A NEURAL CIRCUIT MECHANISM FOR FOOD ODOR PERCEPTION AND APPETITIVE AROUSAL IN *DROSOPHILA* LARVAE

by

YUHAN PU

(Under the Direction of Ping Shen)

ABSTRACT

The sense of smell is crucial to animals for providing external information about food properties and quality, potential threats and pleasure. Odors induce various emotional or cognitive responses which trigger behavior activities. However, understanding how olfactory inputs are perceived by cognitive processing centers and translates into behavior outputs remains challenging. Due to their simple nervous systems and stereotyped olfactory driven behavior, *Drosophila* larvae constitute a well-established model for studying odor representation, odor perception and odor driven behaviors.

Here, we found that after brief exposure to appetitive odorants, 3rd instar fed larvae display feeding rate increase on sugar-rich food. We identified four appetitive odor-responsive dopaminergic neurons (DL2) as a third order olfactory neurons. Activation of those DL2 dopamine neurons mimics the appetitive feeding effect of odor stimulation. Furthermore, we showed that neuropeptide F(NPF), a neuropeptide Y-like neuromodulator and its receptor NPFR1 mediate a gating mechanism for reception of olfactory inputs in DL2 neurons.

Drosophila larvae also display selective recognition of food related odors, indicated as an inverted U function: doses either too high or too low are not appetitive. We found that the four DL2 DA neurons combinatorially integrate olfactory inputs into one dimensional DA signals. The intensity of odor stimuli to DL2 neurons positively regulates DA signal outputs. Then, those odor-induced DA signals relay to downstream target neurons which express D1-like receptor Dop1R1 and NPF. Furthermore, an ensemble of Dop1R1 and its effectors play two functional roles: 1) A Dop1R1/Gβ13F/Irk2-mediated inhibitory effect on the NPF neurons when strong odor stimuli trigger excessive release of odor-evoked DA signals, 2) A Dop1R1/Gαs-mediated default excitatory mechanism that mediates NPF neuronal response to any odor stimuli that are at or above the minimal threshold strength. Thus, through this tuning mechanism, Dop1R1 precisely restrict NPF neuronal response to a narrow range of odor-evoked DA signals levels.

Overall, we have developed a novel behavioral paradigm using *Drosophila* larvae to investigate reception and processing of appetitive olfactory inputs in higher-order olfactory centers, as well as how food related olfactory cues are precisely perceived in downstream reward systems to trigger appetitive behavior output. Our findings may provide a general understanding of molecular and cellular mechanisms underlying DA-modulated appetitive odor perception and odor induced reward anticipation.

INDEX WORDS: *Drosophila melanogaster*, Dopamine, D1 receptor, Neuropeptide F, Olfactory processing, Reward anticipation, Odor driven behaviors, Cognitive control.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

OVERVIEW OF OLFACTORY SYSTEM IN FLIES:

Olfaction is a type of sensory modalities that allows animals to perceive a staggering number and variety of volatile chemicals in their external environment. As an evolutionarily primitive sense, olfaction is critical for survival across the animal kingdom from flies to mammals-- finding food, searching for mates, or avoiding predation all depend on detecting, identifying, and discriminating odors. In many studies, odors induce various mental and physiological responses and behavior activities. However, there is still a big knowledge gap for understanding the rules by which olfactory input is interpreted in a brain and translated into the behavioral output. In contrast with the complexity and redundancy of the mammalian central nervous system, *Drosophila* flies combine a numerically simple brain, a correspondingly moderate behavioral complexity, and the availability of a rich toolbox for transgenic manipulation. Those features make *Drosophila* an excellent model to study odor perception and odor encoding in the central nervous system. In this review. I will focus on introducing the organization of the olfactory system in *Drosophila.*

The olfactory system of *Drosophila* has been well characterized at a single-cell resolution. The larva's olfactory system is much simpler than the adult system, at least in terms of cell number. *Drosophila* Olfactory receptors(ORs) were identified in 1999 by combined difference cloning and bioinformatics approaches (Clyne et al., 1999; Gao and

Chess, 1999; Vosshall et al., 1999). Using in situ hybridization Fishilevich et al. identified 25 larval OR genes expressed in OSNs (Fishilevich et al., 2005). Interestingly, fly ORs represent a novel family of membrane proteins with a distinct seven-transmembrane topology (Benton et al., 2006; Wistrand et al., 2006). Besides the conventional OR, most olfactory receptor neurons also express OR co-receptor Or83b, which is necessary for the proper localization and function of conventional OR (Larsson et al., 2004).The Or83b mutation disrupts behavioral and electrophysiological responses to many odorants, indicating an essential general role of Or83b co-receptor in olfaction (Larsson et al., 2004).

