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Neuropeptide-Gated Perception of Appetitive Olfactory Inputs in *Drosophila* Larvae

Yonghua Wang,[1,2] Yuhan Pu,[1,2] and Ping Shen[1,*]
[1]Department of Cellular Biology and Biomedical and Health Sciences Institute, University of Georgia, 500 D.W. Brooks Drive, Athens, GA 30602, USA
[2]Co-first authors
*Correspondence: pshen@uga.edu
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SUMMARY

Understanding how smell or taste translates into behavior remains challenging. We have developed a behavioral paradigm in *Drosophila* larvae to investigate reception and processing of appetitive olfactory inputs in higher-order olfactory centers. We found that the brief presentation of appetitive odors caused *fed* larvae to display impulsive feeding of sugar-rich food. Deficiencies in the signaling of neuropeptide F (NPF), the fly counterpart of neuropeptide Y (NPY), blocked appetitive odor-induced feeding by disrupting dopamine (DA)-mediated higher-order olfactory processing. We have identified a small number of appetitive odor-responsive dopaminergic neurons (DL2) whose activation mimics the behavioral effect of appetitive odor stimulation. Both NPF and DL2 neurons project to the secondary olfactory processing center; NPF and its receptor NPFR1 mediate a gating mechanism for reception of olfactory inputs in DL2 neurons. Our findings suggest that eating for reward value is an ancient behavior and that fly larvae are useful for studying neurobiology and the evolution of olfactory reward-driven behavior.

INTRODUCTION

The sense of smell is crucial for two vital biological functions, foraging and mating, across evolution. Olfactory information processing in insects and mammals appears to be very similar. In *Drosophila*, environmental odors detected by olfactory receptor neurons are relayed to the glomeruli in the antennal lobe (analogous to the mammalian olfactory bulb), which functions as the primary olfactory center. Processed olfactory information, likely generated via a combinatorial coding mechanism in the antennal lobe, is subsequently transmitted by projection neurons to the secondary olfactory centers in the brain, including the mushroom body and lateral horn, which are responsible for olfactory memory and behavioral organization (Heisenberg, 2003; Masse et al., 2009; Su et al., 2009; Vosshall and Stocker, 2007). At present, the molecular and circuit mechanisms underlying the function and regulation of higher-order olfactory centers remain poorly understood.

Drosophila larvae have a highly evolved nervous system that is also numerically simple. The olfactory system of fly larvae has 21 olfactory receptor neurons unilaterally instead of the 1,300 such neurons found in adults (Vosshall and Stocker, 2007), and each of the 21 neurons relays odor stimulation to one of the 21 uniglomerular projection neurons (Ramaekers et al., 2005). Therefore, genetic tractability, as well as reduced complexity and the availability of well-established cellular and behavioral assays, make the fly larva an excellent model for the neurobiological study of odor-induced behavior.

Neuropeptides are a group of chemically diverse signal molecules involved in the modulation of a multitude of physiological processes and behaviors (Hewes and Taghert, 2001; Nässel and Winther, 2010). In *Drosophila*, evolutionarily conserved neuropeptide pathways have been shown to regulate diverse behaviors (Dierick and Greenspan, 2007; Krashes et al., 2009; Lee et al., 2004; Lingo et al., 2007; Melcher and Pankratz, 2005; Root et al., 2011; Shohat-Ophir et al., 2012; Terhzaz et al., 2007; Wen et al., 2005; Wu et al., 2003; Xu et al., 2008; Yapici et al., 2008). Neuropeptide F (NPF), an abundant signaling peptide in the fly brain, is the fly counterpart of mammalian neuropeptide Y (NPY) (Brown et al., 1999). NPF has been shown to regulate feeding, stress response, ethanol consumption, and memory in *Drosophila* (Krashes et al., 2009; Lingo et al., 2007; Shohat-Ophir et al., 2012; Wen et al., 2005; Wu et al., 2003; Xu et al., 2010). These findings suggest that *Drosophila* presents an excellent opportunity to investigate the roles of conserved signaling peptides in behavioral control.

Food odors can be powerful appetitive cues. Imaging analyses have shown that food odors can activate the brain circuits associated with reward and motivation processing (Bragulat et al., 2010). However, little is understood about how appetitive odors are perceived by the brain and subsequently transformed to appetitive behavior. In this work, we report that brief presentation of appetitive odors caused ad libitum-fed *Drosophila* larvae to impulsively consume sugar-rich food, demonstrating that invertebrate animals engage in appetitive cue-driven feeding. Using this behavioral paradigm, we have investigated how appetitive olfactory reward is perceived and transformed into

appetitive drive in higher-order olfactory centers. We show that deficiencies in an NPF signal blocked appetitive odor-induced feeding by disrupting dopamine (DA)-mediated higher-order olfactory processing. We have identified a small number of dopaminergic neurons that project to the lateral horn region and are likely postsynaptic to the second-order olfactory neurons. NPF neurons also project to the lateral horn, and appetitive odor excitation of these dopaminergic olfactory neurons is gated by NPF via its receptor NPFR1. Our findings suggest that eating for reward value is an ancient behavior and that fly larvae are useful for studying neurobiology and evolution of olfactory reward-driven appetitive behavior.

RESULTS

A Behavioral Paradigm for Appetitive Cue-Driven Feeding

We sought to establish an experimentally amenable invertebrate model to investigate the higher order neural control of reward processing and motivation for seeking food or appetitive motivation. *Drosophila* larvae fed ad libitum normally show a basal level of feeding response to readily accessible palatable food (e.g.,10% glucose agar paste), which is quantifiable by counting the number of larval mouth hook contractions (MHC) during a 30 s test period (Wu et al., 2003, 2005). Although this baseline feeding activity can be significantly enhanced by food deprivation (Wu et al., 2005) (Figure S1A), it remains unclear whether it can be increased through a nonhomeostatic (e.g., reward-driven) mechanism. To test this possibility, we exposed fed larvae to various synthetic and natural odorants that are attractive to flies, including pentyl acetate (PA, with a scent similar to bananas) and balsamic vinegar (Asahina et al., 2009; Fishilevich et al., 2005). Indeed, fed larvae briefly exposed to appetitive olfactory cue(s) showed a significant increase of mouth hook contractions and food ingestion (Figures 1A and 1F; Figure S1). Under our test conditions, PA stimulation of feeding was most effective when the exposure time was limited to 5–10 min. Moreover, PA-stimulated fed larvae continued to display elevated feeding activity for at least 12 min after the removal of PA (Figure 1B).

A key feature of reward-driven eating in mammals is the involvement of readily available palatable food (Lowe and Butryn, 2007; Volkow and Wise, 2005). We found that PA failed to stimulate larval feeding response in the presence of less-accessible solid food (agar block containing 10% glucose) or agar paste (liquid food) low in sugar (Figures 1C and 1D). Therefore, PA-stimulated feeding activity requires food that is not only palatable but also readily available. In addition, the stimulatory effects of an attractive odor and hunger appear to be additive (Figure 1E). For example, the feeding activity of PA-stimulated larvae that fasted for 1 hr was similar to that of nonstimulated control larvae that fasted for 2 hr. The stimulatory effect of PA, however, became undetectable after prolonged food deprivation. These results suggest that in fed or moderately hungry larvae, the homeostatic control of satiation can be transiently overridden by a nonhomeostatic mechanism activated by attractive food odors.

The Higher-Order Olfactory Center Involved in PA-Stimulated Feeding

The *odorant receptor coreceptor* gene (Orco, also known as *or83b*), which is essential for fly odor sensation, is expressed broadly in olfactory neurons (Larsson et al., 2004). We found that a loss-of-function mutation in *or83b* (*or83b*^{1}) abolished larval feeding response to PA stimulation (Figure 2A). *UAS-shi*^{ts1} encodes a temperature-sensitive, dominant negative form of dynamin that inhibits neurotransmission at a restrictive temperature (>29°C) (Kitamoto, 2001). Expression of *UAS-shi*^{ts1} in olfactory receptors, driven by *Or83b-Gal4*, also abolished PA-stimulated feeding at 31°C (Figure 2B; Figure S2D). The *GH146-Gal4* driver labels the projection neurons that relay olfactory information from the AL to the LH and MB (Figures S2A–S2C) (Marin et al., 2005; Stocker et al., 1997). Expression of *UAS- shi*^{ts1} in *GH146-Gal4* neurons also blocked PA-stimulated feeding (Figures 2C and S2E). However, inhibition of the neurotransmission of MB neurons labeled by *OK107-Gal4* had no negative impact on the PA-elicited feeding response (Figures 2C and S2E). These findings suggest that appetitive odor-driven feeding may involve the higher order olfactory processing by the LH and is independent of the MB neurons essential for larval learning and memory (Kahsai and Zars, 2011).

