-GAL4>UAS-dTrpA1, which leads to complete paralysis when globally activated. This cross was used for determination of the threshold temperature specific to our setup (data not shown). This temperature was determined to be about 28$^\circ$C, in agreement with previous work [28]. To activate the dTrpA1 effector, we subjected larvae to a gradual temperature increase in time. We placed a 1-mm-thick layer patch of 1% agarose on the surface of an aluminum slab painted in black, which was connected to a 12 $\times$ 8 cm Peltier device (TE Technology). The temperature was controlled and logged by a commercial thermistor and software provided with the Peltier element (TE Technology). Unrestrained single larvae were monitored for 5 min. Ten seconds into the experiment, the temperature of the aluminum plate was raised from 24$^\circ$C to 31$^\circ$C (0.12$^\circ$/s), yielding a temperature ramp of 23$^\circ$C to 29$^\circ$C at the surface of the agarose substrate. The temperature of the aluminum plate was kept at 31$^\circ$C for 30 s before it decreased back to 24$^\circ$C at a rate of 0.12$^\circ$/s. Behavior was monitored using the same hardware and MATLAB software as for the chemotaxis assay.

**Gradient Quantification**

Following the procedure described in [14], odor profiles were measured at fixed positions on the plate using a FT-IR spectrometer (Tensor 27,Bruker). The absolute concentration of odor in gaseous phase upon application of the Beer-Lambert law (A = ε x C x l) where A denotes the absorbance, ε the molar extinction coefficient, l the length of the section considered and C the average concentration along this section. Molar extinction coefficient of the odorants could not be directly measured at the source concentrations that were used in the behavioral experiments. As explained in Figure S1, we inferred the experimental landscapes by scaling down the gradient reconstructed at the lowest source concentration possible (ethyl butyrate: 30 mM and 1-hexanol: 500 mM).

**Behavioral Analysis for the Secondary Screen**

Behavioral data were classified as described in [2, 15]. To analyze the data shown in Figures 2, 3, 4, and 5, we introduced an additional set of metrics: the percentage of time spent in a zone delineated by an isocline with an odor concentration corresponding to 80% of the gradient’s peak (approximately 15-mm diameter). The tendency of larvae to maintain their ongoing direction of motion was measured through the persistence length of their trajectories—a metrics used in solid-state physics to characterize the stiffness of polymers [46]. This metrics ranges from small values close to zero for winding trajectories to infinity for a straight line. To calculate persistence length of a trajectory, we computed the difference between the headings angles ($\Delta(x)$ at a centroid position of reference and for consecutive centroid positions ($x)$ that were 0.1 mm apart from each other along the trajectory. As for other systems characterized by their persistence length (e.g., DNA or spaghettis), we observed that $\cos(\Delta(x))$ followed an exponential decay. After averaging $\Delta(x)$ over every possible start position and trajectory, the persistence length $P$ was estimated from a least-square fit using the relationship $<\cos(\Delta(x))>=e^{(-1/(x^2))}$ where $<$ denotes the average over all start positions and trajectories.