At the olfactory receptor neurons level, two bilaterally symmetric dorsal organ ganglions each contains 21 olfactory receptor neurons which were identified by electrophysiological and ablation behavior studies (Kreher et al., 2005; Oppliger et al., 2000; Heimbeck et al., 2001). However, ORN number surge from 21 to 1300 in the adult stage, probably due to a predominant long-distance chemical navigation requirement. Then each ORN project purely ipsilaterally into the larval antennal lobe(LAL) which is analogous to the vertebrate olfactory bulb (Fishilevich et al., 2005; Heimbeck et al., 1999). Within the LAL, all of the axons of ORNs expressing the same OR project to the same glomerulus. Thus, each of the 21 ORNs projects to one of 21 distinct glomeruli of the LAL in fly larvae (Ramaekers et al., 2005). Due to the simple connective pattern, odor input was therefore thought to be transmitted faithfully at the level of the ORNs and LAL. Calcium imaging results show that each odorant activates a specific set of glomeruli, creating a glomerular pattern of activation in the AL that is stereotypical between individual flies (Ng et al., 2002; Wang et al., 2003). In those glomeruli, ORNs

target two types of interneurons. The first type is local interneurons (LNs) which provide "horizontal" connections among glomeruli (Ng et al., 2002; Wilson, 2005). The second type is cholinergic projection neurons (PNs) which link individual glomeruli "vertically" with two higher olfactory centers, the mushroom body (MB) calyx and the lateral horn (LH) (Marin, 2005; Stocker, 1994). Single-cell clones in the PN-specific GH146-Gal4 driver showed the connection between glomeruli dendritic arborizations (Stocker et al., 1997). By studying immunoreactivity in PN terminals, it was found that PNs terminate in a stereotypic manner towards the secondary olfactory centers lateral horn region, but their spatial localizations are only partially understood (Ramaekers et al., 2005). PNs activities to odors stimulation could be visualized by functional imaging and recording of spikes. The result showed a clear correlation between the ensemble of activated PNs and the types of odor applied (Ng et al., 2002; Wang et al., 2003; Laurent, 1999). In addition, since most PNs are uniglomerular and receive signals from a single type of ORN, information detected by different ORN channels is not likely to be fully integrated at the level of the PNs. Noticeably, the larvae olfactory pathway lacks cellular redundancy by having a 1:1:1:1 projection pattern from ORNs to AL glomeruli to PNs to MB calyx glomeruli. Its simplest form and straightforward connectivity make *Drosophila* larvae an attractive elementary model for studying olfactory coding.

One of the higher order olfactory centers, the mushroom bodies (MB), have been considered an integrative brain center of *Drosophila* because of its involvement in olfactory learning and memory, and multi-sensory integration (Owald and Waddell, 2015; Das et al., 2016). The MBs are usually described as a network of neuropils which is composed of the neurites from Kenyon cells (Campbell and Turner, 2010). The

Kenyon cells in MBs gets their main olfactory sensory inputs through MB calyx connected by PNs. Connections between the PNs and the Kenyon cell are random; that is, individual flies show distinct wiring patterns (Christiansen et al., 2011; Kazama et al., 2012; Ramaekers et al., 2005). The representation of olfactory information was studied using whole-cell recordings or calcium imaging in the MB neurons following odor stimulation (Turner et al., 2008; Wang et al., 2004). The data obtained using both approaches indicate that the odor response of MB neurons is highly selective and that the odor representation is sparse. For example, a given odorant may be spatially represented by the activity of a small set (~2%) of total KC neurons, indicating a sparse coding scheme for representing the identity of odor (Wang et al., 2004). Furthermore, imaging results showed that the spatial location and distribution of MB neurons showing activation pattern is similar between flies, indicating the stereotyped odor-evoked activity in the MBs (Wang et al., 2004). In the last 30 years, behavioral and genetic studies in different insect species have demonstrated that the MBs play an essential role in olfactory associative learning behaviors (Busto et al., 2010; Davis, 2005; Dubnau et al., 2013; Fiala, 2007; Keene and Waddell, 2005). First, both dopamine and octopamine neurons regulate appetitive and aversive reinforcement through their neurites innervating MB structure (Berry and Davis, 2014; Busto et al., 2010). Second, genes for components of the cAMP signaling pathway, which when mutant produce deficits in olfactory learning and memory, are strongly expressed in Kenyon cells (KCs) (Davis, 2005; Heisenberg, 2003; Müller, 2002). Together with the notion that KCs respond to odor input with sparse coding scheme, supports the concept that MBs encode odor for memory formation.