NPF and Its Receptor NPFR1 in PA-Stimulated Feeding

The conserved NPF system was previously implicated in a hunger-induced drive to procure solid food (Wu et al., 2005). This finding led us to test whether NPF might play a role in reward-driven food motivation. We found that expression of UAS-*kir 2.1* encoding an inward-rectifier potassium channel in *NPF-Gal4* neurons blocked the PA-stimulated feeding response (Figure 3A). In addition, expression of *npfr1RNAi* in the larval nervous system also blocked PA-stimulated feeding (Figure 3B) (Wen et al., 2005). In an effort to identify and characterize the target neurons of NPF, we constructed a new *NPFR1-Gal4* driver. Knockdown of NPFR1 in fed *NPFR1-Gal4*/UAS-*npfr1RNAi* larvae attenuated the PA-stimulated feeding response (Figure 3B). Further, expression of *UAS- shi*^{ts1} in the *NPFR1*-Gal4 neurons also abolished PA-stimulated feeding at 31°C (Figure S3A). Together, these results suggest that the activity of the NPF/NPFR1 pathway is essential for the appetitive drive elicited by olfactory cues.

Dopamine Signaling in PA-Stimulated Feeding

The *NPFR1*-Gal4 is expressed in a broad set of neurons in the larval central nervous system (CNS), including the majority of the DA neurons (Figures S3B and S3C; Table S1; Movie S1). Several lines of evidence suggest that the NPFR1 activity in DA neurons is essential for appetitive odor-driven feeding. First, expression of *Th-Gal80* in *NPFR1-Gal4*/UAS-*npfr1RNAi* larvae, which suppresses *NPFR1-Gal4* function in DA neurons, restored the PA-induced feeding response (Figure 3C). Second, expression of *npfr1*RNAi in *Th-Gal4* neurons also attenuated PA-stimulated feeding response. Finally, this behavioral phenotype of *Th-Gal4*/UAS-*npfr1RNAi* larvae was rescued by feeding with L-dopa, a precursor of dopamine (Figure 3D).

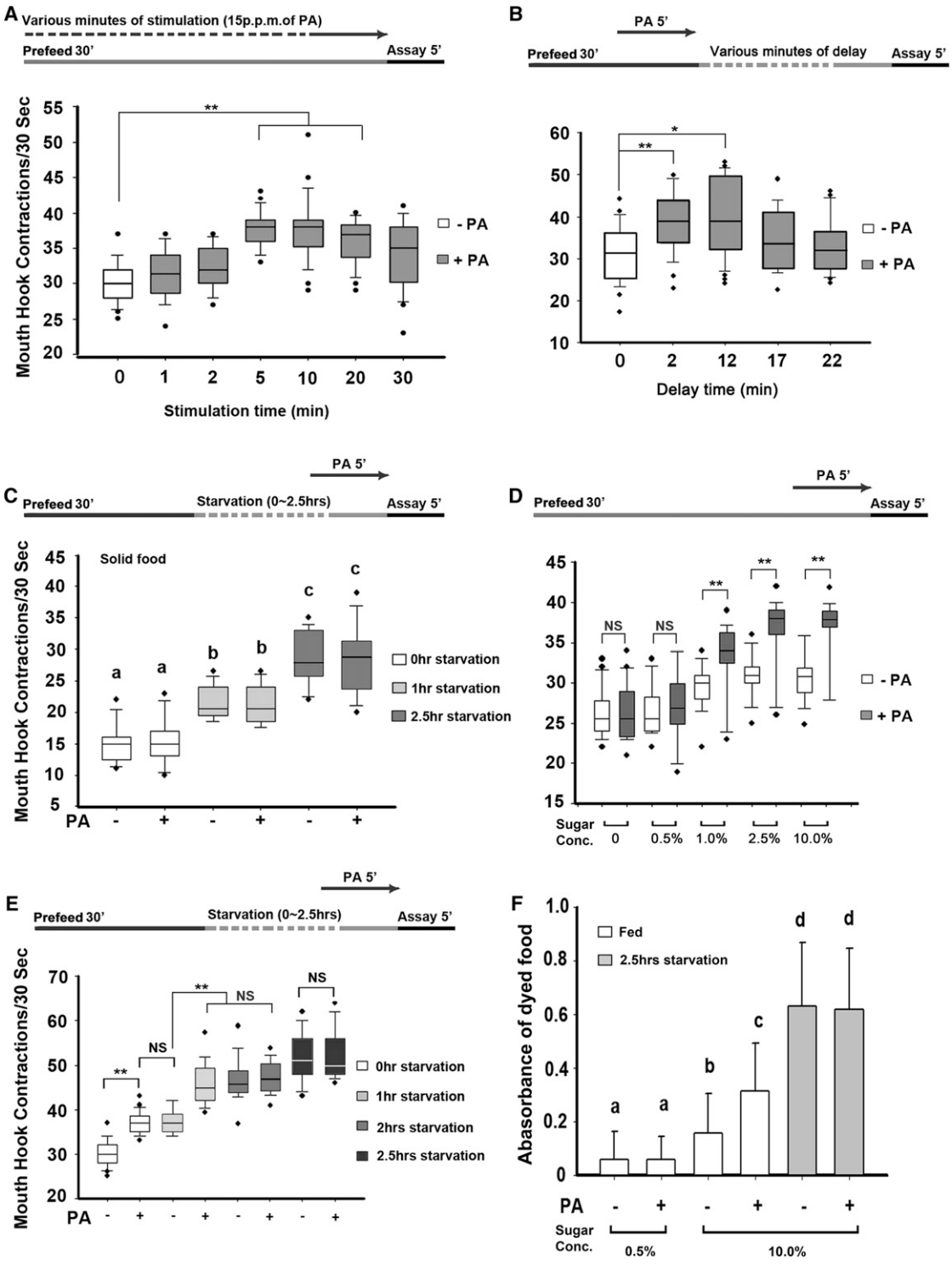

Figure 1. A Behavioral Paradigm for Appetitive Odor-Induced Feeding

Wild-type larvae used in this and the following figures were young third-instar w^{1118} larvae (74 hr AEL).

(A) Larvae were prefed in yeast paste on an apple juice agar plate. After PA exposure (15 ppm), larvae were rinsed with a copious amount of water and transferred to 10% glucose agar paste (liquid food) for the feeding test (see Experimental Procedures for details). Unless indicated otherwise, behavioral phenotypes were quantified under blind conditions, and statistical analyses were performed using one-way ANOVA followed by a Dunn's test in all figures. $^{**}p < 0.001$.

(B) Larvae were exposed to PA during the final 5 min prefeeding. A time delay of up to 22 min was introduced between PA stimulation and the feeding assay by withholding the larvae in yeast paste. $^{*}p < 0.01$; $^{**}p < 0.001$.

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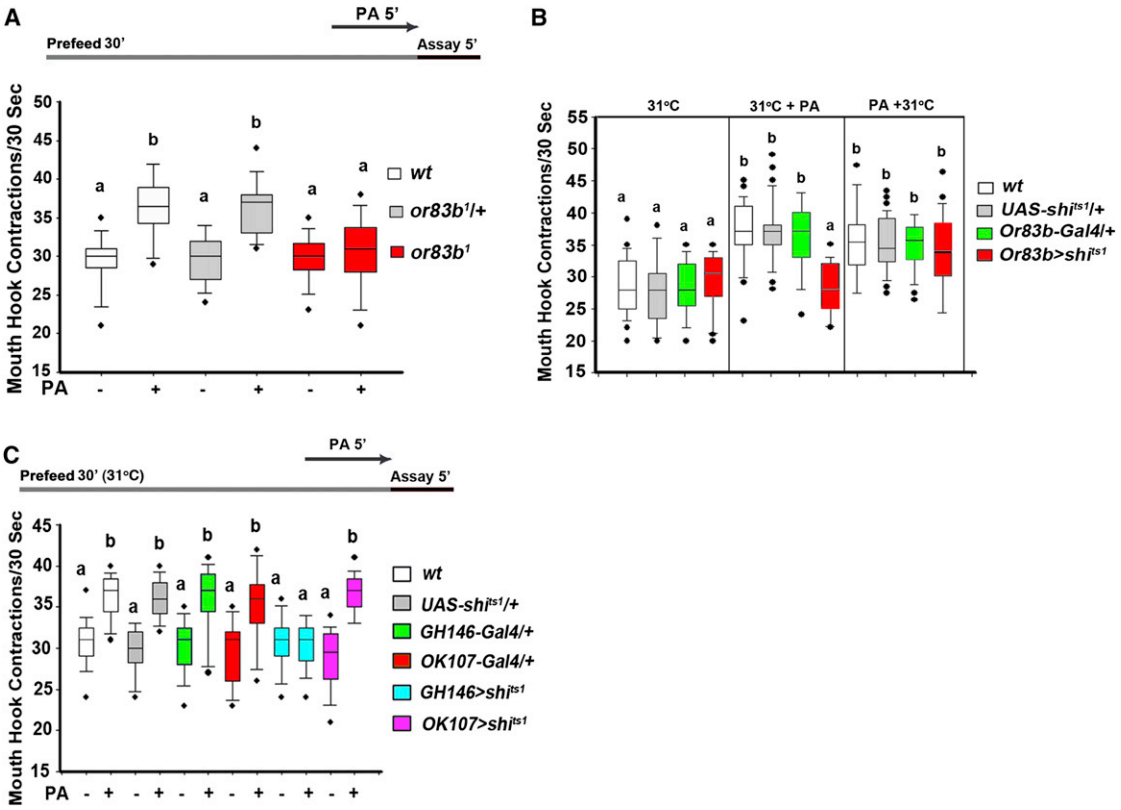

Figure 2. Requirement of Sensory and Processing Neurons in Olfactory Reward-Driven Feeding

(A) PA stimulation increased feeding activity in wild-type and heterozygous but not homozygous *or83b*^{1}mutants.