**Gain-of-Function Manipulations**

For optogenetic gain of function manipulations, GAL4 drivers were crossed to UAS-CsChrimson::mVenus. As a negative control, we used $w$ $\rightarrow$ $x$ $\rightarrow$ UAS-CsChrimson::mVenus. Flies were kept in complete darkness on food supplemented with 0.5 mM all trans-retinal (R2500, Sigma). For optogenetic stimulation we used a red LED with peak emission at 625 nm (PLS 0625-030-S, Mitext Systems). Single larvae were placed on a 2.5% agarose slab. We applied total eight light flashes that are 6 s long and separated by 30 s. For thermogenic gain of function, GAL4 driver lines were crossed to UAS-dTrpA1. As a negative control, we used $w$ $\rightarrow$ $x$ $\rightarrow$ UAS-dTrpA1. As a positive control we used pan-neural elav-GAL4>UAS-dTrpA1, which leads to complete paralysis when globally activated. This cross was used for determination of the threshold temperature specific to our setup (data not shown). This temperature was determined to be about 28$^\circ$C, in agreement with previous work [28]. To activate the dTrpA1 effector, we subjected larvae to a gradual temperature increase in time. We placed a 1-mm-thick layer patch of 1% agarose on the surface of an aluminum slab painted in black, which was connected to a 12 $\times$ 8 cm Peltier device (TE Technology). The temperature was controlled and logged by a commercial thermistor and software provided with the Peltier element (TE Technology). Unrestrained single larvae were monitored for 5 min. Ten seconds into the experiment, the temperature of the aluminum plate was raised from 24$^\circ$C to 31$^\circ$C (0.12$^\circ$/s), yielding a temperature ramp of 23$^\circ$C to 29$^\circ$C at the surface of the agarose substrate. The temperature of the aluminum plate was kept at 31$^\circ$C for 30 s before it decreased back to 24$^\circ$C at a rate of 0.12$^\circ$/s. Behavior was monitored using the same hardware and MATLAB software as for the chemotaxis assay.

**Phototaxis Assay**

To create light gradients with inverted Gaussian geometry, we used a blue LED with peak emission at 470 nm (PLS 0470-030-S, Mitext Systems). Single larvae were introduced on an agarose surface at the position corresponding to the minimum of the light gradient and were tracked for 5 min. The position of the larva was monitored in real time, and the light intensity was continuously updated according to a preset landscape. The light intensity at the agarose surface was determined with a photodiode and a benchtop amplifier (SM05PD7A and PDA200C, Thorlabs).

**Thermotaxis Assay**

To create thermal gradients, we used an aluminum surface that is connected to two identical 12 $\times$ 8 cm Peltier devices (TE Technology) on each side. One side of the aluminum surface was constantly kept at 20$^\circ$C, and the other side was constantly kept at 36$^\circ$C by using the Peltier devices in order to create an axis with a linear thermal gradient. A 1-mm-thick layer patch of 1% agarose was placed on the aluminum surface, and individual larvae were placed in the middle of the gradient. Larvae were tested on the thermal gradient for 150 s. Behavior was monitored using the same hardware and MATLAB software as for the chemotaxis assay.

**Clonal Gain of Function**

w$^+$NP4820::GAL4;ubd-GAL80> males were crossed to hs-flp;+>UAS-CsChrimson::mVenus females. Flies were kept at 21$^\circ$C for all times, and...
5- to 6-day-old larva were tested as it is explained for optogenetic gain of function. Stochastic activity of the heat shock promoter led to expression of CsChrimson::mVenus in subsets of NP4820-labeled neurons. In order to determine thresholds to identify positive and negative clones, Receiver Operator Characteristics (ROC) analysis was used [27, 47]. Briefly, true-positive (TP) and false-positive (FP) rates were computed for different thresholds for w;NP4820-GAL4++; × hs-flp++; UAS-CsChrimson::mVenus larvae. The best performance was observed at a threshold of 100/s for the head angular speed with the criteria that a larva is considered to be TP if its head angular speed exceeded 100/s during at least six of eight light flashes. This threshold was applied to determine positive and negative clones. Later, both positive and negative clones were immunostained against Venus protein using antibodies raised against GFP to identify the neurons expressing the Chrimson protein. Immunostaining was performed as stated above. Comparisons between the behavioral phenotype and neuronal coverage was achieved after completion of all experiments.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2015.04.016.

AUTHOR CONTRIBUTIONS

I.T., J.R., and M.L. designed the research. J.R. and V.S.-K. conducted the primary screen described in Figure 1D. J.R. carried out the experiments described in Figures 2, 3, and 5 (phototaxis). I.T. and J.R. contributed to Figure 4. I.T. contributed to Figures 2, 4, and 5 (thermotaxis) and carried out the clonal analysis shown in Figure 6. I.T. and J.W.T. identified and characterized lines R29F12 and R23F01. I.T., J.R., and A.G.-M. analyzed behavioral data. I.T., J.R., and M.L. interpreted the results and wrote the manuscript.

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