Another higher olfactory center, the lateral horn (LH) is an area defined by the terminal arborizations of PNs in the lateral protocerebrum (Ernst et al., 1977; Homberg et al., 1987). There are several hypotheses about the roles the LH plays in olfaction and olfactory-driven behavior. First, Perez-Orive et al. proposed that a subset of GABAergic interneurons in the LH (LHNs) inhibit Kenyon cells firing in the MBs and help maintain the sparseness and specific odor-elicited spiking in KCs (Perez-Orive et al., 2002). However, it is debatable about the inhibitory source of KCs from giant GABAergic neuron or LHNs (Gupta et al., 2012; Kee et al., 2015). Second, a recent study on locust also suggests LH may contribute to odor concentration coding, bilateral processing, and multimodal integration (Gupta et al., 2012). Third, several behavioral studies indicate that the LH is involved with encoding innate olfactory preferences. For example, ablated the MB cells in *Drosophila* was linked to deficits in olfactory learning but had little effect on innate olfactory-driven courtship behaviors (de Belle and Heisenberg, 1994; Kido and Ito, 2002). However, reducing olfactory input by expressing tetanus toxin in the majority of PNs leads to defects in odor detection in male courtship behavior. Those two pieces of evidence together support the argument that the LH could mediate innate, unlearned responses (Heimbeck et al., 2001). In addition, consistent with this proposal, anatomical analysis showed stereotypical clustering of PN arborizations in the LH (Marin et al., 2002; Tanaka et al., 2004; Wong et al., 2002). Furthermore, odor evoked calcium imaging results in the LH area are spatially stereotyped and reproducible among different individual flies (Strutz et al., 2014). Another interesting finding is that odor response profiles in adult flies showed fruit odors and pheromones are represented in distinct region of the LH (Jefferis et al., 2007) indicating the LH is organized according to

biological values of olfactory input. Together, those features support the notion that the LH is a crucial olfactory center for integrating biological values of odor towards innate decision to drive innate olfactory-driven behaviors in *Drosophila*.

DOPAMINE SYSTEM IN HUMAN AND *DROSOPHILA*

Dopamine (DA) is a highly conserved catecholamine neurotransmitter throughout evolution (Yamamoto and Vernier, 2011). Many human studies associated DA with diverse cognitive functions such as reward prediction, attention and working memory that are crucial for execution of goal-directed behavior. Imbalanced dopamine (DA) signaling, either too low or too high, is detrimental to cognitive functions. Just like humans, alternation in DA signaling and DA receptors are implicated with various behavioral changes including olfactory memory, arousal, motivation, attention, and appetite in *Drosophila* (Yamamoto and Seto, 2014). Those findings indicate that DA are a prominent regulator to precisely modulate neuronal circuits which are crucial for goal-directed behaviors. In this section, I will focus the introduction of the DA system and DA receptors in both human and *Drosophila*.

Dopamine and dopamine receptor in human

Dopamine (DA) was first discovered in the late 1950s by Swedish pharmacologist Arvid Carlsson. In his experiment, Carlsson showed that DA — the chemical converted from L-dopa, acts as a neurotransmitter in the brain (Abbott, 2007; Carlsson, 2001). As a catecholamine neurotransmitter, DA is synthesized through hydroxylation of tyrosine to L-DOPA, through a process that is catalyzed by rate-limiting enzyme Tyrosine hydroxylase (TyrH or TH) (Molinoff and Axelrod, 1971). In the human central nervous system (CNS), the total number of DA neurons, determined by TH immunostaining, is

around 400,000 and thus make up less than 1% of the total number of brain neurons (Schultz, 2007). Dopaminergic neurons are restricted to small populations in the olfactory bulbs, the hypothalamus, and the midbrain, but these neurons extend processes to most brain regions throughout the CNS. Dysregulation of DA pathway can lead to pathological states including Parkinson's disease, Huntington's disease, schizophrenia, and attention deficit hyperactivity disorder (Kienast and Heinz, 2006).

Dopaminergic signaling is mediated through canonical seven transmembrane domain GPCRs at the cell surface (J A Gingrich and Caron, 2003). G-proteins consist of α, β and γ subunits and binding of DA to its receptor causes a GDP to GTP exchange in the α subunit resulting in its activation and release from the βγ heterodimer so as to recruit downstream effectors (New and Wong, 2007). Based on pharmacological profiles and coupling to specificity to heterotrimeric G-proteins, DA receptors are classified as either D1-like receptors (D_1 and D_5) or D2-like receptors (D_2, D_3 and D_4) (Kebabian and Calne, 1979; Niznik, 1987; Spano et al., 1978). D1-like receptors signal by coupling to Gαs and $G\alpha/_{olf}$ proteins. Activation of D1 receptor stimulate adenylate cyclase (AC), thus triggering the second messenger cyclic AMP accumulation which results in the regulation of PKA and potentially other exchange proteins activated by cAMP (Epac1 and Epac2) (Neve et al., 2004; Simon et al., 1991). Furthermore, under certain circumstances, there is evidence that alternative coupling of D1 receptors to $G\alpha_q$/PLC mediated signaling (Medvedev et al., 2013; Pacheco and Jope, 2002; Undie et al., 1994). In addition, D1-like receptors were also found to mediate the G protein-gated K^+ channel (GIRK) through disassociated Gβγ to regulate neurons depolarization (Witkowski et al., 2008). D2-like receptor signaling is mediated through inhibitory Gαi/o. Gαi activation

inhibits AC thus leading to inhibition of cyclic AMP accumulation and decreasing the phosphorylation of PKA substrates. In addition, several results have shown that concomitant activation of D_1 and D_2 receptors within a heterodimeric complex can also regulate Gαq/PLC/IP3 mediated signaling thus it is linked to an increase in intracellular calcium levels, likely via multiple mechanisms (Medvedev et al., 2013, Hasbi et al., 2010; Lee et al., 2004; Perreault et al., 2014; Rashid et al., 2007). Besides the AC activation, other studies also have shown that there are various other effectors which are regulated by activation of DA receptors including ion channels, mitogen activated protein kinases (MAPKs), and sodium proton exchangers (Neve et al., 2004; Yan et al., 1999).