(B) Larvae were incubated for 10 min at the restrictive temperature of 31°C, either before (middle panel) or after (right panel) PA stimulation. At the permissive temperature of 23°C, *Or83b-Gal4*/UAS- *shi*^{ts1} larvae were normal in PA-stimulated feeding response (Figure S2D).

(C) At 31°C, expression of UAS- *shi*^{ts1} in *GH146-Gal4*, but not *OK107-Gal4*, neurons attenuated PA-stimulated feeding activity in fed larvae (Figure S2E).

Different letters indicate statistically significant differences; $p < 0.01$.

Roles of D1-like Receptors in PA-Stimulated Feeding

We also found that an oral treatment of wild-type larvae with 3IY, an inhibitor of tyrosine hydroxylase, attenuated a PA-elicited feeding increase (Figure 3E; Figure S3D), suggesting that the NPF system mediates the PA-stimulated feeding response through positive regulation of DA signaling. *Drosophila* genome contains four DA receptor genes, including two members of the D1 family, DopR and DopR2, one D2-like receptor, D2R, and a noncanonical receptor, DopEcR, that can be activated by either dopamine or steroids (Draper et al., 2007; Inagaki et al., 2012; Srivastava et al., 2005). Using both genetic and RNA interference analyses, we have identified at least one DA receptor DopR that is required for the odor enhancement of appetite. A loss-of-function *DopR* mutation (*DopR*^{f02676}) has been characterized (Inagaki et al., 2012; Kim et al., 2007; Kong et al., 2010; Lebestky et al., 2009). Fed *DopR*^{f02676} larvae failed to display PA-induced food response (Figure 3E). In addition, fed *elav-Gal4*/UAS-*DopRRNAi* larvae that express *DopR* RNAi in the nervous system also showed attenuated PA-induced food response (Figure S3E). These results have revealed an essential role of the DA/DopR pathway in PA-induced feeding behavior. To provide evidence that DA signaling is acutely required for the feeding behavior, we transiently inhibited neurotransmission of DA neurons in *Th-Gal4*/UAS-*shi*^{ts1} larvae. Indeed, at the restricted temperature, PA failed to elicit the feeding response in *Th-Gal4*/UAS-*shi*^{ts1} larvae (Figure 3F; Figure S3F).

(C) Larvae fasted for up to 2.5 hr in water. Larvae were exposed to PA during the final 5 min of fasting before testing their feeding response to 10% glucose agar block (solid food). Different letters indicate statistically significant differences. $p < 0.001$.

(D) Larvae continued to display feeding activity in liquid media low in or free of sugar immediately after their removal from palatable food and rinsing. PA stimulation elicited feeding responses to liquid food containing ≥1% glucose. **$p < 0.001$. NS, no significance.

(E) Stimulating effects of PA on larvae that fasted for up to 2.5 hr. **$p < 0.001$.

(F) A group of 20 third-instar larvae (74 hr AEL) were allowed to feed in dyed liquid food for 2 min. The amount of ingested food increased after PA stimulation or food deprivation. n = 10 trials for each data point. Different letters indicate statistically significant differences. $p < 0.01$.

See also Figure S1.

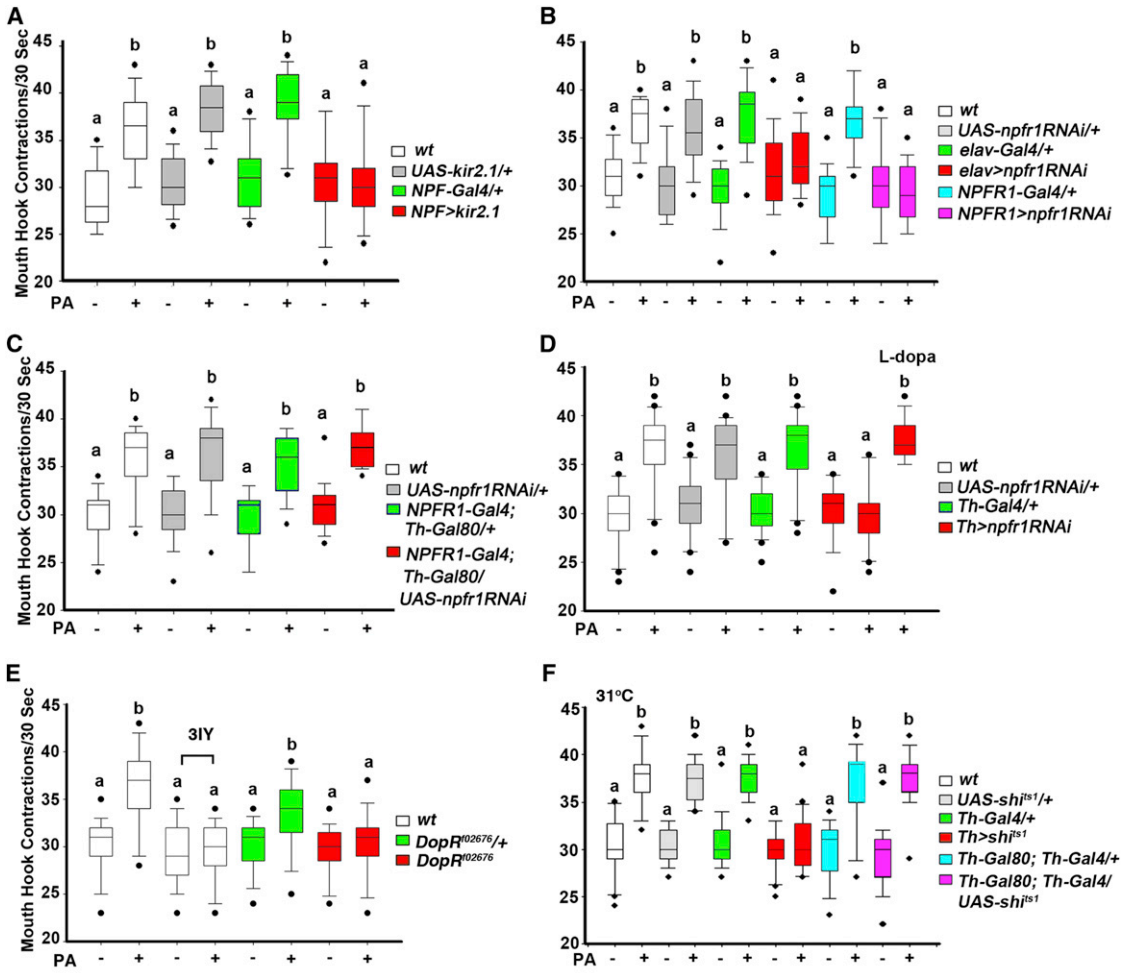

Figure 3. Olfactory Reward-Driven Feeding Requires the NPF/NPFR1 and DA/DopR Pathways

(A) *NPF-Gal4*/UAS- *kir2.1* larvae failed to show PA-stimulated feeding response.

(B) Expression of *npfr1*RNAi by *elav-Gal4* or *NPFR1-Gal4* attenuated PA-stimulated feeding response.

(C) The PA-stimulated feeding response of *NPFR1-Gal4*/UAS- *npfr1RNAi*/*Th-Gal80* larvae was restored to the normal level.

(D) *Th-Gal4* is broadly expressed in DA neurons. Expression of *npfr1*RNAi by *Th-Gal4* attenuated the PA-stimulated feeding response, which can be rescued by feeding L-dopa, the dopamine precursor, to the fed experimental larvae.

(E) Feeding wild-type larvae 3IY, an inhibitor of tyrosine hydroxylase, attenuated the PA stimulatory effect (Figure S3D). A loss-of-function mutation ($DopR^{f02676}$) of the D1-like receptor gene attenuated the PA-stimulated feeding increase (Figure S3E).

(F) Incubation of *Th-Gal4* /*UAS-shi*^{ts1} larvae at 31°C blocked PA stimulated feeding increase. Introduction of *Th-Gal80*, which inhibits *Th-Gal4* activity, restored the PA effect (Figure S3F).

Different letters indicate statistically significant differences; $p < 0.01$.