Dopamine and dopamine receptor in *Drosophila*

As most genes involved in DA synthesis, transport, secretion, and signaling are conserved between flies and mammals, *Drosophila* are a powerful genetic model organism to study the DA-related neurobehavioral disorders (Yamamoto and Seto, 2014). Similar to mammals, in *Drosophila* tyrosine hydroxylase (TH) catalyzes the first step in DA biosynthesis. The *Drosophila* TH gene (DTH, CG10118) is specifically expressed in all dopaminergic cells. Friggi-Grelin et al. (2003) generated TH-GAL4 which contain regulatory sequences from the DTH 5' flanking and downstream coding regions (Friggi-Grelin et al., 2003). The expression pattern of the TH-GAL4 is broadly similar but not completely identical to the expression of endogenous TH (Mao and Davis, 2009). After examining TH-Gal4 targets neuron pattern together with anti-TH immunostaining, the dopaminergic cell pattern has been well characterized at single cell level both in the *Drosophila* larval CNS and adult CNS ((Selcho et al., 2009, Mao and Davis, 2009). In the larval CNS, approximately 90 putative DA neurons have been described in the CNS of

third instar larvae (Selcho et al., 2009). Nearly all neuropil regions receive dense dopaminergic innervation, including some higher brain centers, such as the protocerebra, suboesophageal ganglion (SOG), mushroom bodies (MBs) and central complex which is a structure only in adult flies CNS (Selcho et al., 2009; Mao and Davis, 2009). In the larval brain lobe, there are three bilaterally symmetrical clusters of DA neurons in the brain named DL1, DL2 and DM (Monastirioti, 1999; Selcho et al., 2009). In the DL1, seven to eight cell bodies were labeled with TH-Gal4 and their neurites innervate MBs (Mao and Davis, 2009). DM neurons were found innervating the horizontal lobe of MB. DL2 cluster consist of about six neurons per hemisphere labeled by both TH-GAL4 and anti-TH- immunostaining. Single cell analysis showed DL2 cluster neurons innervate both the ipsilateral and contralateral side. Their primary neuritis projected dorsally, bifurcated and terminated widely in the dorsolateral protocerebrum including the lateral horn (LH). Among those, some DL2 neurons remained strictly ipsilateral to the LH region (Mao and Davis, 2009).

In mammals, DA has been classically implicated in pleasure, addiction, learning and motivation (Colombo, 2014). For example, phasic DA neurons responses could be triggered by many types of rewards and reward-related sensory cues and it could be used as a teaching signal in reinforcement learning or as an incentive signal that promotes immediate reward seeking (Schultz, 1998; Petrovich, 2011; Schultz et al., 1997; Berridge and Robinson, 1998). Likewise, fly studies indicate that particular dopamine clusters and even individual DA neurons likely form valence-specific circuit motifs that are engaged by conditioned or innate values of a stimulus, and whose function can be modified by the internal state (Azanchi et al., 2013; Cohn et al., 2015; Lin et al., 2000; Waddell, 2013).

For example, a single dopaminergic neuron TH-VUM located in the SOG, is necessary and sufficient to promote proboscis extension to sucrose, its tonic activity is increased in starved flies (Marella et al., 2012). DA also tunes the sensory perception of appetitive cues (Bernays, 1977). In addition, DA acts directly on sugar-sensing taste neurons to enhance taste reactivity in starved flies (Inagaki et al., 2012). As in human, abnormal status of DA system in the *Drosophila* has been associated with several behavioral defects, including locomotion control (Pendleton et al., 2002), sleep and arousal (Andretic et al., 2005; Foltenyi et al., 2007; Kume et al., 2005; Lebestky et al., 2009), courtship behavior (Liu, 2008, Neckameyer,1998), and olfactory classical conditioning (Davis, 2005; Kim et al., 2007; Schwaerzel et al., 2003) and food memory formation, consolidation, and deprivation-state dependent food memory retrieval(Krashes et al., 2009).

In *Drosophila*, four G-protein coupled DA receptors have been identified two D1-like receptors Dop1R1 (CG9652) (Gotzes et al., 1994), Dop1R2 (CG18741) (Feng et al., 1996) and one D2-like receptors, DD2R (CG9569) (Hearn et al., 2002), and one non-canonical receptor dopamine Ecdysteroid receptor DopECR (CG18314) (Srivastava, 2005). Dop1R1(CG9652) was isolated using a low stringency hybridization approach (Gotzes et al., 1994). The amino acid sequence of Dop1R1 is approximately 70% homologous to the human D1/D5 receptors (Sugamori et al., 1995). Similar to humans, Dop1R1 receptor acts through activation of the adenylyl cyclase (AC) pathway. When expressed in HEK 293 cells, Dop1R1 stimulates cAMP production in response to application of dopamine as well as D1 receptor agonist SKF 38393 (Gotzes et al., 1994; Sugamori et al., 1995). Also, there is evidence of direct coupling of Dop1R1 both to the

activation of AC and to the initiation of an intracellular Ca^{2+} signal (Richardson et al., 2003). Recently, it was reported that Dop1R1 together with Dop1R2 could mediate odor-induced Ca^{2+} influx in the mushroom body (Cohn et al., 2015). In both larval and adult CNS strong Dop1R1 immunoreactivity was present in the neuropil of the MBS(Kim et al., 2003) and soma in the optic lobe in adult flies (Gotzes et al., 1994). In behavioral studies, Dop1R1 has been implicated with regulation of arousal state (Kume et al., 2005; Lebestky et al., 2009) and memory formation (Berry et al., 2012; Kim et al., 2007). Also, the Dop1R1 receptor mutants exhibit little or no learning behavior after olfactory classical conditioning (Kim et al., 2007). The phenotype of Dop1R1 mutants is rescued with the expression of a wild-type form of the Dop1R1 receptor specifically in the MB neurons (Kim et al., 2007).

THE NEUROPEPTIDE Y-LIKE SIGNALING SYSTEM

Over the last three decades Neuropeptide Y(NPY) has been implicated as a major feeding regulator for both metabolic driven feeding (Currie, 2003; Mercer et al., 2011) and hedonic feeding (Pandit et al., 2014). NPY has also been thought to related some nonfeeding-related functions such as encoding motivational states (Quarta and Smolders, 2014), reward threshold regulation (Pleil et al., 2015), mood and emotion regulation (Heilig, 2004), and regulating goal-directed behavior (Quarta and Smolders, 2014). Its homolog in *Drosophila*, Neuropeptide F (NPF) is also implicated with encoding food motivated, metabolic-driven feeding, rewarding related behavior and state-dependent appetitive memory retrieval (Larhammar, 1996). All these findings indicate that neuropeptide-Y like system is a crucial neural modulator encoding the motivation of feeding behavior.

Neuropeptide Y and NPY receptors in mammals

In mammals, Neuropeptide Y was first discovered and isolated from porcine the hypothalamus in 1982 (Lundberg et al., 1982; Tatemoto, 1982). Neuropeptide Y (NPY) is a 36-amino acid peptide with structural similarities to peptide YY (PYY) and pancreatic polypeptide (PP) (Tatemoto, 1982; 2004). The highest level of NPY immunoreactivity is found in hypothalamus, particularly in the paraventricular nucleus (PVN), arcuate nucleus (ARC), suprachiasmatic nucleus (SCN), and dorsomedial nucleus (VMH) (Chronwall et al., 1985).

Pharmacological and behavioral studies have implicated hypothalamic NPY as a prominent stimulator for feeding and reward behavior. For example, chronic administration of NPY in the paraventricular nucleus induces uncontrolled palatable food intake in rats, thus causing obesity (Clark et al., 1984; Levine and Morley, 1984; Stanley, 2005). Besides triggering palatable food overconsumption, central NPY also enhances motivations to obtain a food reward (Gruninger et al., 2007). The central effects of NPY on hedonic feeding include increasing lever pressing number to obtain food (Jewett et al., 1995; 1992) and overriding the associated aversive consequences, like a light electric shock or exposure to quinine-adulterated milk solution (Flood and Morley, 1991). Moreover, NPY infusion in the perifornical hypothalamus enhanced food motivation together with conditioned place preference (Brown et al., 2000). In addition, injection of NPY into the nucleus accumbens of rats is rewarding (Josselyn and Beninger, 1993) and NPY administration relieves the negative affective states of drug withdrawal and depression (Redrobe et al., 2002; Stogner and Holmes, 2000) . Thus, those findings

indicate that NPY drive both homeostatic and hedonic aspects of food intake via different neuronal network.

NPY affects a wide variety of physiological functions via the activation of at least six different, cloned Y-receptors: Y_1, Y_2, y_3, Y_4, Y_5, and y_6 (Blomqvist and Herzog, 1997; Gehlert et al., 1996; Larhammar et al., 1993). The neuropeptide y3 and y6 receptors have both been hypothesized in human (Currie and Coscina, 1996; Matsumoto et al., 1996; Movafagh, 2006). Neuropeptide Y receptors, Y1, Y2, Y4, Y5 are found throughout the body but they have distinct tissue expression profiles (Durkin et al., 2000; Jacques et al., 1996; Kopp et al., 2002; Larsen and Kristensen, 1998).

Despite the low homology among NPF receptor subtypes (Larhammar, 1996), all NPY receptors couple to the Gαi/Gαo cascade where the Gαi subunit inactivates adenylyl cyclase and subsequent inhibition of cyclic AMP accumulation (Holliday et al., 2004). In addition, NPY receptors also couple to phospholipase C to induce release of Ca^{2+} from intracellular stores (Gehlert, 2004). It is also reported that NPY activates ERK by a pathway involving phosphatidylinositol-3-kinase, whereas PKC may not be involved (Keffel et al., 1999).