Functional Mapping of DA Neurons

There are approximately 70 DA neurons in the *Drosophila* larval central nervous system (CNS) (Monastirioti et al., 1996; Selcho et al., 2009) (Figure S4A). *Tsh-Gal80* is expressed in the larval thoracic and abdominal ganglia (Yu et al., 2010). Because a large number of DA neurons are present in the larval ventral ganglia, we introduced *Tsh-Gal80* into the *Th-Gal4*/ UAS- *shi*^{ts1} larvae to suppress *shi*^{ts1} expression in the thoracic and abdominal DA neurons (Figures S4B–S4F). The *Th-Gal4*/ *UAS-shi*^{ts1}/*Tsh-Gal80* larvae remained deficient in PA-stimulated feeding response, suggesting that DA neurons in the protocerebrum and/or subesophageal ganglia (SOG) may be responsible for appetitive odor-driven feeding. There are three paired clusters of DA neurons named DM, DL1, and DL2 in the brain of third-instar larvae (Friggi-Grelin et al., 2003; Selcho et al., 2009). To determine which subset(s) of DA neurons are responsible for the PA-stimulated feeding response, we induced lesions in targeted protocerebral DA neurons using focused laser beams (Xu et al., 2008) (Figure 4A; Figures S5A and S5B). We found that lesions in the DL2 and DL1 neurons or DL2 neurons alone (in both brain lobes) abolished a PA-elicited feeding increase, suggesting that DL2 neurons are required for PA-stimulated feeding. The DL2 neurons form a two- and four-cell cluster (Figures S4G–S4L; Movie S2). The presumptive dendrites of DA

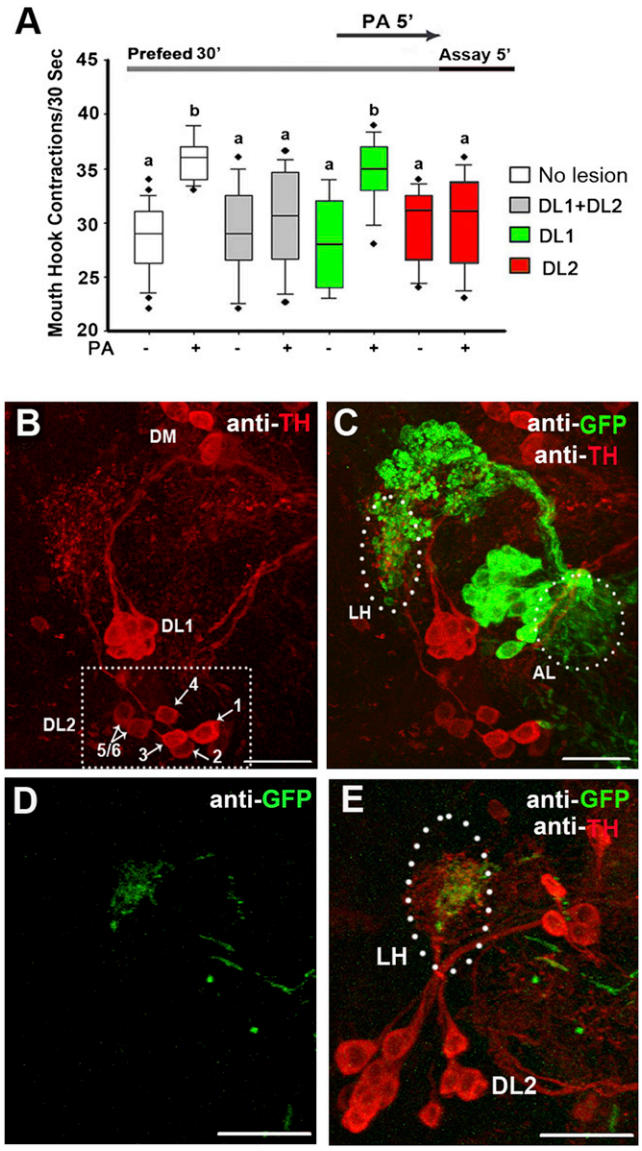

Figure 4. Functional and Anatomical Analyses of DA Neurons in the Larval Central Nervous System

(A) Targeted lesions in selected DA neurons of living second-instar *Th-Gal4/UAS-nlsGFP* larvae were induced using the laser beam. After recovery, PA-stimulated feeding responses of fed third-instar larvae (74 hr AEL) were quantified. Different letters indicate statistically significant differences; $p < 0.01$.

(B) Immunofluoresce of anti-TH in DL2, DL1, and DM neurons. DL2 neurons are marked by dotted squares and named from 1 to 6 by their soma positions (Movies S2 and S3). Scale bar, 20 μm.

(C) Immunofluorescence of anti-TH in DL2, DL1, and DM neurons (red) and GFP in *GH146-Gal4* neurons (green). The overlapping fluorescence (yellow) in the lateral horn (LH, dotted ellipses) region suggested the presence of synaptic connections (also see Movie S3). The antenna lobe (AL) is marked by dotted circles. Scale bar, 20 μm.

(D and E) Synaptic connections between *GH146-LexA* and *Th-Gal4* neurons in the LH region are shown using GRASP technique. Immunofluorescence of split GFP is green and anti-TH is red. The LH is marked by dotted ellipses. Genotype: *GH146-LexA*; *Th-Gal4/ UAS-mCD4::spGFP$^{1-10}$*; *LexAop-mCD4::spGFP^{11}*. Scale bar, 20 μm.

See also Figures S4, S5A, and S5B.

neurons in the four-cell cluster (labeled DL2-1, DL2-2, DL2-3, and DL2-4) may form synaptic connections with projection neurons in the LH region, as evidenced by the overlapping yellow fluorescence (Figures 4B and 4C; Movie S3) and further supported using the GFP Reconstitution Across Synaptic Partners (GRASP) technique that utilizes two complementary fragments of GFP (Feinberg et al., 2008; Gordon and Scott, 2009) (Figures 4D and 4E). Mosaic analyses using the FLP-Out Gal80 technique (Gordon and Scott, 2009; Marella et al., 2012) revealed that DA neurons from the four-cell cluster (DL2-1 to 4), but not DL2-5 and 6 neurons, project ipsilaterally to the LH region, and their dendritic and axon arbors show restricted distribution in the LH region (hence these four neurons are named as DL2-LH; Figures 5A–5D; Movies S4, S5, S6, and S7). We also used the FLP-Out Gal80 technique to selectively express TrpA1 in a small subset(s) of DA neurons (see the Experimental Procedures for details). The fed experimental larvae were individually assayed and subsequently examined for the GFP expression in subsets of *Th-Gal4* neurons. We found that activation of one or two DL2-LH neurons by TrpA1 expression in fed larvae was sufficient to mimic the stimulating effect of PA to induce elevated feeding, whereas activation of DM, DL1, or DL2-5/6 neurons failed to do so (Figure 5E; Figure S5C).

NPF-Gated Excitation of DA Neurons in the LH

To better understand the actions of NPF/NPFR1 on DL2-LH neurons, we performed neuroanatomical analysis of NPF and *NPFR1-Gal4* neurons. Immunofluorescence staining showed that several projections of the lateral NPF neurons are juxtaposed to the processes of DL2-LH neurons in the LH (Figures 6A and 6B; Movie S8). Furthermore, at least three of the four DL2-LH neurons, whose activation mimicked the effect of PA stimulation, are marked by *NPFR1-Gal4* (Figures 6C–6E). We also found that in *Th-Gal4*/UAS-*GCaMP3* larvae that express a Ca^{2+} indicator, DL2 neurons, especially DL2-2 and DL2-3 neurons, displayed increased Ca^{2+} influx in response to PA stimulation (Figures 7A and 7B; Movie S9). PA-stimulated increases of Ca^{2+} influx were also observed at the neuronal processes in the LH. To provide direct evidence that DL2-LH neurons are the targets of NPF action, we knocked down *npfr1* activity in fed *Th-Gal4/UAS-GCaMP3* larvae. The DL2-LH, but not DL1, neurons in these larvae failed to display PA-induced excitation, confirming that *npfr1* activity is required for this effect (Figures 7C and S5E). These results suggest that reception of olfactory inputs by DA neurons is gated by the NPF/NPFR1 pathway. In addition, the *Th-Gal4/UAS-npfr1RNAi* larvae showed normal chemotactic response to PA, suggesting that they have normal odor acuity (Figure S5D).