Neuropeptide F and NPF receptors in *Drosophila*

Drosophila Neuropeptide F (dNPF, CG10324) is considered as the first insect neuropeptide that belongs to the NPY family (Brown et al., 1999; Larhammar et al., 1993). They are termed neuropeptide F (NPF) as the C-terminal tyrosine (Y) of vertebrates is exchanged to phenylalanine (F) in invertebrates (Brown et al., 1999). Using *in situ* hybridization and immunocytochemistry, the NPF transcripts are detected only in four neurons in the protocerebral region and two additional NPFergic neurons are

consistently detected on the ventromedial surface of the subesophageal ganglion (SOG) in the CNS. Also, a number of endocrine cells are found by in situ hybridization in midguts of larvae and adults (Shen and Cai, 2001; Wu et al., 2003).

Consist with its mammalian orexigenic counterpart, *Drosophila* NPF signaling is involved in regulation of multiple feeding-related behaviors. First, the CNS NPF expression of feeding larvae can be modulated by exposure to fructose and glucose, suggesting that the dNPF neuronal circuit is an integral part of the sensory system that mediates food signaling (Shen and Cai, 2001). Second, NPF is important for regulating the transition between feeding and non-feeding wandering behavior in third-instar fly larvae, emphasizing its role in regulation of developmental dependent feeding behavior. Ectopically overexpressing NPF cDNA in larvae abolishes the food-avoidance and migration toward food-free habitat (Wu et al., 2003). Third, NPF signaling is also mediating food choice in feeding is larvae. Several pieces of evidence showed deprivation-motivated feeding in regulated by NPF, as silencing NPF signaling causes abolished larval motivated feeding during hunger state (Wu et al., 2005b). Consistent with this notion, overexpression the NPF receptors (NPFR1), causes well-fed larvae to eat bitter-tasting food that wild-type larvae will only consume if they are hungry (Wu et al., 2005b). NPF signaling is also required in food deprivation-dependent feeding in low temperature (Lingo et al., 2007). Besides its functional role in feeding regulation, NPF circuitry acts as a motivational switch for appetitive memory retrieval via mediating PPL1 DA neurons activities (Krashes et al., 2009). Recently, NPF neurons innervate food-odor direct foraging behavior by responding to specific food-related odor in both

fed and hungry flies (Beshel and Zhong, 2013). In conclusion, those findings indicate that NPF regulates feeding behavior.

In addition to its involvement in feeding related behaviors, current data also suggests that the NPF system is involved in representing the state of reward system in flies (Shohat-Ophir et al., 2012). The regulatory roles of NPF are also found in other behaviors including foraging, ethanol sensitivity, nociception, aggression, reproduction, clock function, and learning (Shen and Cai, 2001; ssel and Wegener, 2011; Wen et al., 2005; Wu et al., 2005b; 2005a; Xu et al., 2010).

A pharmacological study revealed that NPF signals through *NPFR1*(CG1147) (Garczynski et al., 2002). Based on ClustalW alignment with the vertebrate NPY receptors, *Drosophila* NPFR1 is most closely related to the Y_2 receptor subtype (Garczynski et al., 2002). *NPFR1* immunoreactivity was detected both in the neurons and neuropils of the brain lobes, subesophageal ganglia and ventral nerve cord in the CNS (Xu et al., 2010). In addition, NPFR1 peptide antibody also has been detected in ventral epidermis of the thoracic segments (Xu et al., 2008). NPFR1 receptor signaling was first tested by peptide binding assay. Similar to homologous partner, NPFR1 acts via Gαi G protein which inhibits adenylyl cyclase (AC). (Garczynski et al., 2002; Balasubramaniam, 1997; Blomqvist and Herzog, 1997). Cellular assay also suggests that NPFR1 activation suppress TRP channel-mediated Ca^{2+} influx in primary sensory neurons and in human cells through a yet uncharacterized PKA-independent mechanism(s) (Xu et al., 2010).

OBJECTIVES

The sense of smell is crucial for two vital biological functions across evolution foraging and mating. Food odors can be powerful appetitive cues for directing animal feeding behavior. 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 encoded and perceived by the brain and subsequently translate to appetitive behavior.

The genetically-tractable *Drosophila* constitutes a well-established model organism for studying odor decoding and olfactory driven behavior. *Drosophila* has a relatively simpler yet evolutionarily conserved olfactory pathway, allowing easier identification and functional characterization of neurons and neural circuits underlying complex biological processes. Furthermore, *Drosophila* displays a robust and stereotyped behavior under the food-related odor stimuli. It presents an excellent opportunity to investigate the neural and molecular mechanisms underlying appetitive odor encoding and odor-induced reward anticipation.