DISCUSSION

We have shown that *Drosophila* larvae display appetitive odor-driven feeding of sugar-rich food, demonstrating that an invertebrate organism consumes food for its reward value, similar to mammals. Using this behavioral paradigm, we have identified a circuit mechanism, mediated by conserved NPF and DA systems, for higher-order olfactory processing in the lateral horn of the larval brain (Figure 7D). Our findings suggest that fly larvae

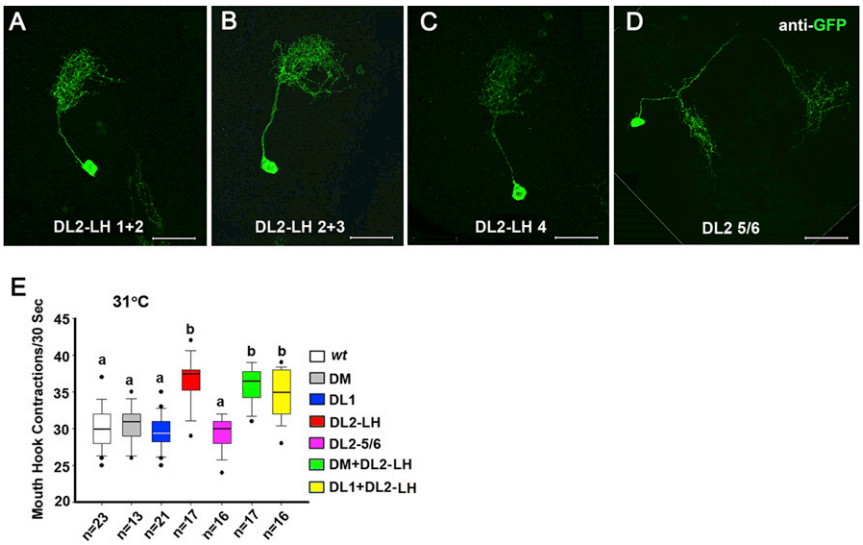

Figure 5. Activation of a Subset of DA Neurons is Sufficient to Mimic PA Stimulation

(A–C) The effects of stimulating one or two defined DA neurons on the PA-induced feeding response were analyzed using the FLP-Gal80 technique. Examples of the processes of four DA neurons (DL2-1 to DL2-4; named as DL2-LH) show restricted distribution to the LH region. (A) An example of the projection of two DL2-LH neurons (DL2-LH1 and DL2-LH2) (see Movie S4). (B) An example of the projection of two DL2-LH neurons (DL2-LH2 and DL2-LH3) (see Movie S5). (C) An example of the projection of one DL2-LH neurons (DL2-LH4) (see Movie S6). Scale bars, 20 μm.

(D) An example of the projection of two other DL2 neurons (DL2-5/6). (See Movie S7.)

(E) Quantification of feeding activities of fed larvae (*hsFLP;;Th-Gal4,UAS-mCD8-GFP/UAS-dTrpA1; tub > Gal80 >*) expressing dTrpA1 in the subset of DA neurons in the absence of PA (see 23°C controls in Figure S5C). Larvae were individually assayed for feeding behavior followed by examining GFP-labeled DA neurons in the brain. DL2-LH: larvae showing one or two DL2 neurons from the four-cell cluster that project ipsilaterally to the LH region. DM and DL1: larvae displaying one or two DM and DL1 neurons, respectively. DM+DL2-LH and DL1+DL2-LH: larvae displaying one or two DM and DL1 neurons, plus one or two DL2-LH neurons. Different letters indicate statistically significant differences. $p < 0.01$.

can be a useful model for elucidating the molecular and neural mechanisms underlying the perception of olfactory reward and behavioral organization.

Role of DA Neurons in Odor Perception

Animals have innate abilities to selectively associate various attractive olfactory cues with anticipated changes in their surroundings, such as the emergence of favored energy sources or approaching mates. We have found that a small number of DA neurons (DL2-LH) play a direct role in organizing an enhanced appetite for the favored sugar-rich liquid medium in response to an attractive food cue. Neuroanatomical and functional imaging evidence suggest that these DA neurons are likely postsynaptic to the second-order olfactory neurons; they may form synaptic connections in the lateral horn, one of the two higher-order olfactory centers in the insect brain. We have also shown that blocking the mushroom body, the other higher-order olfactory center of the insect brain, had no adverse effect on the appetitive odor induction of appetite. Together, these observations suggest that DL2-LH neurons define an integration mechanism that mediates the experience-independent conversion of appetitive olfactory codes into motivational states specific for the feeding of highly rewarding food in fed animals. Interestingly, it has been shown that in adult flies, transformation of pheromones to sex drive in the lateral horn involves other neurotransmitters, such as GABA instead of DA (Ruta et al., 2010). Therefore, the neurochemicals and signaling mechanisms underlying the olfactory circuits for feeding and mating may be rather different. Future work will determine how DA neurons function in the reception and processing of appetitive odor inputs.

The Potential Role of DopR in Appetitive Motivation

We have obtained evidence that the D1-like DA receptor DopR is required for the appetitive odor-driven feeding response. However, the functional significance of DopR remains unclear. It is possible that DopR may define a downstream neural mechanism that determines the motivational state for the feeding response in fed larvae to highly rewarding food. DopR may exert such an effect through regulation of the signaling activity of a neurotransmitter(s)/neuropeptide(s). Therefore, future investigation of the DopR activity may lead to the discovery of a yet uncharacterized motivation circuit for reward-driven feeding behavior in fly larvae.

The NPF System Mediates a Gating Mechanism in DA Neurons

We found that NPF neurons project to the lateral horn region and that NPF signaling is required for appetitive odor-induced feeding. Our evidence also suggests that NPF directly acts on DL2-LH neurons via NPFR1. Because knockdown of NPFR1 signaling blocked excitation of DL2-LH neurons by appetitive odor and larval appetitive odor-induced feeding, this observation indicates that the NPF/NPFR1 pathway has a previously uncharacterized role in gating odor excitation of the DA neurons. Both NPF and NPFR1 activities are modulated by various physiological states (Shohat-Ophir et al., 2012; Wu et al., 2005), suggesting that the NPF/NPFR1 pathway could be well-suited for coupling physiological changes with DA signaling in the olfactory reward circuit.

Two Opposite Effects of NPFR1 on DA Neurons

The *NPFR1-Gal4* is expressed in most of the DA neurons in the larval CNS, suggesting that NPFR1 likely functions in diverse DA neuronal pathways. It has been reported that activation of NPFR1 inhibits the activity of DA neurons in the mushroom body, resulting in hunger-induced expression of appetitive memory (Krashes et al., 2009). However, our evidence suggests that NPFR1 expression in the LH-projecting DA neurons may

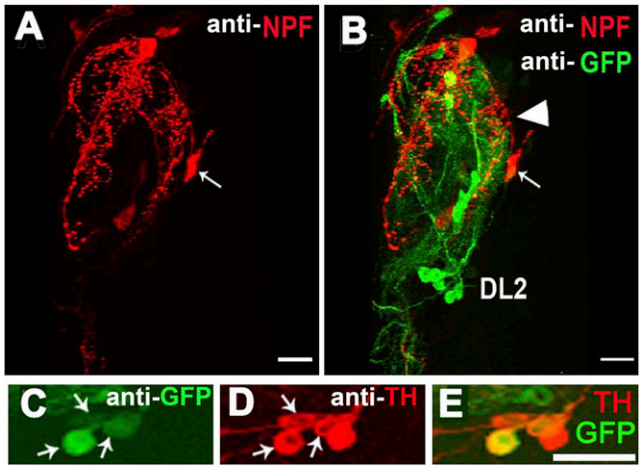

Figure 6. Anatomical Analysis of NPF, NPFR1, and DA Neurons in the LH

(A and B) Immunofluorescence of anti-GFP in *Th-Gal4* neurons (green) and anti-NPF (red). Lateral view. Arrow: Dorsal lateral NPF neuron. Arrowhead: LH region (also see Movie S8). Scale bar, 20 μm. Genotype: *Th-Gal4/UAS-mCD8GFP*.

(C–E) Colocalization of *NPFR1-Gal4* neurons (green) and DA neurons (red). Arrows indicate the three overlapping neurons. Scale bar, 20 μm. Genotype: *NPFR1-Gal4/UAS-mCD8GFP*.

enhance the activity of these neurons because TrpA1-mediated excitation of DL2-LH neurons elicited PA-stimulated feeding in fed larvae, whereas knockdown of *npfr1* activity in DA neurons attenuated larval appetitive odor-induced feeding. Thus, NPFR1 can exert two opposite effects in functionally distinct DA neurons. It remains to be determined whether these opposing effects of NPFR1 may reflect the difference in the cellular properties of two subpopulations of DA neurons or downstream effectors (e.g., the G protein subunits) of the NPFR1 pathway.

The Potential Roles of NPY and DA in the Mammalian Olfactory System

It has been reported that at least 70% of patients with idiopathic Parkinson's disease have loss of or defective sense of smell (Hawkes, 1995). These clinical findings have raised an interesting possibility that DA may be an important neural substrate for olfaction. In mammals, the NPY system has been implicated in modulating DA neurons from midbrain and other brain sites. DA and NPY neurons are also found in the higher order centers of the olfactory and vomeronasal systems in diverse vertebrate species, but their neurobiological significance remains unclear (Ubeda-Bañon et al., 2008). These observations have raised the question of whether dopamine and NPY/NPF systems may play parallel roles in higher-order olfactory processing in both vertebrates and invertebrates. We suggest that the study of NPF/DA-mediated olfactory processing in *Drosophila* larvae may yield useful mechanistic insights into the general understanding of how the brain controls appetitive behaviors in diverse animals.