After a brief exposure to an appetitive odor, *Drosophila* larvae show increased feeding activity on readily accessible palatable food. This behavioral phenotype demonstrates that invertebrate animals engage in appetitive cue-driven feeding and have an innate cognitive ability to selectively attribute anticipated sugar reward to discrete olfactory cues. In this study, I demonstrate a *Drosophila* model to identify the neural circuit of food odor processing and to understand how appetitive odorants are converted into reward cues to promote feeding behavior. There are three major objectives of this dissertation:

Objective 1. Delineate the neurobiological basis of the feeding response under a brief external appetitive odor stimuli, and identify neurons and neural substrates that are important to represent appetitive odor inputs in larval brain. The results of these studies are presented in Charter II of this dissertation in a publication format and have been published in *Cell Reports* (Wang et al., 2013).

Objective 2. Characterize the inverted U shaped appetizing effects under various doses of monomolecular odorants or natural food odorants stimulation and investigate the functional role of DL2 dopamine neurons in various appetitive odor encoding. Elucidate the correlations between intensity of appetitive odor stimulation and odor-induced DA signaling in transforming odor input into reward cues. Reveal functional roles of D1-like receptor, *Dop1R1,* in regulating odor-induced DA signaling and inverted U shape appetizing effects. The results of these studies are presented in Charter III of this dissertation.

Objective 3. Dissect the molecular and circuit pathways of Neuropeptide F signaling in promoting appetitive arousal and investigate *Dop1R1*-gated NPF neurons activities regulated by odor-induced DA signaling in an inverted U manner. Briefly demonstrate DM NPF neurons regulate attention span and arousal stage.

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CHAPTER 2

NEUROPEPTIDE-GATED PERCEPTION OF APPETITIVE OLFACTORY INPUTS IN *DROSOPHILA* LARVAE

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ABSTRACT

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; Na¨ssel 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 pep- tide 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), 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,2009, Fishilevich, 2005). Indeed, fed larvae briefly exposed to appetitive olfactory cue(s) showed a significant increase of mouth hook contractions and food ingestion (Figures 2.1A and2.1 F). 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 2.1B).

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 2.1C and 2.1D). 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 2.1E). For example, the feeding activity of PA-stimulated larvae that fasted for 1 hr was similar to that of non-stimulated 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 2.2A). *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 2.2B). The *GH146-Gal4* driver labels the projection neurons that relay olfactory information from the AL to the LH and MB(Marin:2005bt, Vosshall:2007hh}. Expression of *UAS- shi*^{ts1} in *GH146-Gal4* neurons also blocked PA-stimulated feeding (Figures 2.2C). However, inhibition of the neurotransmission of MB neurons labeled by *OK107-Gal4* had no negative impact on the PA-elicited feeding response (Figures 2.2C). 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, 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-*kir2.1*

encoding an inward-rectifier potassium channel in *NPF-Gal4* neurons blocked the PA-stimulated feeding response (Figure 2.3A). In addition, expression of *npfr1RNAi* in the larval nervous system also blocked PA-stimulated feeding (Figure 2.3B) (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 2.3B). Further, expression of *UAS- shi^{ts1}* in the *NPFR1*-Gal4 neurons also abolished PA-stimulated feeding at 31 ^{O}C . 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. 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 2.3C). 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 2.3D).

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 2.3E), 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 (Inagaki et al., 2012; Srivastava, 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., 2014; Kim et al., 2007; Lebestky et al., 2009; Weber et al., 2010). Fed $DopR^{f02676}$ larvae failed to display PA-induced food response (Figure 2.3E). In addition, fed *elav- Gal4*/UAS-*DopRRNAi* larvae that express *DopR* RNAi in the nervous system also showed attenuated PA-induced food response. 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 2.3F).

Functional Mapping of DA Neurons

There are approximately 70 DA neurons in the *Drosophila* larval central nervous system (CNS) (Monastirioti, 1999; Selcho et al., 2009). 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. 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 2.4A). 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. The presumptive dendrites of DA 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 2.4B and C) and further sup- ported 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 2.4D and 2.4E). 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 2.5A–D). 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 2.5E).

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 juxta- posed to the processes of DL2-LH neurons in the LH (Figures 2.6A and 2.6B). Furthermore, at least three of the four DL2-LH neurons, whose activation mimicked the effect of PA stimulation, are marked by *NPFR1-Gal4* (Figures 2.6C–2.6E). 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 2.7A and 2.7B). 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 2.7C). 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.

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 2.7D). Our findings suggest that fly larvae 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 neuro- chemicals 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 mech- anism 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 investi- gation 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 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 under- standing of how the brain controls appetitive behaviors in diverse animals.

METHODS

Fly Stocks and Larval Growth

All flies are in the w1118 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-shits1 (Kitamoto, 2001), UAS-dTrpA1 (Hamada et al., 2008), UAS-GcaMP3 (Tian et al., 2009), UAS-Denmark (Nicolaı¨et al., 2010), Or83b-LexA, UAS-CD4::spGFP1-10, LexAop-CD4::spGFP11, 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, or83b1, or83b2 (Larsson et al., 2004), DopRf02676

(Kong et al., 2010; Lebestky et al., 2009), and tbhmM18 (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 ml 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 (Edge- comb 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 ml 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 ml 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-shits1 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 50 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-ex- pressing 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 2.6.