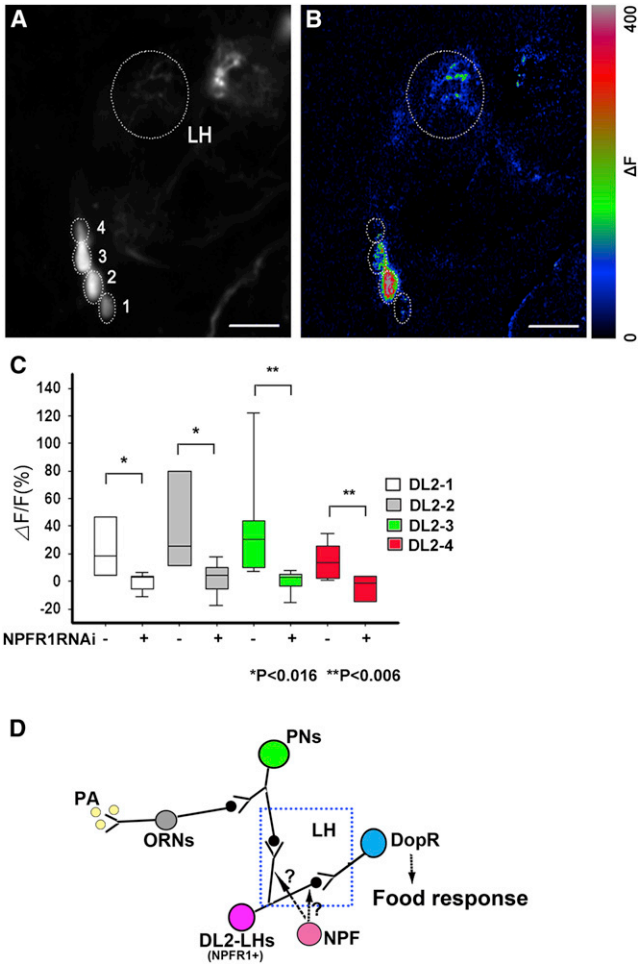

Figure 7. The NPF/NPFR1 Pathway Modulates the Activity of DL2-LH Neurons

(A) The four DL2-LH neurons labeled by *GCaMP3* in the brain of third-instar *Th-Gal4*/UAS-*GCaMP3* larvae. LH is marked by a dotted circle. Scale bar, 20 μm.

(B) Ca^{2+} imaging analysis revealed PA-induced fluorescence increases in DL2-LH neurons (ΔF) (see Movie S9). Scale bar, 20 μm.

(C) Quantification of fluorescence changes (ΔF/F) in the soma of DL2-LH neurons with or without expressing NPFR1RNAi, n = 8. Statistical analysis was performed using the Mann-Whitney test. *p < 0.016; **p < 0.006 (Figure S5E).

(D) A working model describing a proposed neural circuit for PA-induced appetitive response. PA excites larval olfactory receptor neurons (ORNs), which relay the odor information to projection neurons (PNs). PNs transduce odor representations to the higher-order olfactory center (the lateral horn, LH). Four DA neurons (DL2-LH) that are responsive to PA may form synaptic connections with PNs in the LH region. NPF modulates DL2-LH neuronal activity via its receptor NPFR1. NPFR1 signaling may be required for the reception of olfactory inputs or transmission of DA-coded signal outputs by DL2-LH neurons or both. DL2-LH neurons may directly signal to yet uncharacterized LH-projecting DopR neurons, thereby transforming processed food odor information to appetitive drive.

See also Figure S5D.

EXPERIMENTAL PROCEDURES

Fly Stocks and Larval Growth

All flies are in the w^{1118} background. Larvae were reared at 23°C as previously described (Wu et al., 2003, 2005). Briefly, eggs were collected onto an apple

juice agar plate with yeast paste for 2 hr to obtain synchronized larvae. After becoming second instars (50 hr after egg laying [AEL]), larvae were transferred to fresh yeast paste on apple juice agar. The early third-instar larvae (~74 hr AEL) were fed with yeast paste before being used for behavioral and other experiments. The transgenic flies used include *Th-Gal4* (Friggi-Grelin et al., 2003), *Tdc2-Gal4* (Cole et al., 2005), *GH146-Gal4*, *OK107-Gal4*, *Or83b-Gal4*, *GH146-LexA* (Lai and Lee, 2006), *UAS-shi^{ts1}* (Kitamoto, 2001), *UAS-dTrpA1* (Hamada et al., 2008), *UAS-GcaMP3* (Tian et al., 2009), *UAS-Denmark* (Nicolaï et al., 2010), *Or83b-LexA*, *UAS-CD4::spGFP$^{1-10}$*, *LexAop-CD4::spGFP^{11}*, *Tub > Gal80 >* (Gordon and Scott, 2009), *Th-Gal80* (Sitaraman et al., 2008), and *Tsh-Gal80* lines (Yu et al., 2010). *UAS-DopRRANi* (KK107058), *UAS-DopR2RNAi* (KK105324), and *UAS-DopEcRRNAi* (Kk103494) were obtained from the VDRC stock center. *UAS-D2RRNAi* (JF02025) was from the *Drosophila* RNAi Screening Center. The mutant flies, *or83b^{1}*, *or83b^{2}* (Larsson et al., 2004), *DopR^{f02676}* (Kong et al., 2010; Lebestky et al., 2009), and *tβh^{mM18}* (Monastirioti et al., 1996), were described previously.

Behavioral Experiments

Assays for quantification of mouth hook contraction rate in liquid or solid food were previously described (Wu et al., 2003, 2005). The food ingestion assay was carried out by feeding a group of 20 larvae 10% glucose liquid media containing 1% food dye FD&C Blue No. 1 (Sigma-Aldrich, St. Louis) for 2 min. After rinsing with a copious amount of water, larvae were quickly frozen in liquid nitrogen and homogenized in 100 μl 0.1 M phosphate buffer (pH 7.2). The homogenates were centrifuged for 13,000 rpm for 10 min, and the supernatants were analyzed spectrophotometrically for absorbance at 625 nm (Edgecomb et al., 1994). Homogenates of control larvae fed in undyed food were used for establishing the baseline of absorbance.

Odor stimulation of fly larvae was performed inside a sealed 1.5 l glass chamber with 15 ppm of pentyl acetate (PA) (Sigma-Aldrich, 628-63-7), which is attained by adding 5 μl PA to a small container at the bottom of the chamber. After incubation for 2 min, the PA container was quickly removed to keep the level of PA fumes at about 15 ppm. Similarly, the odor levels of balsamic vinegar, 1-hexonal (Sigma-Aldrich, 111-27-3), and geranyl acetate (Sigma-Aldrich, 105-87-3) were adjusted to 5, 20, and 5 ppm, respectively. The odor concentrations were measured with a photoionization detector (Rae Systems, San Jose, CA, USA, MiniRAE 3000).

Larvae were prefed for a total of 30 min, including a feeding time in the presence of odor cues. For odor treatment, about 25 larvae were transferred to a 35 mm petri dish containing 100 μl yeast paste, which was immediately placed inside the odor stimulation chamber. The petri dish was covered with a piece of mesh and a wet tissue to prevent larvae from escaping. After stimulation, the larvae were rinsed with a copious amount of water and transferred to the liquid food for the feeding test. After acclimating for 1 min, larvae were videotaped for 4 min. The mouth hook contractions of each larva were counted over a 30 s test period. For food deprivation, larvae were held on wet paper for a desired time period. To express *UAS-shi^{ts1}* and *UAS-dTrpA1* at 31°C before odor stimulation, larvae were fed in warm yeast paste in a 31°C incubator for a desired period and rinsed with 31°C water for subsequent feeding assays.

3IY and L-Dopa Feeding

The protocols for 3IY and L-dopa treatment were modified from Bainton et al. (2000) and Neckameyer (1996). Synchronized larvae were fed in yeast paste containing 10 mg/ml of the TH inhibitor 3-iodo-tyrosine (3IY, Sigma-Aldrich) for 6 hr or containing 0.5 mg/ml of L-Dopa (Sigma-Aldrich) for 2 hr before the behavioral test.

Molecular Cloning

To construct the *NPFR1-Gal4* driver, a 1.6 kb DNA fragment containing the 5′ regulatory region and part of the first exon was amplified by genomic PCR and cloned into the pCaSpeR-Gal4 vector at the EcoR I site.

Mosaic Analysis

Activation of individual *Th-Gal4* neurons in third-instar larvae was achieved by using the FLP-out Gal80 technique (Gordon and Scott, 2009; Marella et al., 2012). First-instar larvae (*hsFLP;;Th-Gal4,UAS-mCD8-GFP/UAS-dTrpA1; tub > Gal80 >*) were heat-treated for 10 min at 37°C to induce *Th-Gal4*-expressing clones. At 74 hr AEL, the larvae were incubated in 31°C for 30 min to activate the dTrpA1-expressing neurons. About 700 fed larvae were randomly picked, and their feeding responses to liquid food were scored individually in the absence of PA. Each larva was dissected to visualize the mCD8GFP/dTrpA1-expressing neurons following the behavioral assay. Based on the anatomical analysis, the feeding responses of fed larvae expressing dTrpA1 in one or a small number of DA neurons were collected for the analysis in Figure 6.

Targeted Lesion of Th-Gal4 Neurons

The 337 nm nitrogen laser unit (Micro Point, SRS Stanford Research System, Sunnyvale, CA, USA, model 337-USAS) was calibrated and performed as previously described (Xu et al., 2008). The *Th-Gal4* neurons were shown by a nucleus GFP (*UAS-nlsGFP*). Briefly, 6 to 9 s instar larvae (48 hr AEL) were transferred onto a microscope slide containing 150 μl water. The larvae were then exposed to 250 μl ether in a 90 mm petri dish for 3 min. A coverslip was placed on the immobilized larvae for laser treatment. The laser beam was focused on the nucleus, and three bursts of 30 shots were fired at a rate of 3 shots per second. Treated neurons showed invisible GFP signals (Figures S5A and S5B). The larvae were allowed to recover for 24 to 28 hr on fresh food before behavioral assays. After being assayed individually for feeding behavior, larvae were dissected to examine the GFP expression pattern. Those larvae that showed diminished GFP signals in neurons of interest were analyzed. Larvae from the control group were handled in the same way, except without laser treatment.

Calcium Imaging

Th-Gal4/UAS-GcaMP3 larvae (74 hr AEL) were used for calcium imaging odor excitation of *Th-Gal4* neurons. To knockdown the activity of NPFR1, UAS-*npfr1RNAi* was coexpressed with UAS-*GcaMP3* driven by *Th-Gal4*. Briefly, the larva was cut at the thoracic segment to keep the anterior part of the larva intact. The mouthpart of the preparation was inserted into a small hole in a plastic coverslip to expose larval sensory organs to air (Asahina et al., 2009). Low melting agarose (1.5%; Sigma-Aldrich) was used to seal the gap. After chilling for 2 min on ice, the preparation was incubated in adult hemolymph-like (AHL) saline (Wang et al., 2003) for imaging odor response. Imaging was performed using a Zeiss LSM510 META confocal microscope under a 40× water immersion lens. Images were captured at 1.57 s per frame with a resolution of 512 × 512 pixels. A z stack of images (512 × 512 pixels) was collected for verification of DL2 neurons after each experiment. To apply odor, 15 ppm of PA was applied through a 2 ml plastic syringe (Becton Dickinson, Franklin Lakes, NJ, USA) with a needle. The tip of the needle was positioned about 2 cm away from the sample. The delivery speed is around 0.5 ml/second. Imaging data were collected from intact larval brains showing odor-stimulated fluorescence changes at the LH region and identifiable DL2 neurons and processed using ImageJ. F values represent the average fluorescence intensity of five frames immediately prior to the delivery of odor. The peak fluorescence (F_s) was calculated as the average intensity of two frames after odor stimulation. The change in fluorescence (ΔF) = $F_s - F$. Pseudocolored images were generated by ImageJ (U.S. National Institutes of Health, http://rsbweb.nih.gov/ij/).

Immunostaining

Dissection of intact CNS tissues of larvae (74 hr AEL) was performed in cold PBS and fixed in 4% fresh paraformaldehyde solution for 30 min at room temperature. The tissues were then washed with PBS/Triton (PBT) (0.3% Triton X-100 in PBS) five times (15 min each), blocked 30 min with PBT containing 5% normal goat serum, and incubated with primary antibody in blocking buffer overnight at 4°C. After washing with PBT five times, the tissues were incubated with the secondary antibody in PBT overnight at 4°C. Images were collected using a Zeiss LSM510 META confocal microscope and processed with ImageJ and Adobe Photoshop. Antibodies include chicken anti-GFP (Invitrogen, Carlsbad, CA, USA; 1:1,000), rabbit anti-DsRed (Clontech, Mountain View, CA, USA; 1:200), mouse anti-FasII (the Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA, USA; 1:500), rabbit anti-Tyrosine hydroxylase (gift from Wendy Neckameyer; 1:500), and rabbit anti-NPF (1:2,000) (Wu et al., 2003). Alexa Fluor-488 goat anti-chicken

(Invitrogen; 1:2,000), Alexa Fluor-568 goat anti-rabbit (Invitrogen; 1:2,000), and Alexa Fluor-568 goat anti-mouse (Invitrogen; 1:2,000) were used as secondary antibodies.

Statistical Analysis

The statistical analyses were performed using one-way ANOVA, followed by Dunn's post hoc test in all figures, except in Figure 7, where the Mann-Whitney test is used.

For further details, please refer to the Extended Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, five figures, one table, and nine movies and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2013.02.003.

LICENSING INFORMATION

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REFERENCES

Asahina, K., Louis, M., Piccinotti, S., and Vosshall, L.B. (2009). A circuit supporting concentration-invariant odor perception in Drosophila. J. Biol. *8*, 9.

Bainton, R.J., Tsai, L.T., Singh, C.M., Moore, M.S., Neckameyer, W.S., and Heberlein, U. (2000). Dopamine modulates acute responses to cocaine, nicotine and ethanol in Drosophila. Curr. Biol. *10*, 187–194.

Bragulat, V., Dzemidzic, M., Bruno, C., Cox, C.A., Talavage, T., Considine, R.V., and Kareken, D.A. (2010). Food-related odor probes of brain reward circuits during hunger: a pilot FMRI study. Obesity (Silver Spring) *18*, 1566–1571.

Brown, M.R., Crim, J.W., Arata, R.C., Cai, H.N., Chun, C., and Shen, P. (1999). Identification of a Drosophila brain-gut peptide related to the neuropeptide Y family. Peptides *20*, 1035–1042.

Cole, S.H., Carney, G.E., McClung, C.A., Willard, S.S., Taylor, B.J., and Hirsh, J. (2005). Two functional but noncomplementing Drosophila tyrosine decarboxylase genes: distinct roles for neural tyramine and octopamine in female fertility. J. Biol. Chem. *280*, 14948–14955.

Dierick, H.A., and Greenspan, R.J. (2007). Serotonin and neuropeptide F have opposite modulatory effects on fly aggression. Nat. Genet. *39*, 678–682.

Draper, I., Kurshan, P.T., McBride, E., Jackson, F.R., and Kopin, A.S. (2007). Locomotor activity is regulated by D2-like receptors in Drosophila: an anatomic and functional analysis. Dev. Neurobiol. *67*, 378–393.

Edgecomb, R.S., Harth, C.E., and Schneiderman, A.M. (1994). Regulation of feeding behavior in adult Drosophila melanogaster varies with feeding regime and nutritional state. J. Exp. Biol. *197*, 215–235.

Feinberg, E.H., Vanhoven, M.K., Bendesky, A., Wang, G., Fetter, R.D., Shen, K., and Bargmann, C.I. (2008). GFP Reconstitution Across Synaptic Partners (GRASP) defines cell contacts and synapses in living nervous systems. Neuron *57*, 353–363.

Fishilevich, E., Domingos, A.I., Asahina, K., Naef, F., Vosshall, L.B., and Louis, M. (2005). Chemotaxis behavior mediated by single larval olfactory neurons in Drosophila. Curr. Biol. *15*, 2086–2096.

Friggi-Grelin, F., Coulom, H., Meller, M., Gomez, D., Hirsh, J., and Birman, S. (2003). Targeted gene expression in Drosophila dopaminergic cells using regulatory sequences from tyrosine hydroxylase. J. Neurobiol. *54*, 618–627.

Gordon, M.D., and Scott, K. (2009). Motor control in a Drosophila taste circuit. Neuron *61*, 373–384.

Hamada, F.N., Rosenzweig, M., Kang, K., Pulver, S.R., Ghezzi, A., Jegla, T.J., and Garrity, P.A. (2008). An internal thermal sensor controlling temperature preference in Drosophila. Nature *454*, 217–220.

Hawkes, C.M. (1995). Diagnosis and treatment of Parkinson's disease. Anosmia is a common finding. BMJ *310*, 1668.

Heisenberg, M. (2003). Mushroom body memoir: from maps to models. Nat. Rev. Neurosci. *4*, 266–275.

Hewes, R.S., and Taghert, P.H. (2001). Neuropeptides and neuropeptide receptors in the Drosophila melanogaster genome. Genome Res. *11*, 1126–1142.

Inagaki, H.K., Ben-Tabou de-Leon, S., Wong, A.M., Jagadish, S., Ishimoto, H., Barnea, G., Kitamoto, T., Axel, R., and Anderson, D.J. (2012). Visualizing neuromodulation in vivo: TANGO-mapping of dopamine signaling reveals appetite control of sugar sensing. Cell *148*, 583–595.

Kahsai, L., and Zars, T. (2011). Learning and memory in Drosophila: behavior, genetics, and neural systems. Int. Rev. Neurobiol. *99*, 139–167.

Kim, Y.C., Lee, H.G., and Han, K.A. (2007). D1 dopamine receptor dDA1 is required in the mushroom body neurons for aversive and appetitive learning in Drosophila. J. Neurosci. *27*, 7640–7647.

Kitamoto, T. (2001). Conditional modification of behavior in Drosophila by targeted expression of a temperature-sensitive shibire allele in defined neurons. J. Neurobiol. *47*, 81–92.

Kong, E.C., Woo, K., Li, H., Lebestky, T., Mayer, N., Sniffen, M.R., Heberlein, U., Bainton, R.J., Hirsh, J., and Wolf, F.W. (2010). A pair of dopamine neurons target the D1-like dopamine receptor DopR in the central complex to promote ethanol-stimulated locomotion in Drosophila. PLoS ONE *5*, e9954.

Krashes, M.J., DasGupta, S., Vreede, A., White, B., Armstrong, J.D., and Waddell, S. (2009). A neural circuit mechanism integrating motivational state with memory expression in Drosophila. Cell *139*, 416–427.

Lai, S.L., and Lee, T. (2006). Genetic mosaic with dual binary transcriptional systems in Drosophila. Nat. Neurosci. *9*, 703–709.

Larsson, M.C., Domingos, A.I., Jones, W.D., Chiappe, M.E., Amrein, H., and Vosshall, L.B. (2004). Or83b encodes a broadly expressed odorant receptor essential for Drosophila olfaction. Neuron *43*, 703–714.

Lebestky, T., Chang, J.S., Dankert, H., Zelnik, L., Kim, Y.C., Han, K.A., Wolf, F.W., Perona, P., and Anderson, D.J. (2009). Two different forms of arousal in Drosophila are oppositely regulated by the dopamine D1 receptor ortholog DopR via distinct neural circuits. Neuron *64*, 522–536.

Lee, K.S., You, K.H., Choo, J.K., Han, Y.M., and Yu, K. (2004). Drosophila short neuropeptide F regulates food intake and body size. J. Biol. Chem. *279*, 50781–50789.

Lingo, P.R., Zhao, Z., and Shen, P. (2007). Co-regulation of cold-resistant food acquisition by insulin- and neuropeptide Y-like systems in Drosophila melanogaster. Neuroscience *148*, 371–374.

Lowe, M.R., and Butryn, M.L. (2007). Hedonic hunger: a new dimension of appetite? Physiol. Behav. *91*, 432–439.

Marella, S., Mann, K., and Scott, K. (2012). Dopaminergic modulation of sucrose acceptance behavior in Drosophila. Neuron *73*, 941–950.

Marin, E.C., Watts, R.J., Tanaka, N.K., Ito, K., and Luo, L. (2005). Developmentally programmed remodeling of the Drosophila olfactory circuit. Development *132*, 725–737.

Masse, N.Y., Turner, G.C., and Jefferis, G.S. (2009). Olfactory information processing in Drosophila. Curr. Biol. *19*, R700–R713.

Melcher, C., and Pankratz, M.J. (2005). Candidate gustatory interneurons modulating feeding behavior in the Drosophila brain. PLoS Biol. *3*, e305.

Monastirioti, M., Linn, C.E., Jr., and White, K. (1996). Characterization of Drosophila tyramine beta-hydroxylase gene and isolation of mutant flies lacking octopamine. J. Neurosci. *16*, 3900–3911.

Nässel, D.R., and Winther, A.M. (2010). Drosophila neuropeptides in regulation of physiology and behavior. Prog. Neurobiol. *92*, 42–104.

Neckameyer, W.S. (1996). Multiple roles for dopamine in Drosophila development. Dev. Biol. *176*, 209–219.

Nicolaï, L.J., Ramaekers, A., Raemaekers, T., Drozdzecki, A., Mauss, A.S., Yan, J., Landgraf, M., Annaert, W., and Hassan, B.A. (2010). Genetically encoded dendritic marker sheds light on neuronal connectivity in Drosophila. Proc. Natl. Acad. Sci. USA *107*, 20553–20558.

Ramaekers, A., Magnenat, E., Marin, E.C., Gendre, N., Jefferis, G.S., Luo, L., and Stocker, R.F. (2005). Glomerular maps without cellular redundancy at successive levels of the Drosophila larval olfactory circuit. Curr. Biol. *15*, 982–992.

Root, C.M., Ko, K.I., Jafari, A., and Wang, J.W. (2011). Presynaptic facilitation by neuropeptide signaling mediates odor-driven food search. Cell *145*, 133–144.

Ruta, V., Datta, S.R., Vasconcelos, M.L., Freeland, J., Looger, L.L., and Axel, R. (2010). A dimorphic pheromone circuit in Drosophila from sensory input to descending output. Nature *468*, 686–690.

Selcho, M., Pauls, D., Han, K.A., Stocker, R.F., and Thum, A.S. (2009). The role of dopamine in Drosophila larval classical olfactory conditioning. PLoS ONE *4*, e5897.

Shohat-Ophir, G., Kaun, K.R., Azanchi, R., Mohammed, H., and Heberlein, U. (2012). Sexual deprivation increases ethanol intake in Drosophila. Science *335*, 1351–1355.

Sitaraman, D., Zars, M., Laferriere, H., Chen, Y.C., Sable-Smith, A., Kitamoto, T., Rottinghaus, G.E., and Zars, T. (2008). Serotonin is necessary for place memory in Drosophila. Proc. Natl. Acad. Sci. USA *105*, 5579–5584.

Srivastava, D.P., Yu, E.J., Kennedy, K., Chatwin, H., Reale, V., Hamon, M., Smith, T., and Evans, P.D. (2005). Rapid, nongenomic responses to ecdysteroids and catecholamines mediated by a novel Drosophila G-protein-coupled receptor. J. Neurosci. *25*, 6145–6155.

Stocker, R.F., Heimbeck, G., Gendre, N., and de Belle, J.S. (1997). Neuroblast ablation in Drosophila P[GAL4] lines reveals origins of olfactory interneurons. J. Neurobiol. *32*, 443–456.

Su, C.Y., Menuz, K., and Carlson, J.R. (2009). Olfactory perception: receptors, cells, and circuits. Cell *139*, 45–59.

Terhzaz, S., Rosay, P., Goodwin, S.F., and Veenstra, J.A. (2007). The neuropeptide SIFamide modulates sexual behavior in Drosophila. Biochem. Biophys. Res. Commun. *352*, 305–310.

Tian, L., Hires, S.A., Mao, T., Huber, D., Chiappe, M.E., Chalasani, S.H., Petreanu, L., Akerboom, J., McKinney, S.A., Schreiter, E.R., et al. (2009). Imaging neural activity in worms, flies and mice with improved GCaMP calcium indicators. Nat. Methods *6*, 875–881.

Ubeda-Bañon, I., Novejarque, A., Mohedano-Moriano, A., Pro-Sistiaga, P., Insausti, R., Martinez-Garcia, F., Lanuza, E., and Martinez-Marcos, A. (2008). Vomeronasal inputs to the rodent ventral striatum. Brain Res. Bull. *75*, 467–473.

Volkow, N.D., and Wise, R.A. (2005). How can drug addiction help us understand obesity? Nat. Neurosci. *8*, 555–560.

Vosshall, L.B., and Stocker, R.F. (2007). Molecular architecture of smell and taste in Drosophila. Annu. Rev. Neurosci. *30*, 505–533.

Wang, J.W., Wong, A.M., Flores, J., Vosshall, L.B., and Axel, R. (2003). Two-photon calcium imaging reveals an odor-evoked map of activity in the fly brain. Cell *112*, 271–282.

Wen, T., Parrish, C.A., Xu, D., Wu, Q., and Shen, P. (2005). Drosophila neuropeptide F and its receptor, NPFR1, define a signaling pathway that acutely modulates alcohol sensitivity. Proc. Natl. Acad. Sci. USA *102*, 2141–2146.

Wu, Q., Wen, T., Lee, G., Park, J.H., Cai, H.N., and Shen, P. (2003). Developmental control of foraging and social behavior by the Drosophila neuropeptide Y-like system. Neuron *39*, 147–161.

Wu, Q., Zhang, Y., Xu, J., and Shen, P. (2005). Regulation of hunger-driven behaviors by neural ribosomal S6 kinase in Drosophila. Proc. Natl. Acad. Sci. USA *102*, 13289–13294.

Xu, J., Sornborger, A.T., Lee, J.K., and Shen, P. (2008). Drosophila TRPA channel modulates sugar-stimulated neural excitation, avoidance and social response. Nat. Neurosci. *11*, 676–682.

Xu, J., Li, M., and Shen, P. (2010). A G-protein-coupled neuropeptide Y-like receptor suppresses behavioral and sensory response to multiple stressful stimuli in Drosophila. J. Neurosci. *30*, 2504–2512.

Yapici, N., Kim, Y.J., Ribeiro, C., and Dickson, B.J. (2008). A receptor that mediates the post-mating switch in Drosophila reproductive behaviour. Nature *451*, 33–37.

Yu, J.Y., Kanai, M.I., Demir, E., Jefferis, G.S., and Dickson, B.J. (2010). Cellular organization of the neural circuit that drives Drosophila courtship behavior. Curr. Biol. *20*, 1602–1614.