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 ml water. The larvae were then exposed to 250 ml 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. 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 403 water immersion lens. Images were captured at 1.57 s per frame with a resolution of 512 3 512 pixels. A z stack of images (512 3 512 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 (Fs) was calculated as the average intensity of two frames after odor stimulation. The change in fluorescence (DF) = Fs 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 Figure2.7, where the Mann-Whitney test is used.

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Figure 2.1. A Behavioral Paradigm for Appetitive Odor-Induced Feeding

Wild-type larvae used in this and the following figures were young third-instar w1118 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. $^{*}p < 0.01$; $^{**}p < 0.001$.

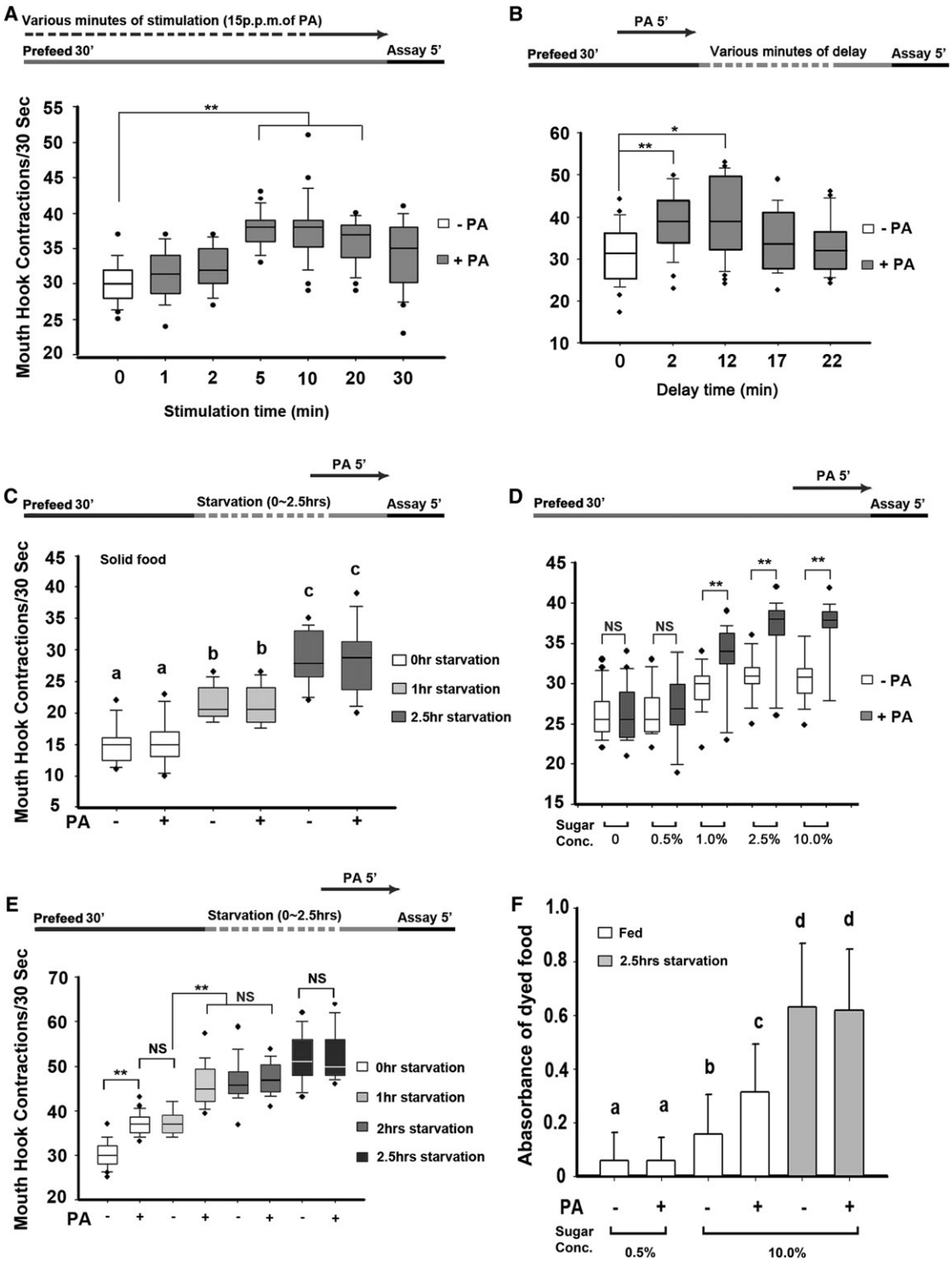

A
Various minutes of stimulation (15p.p.m.of PA)
Prefeed 30'
Assay 5'
Mouth Hook Contractions/30 Sec
Stimulation time (min)
- PA
+ PA
B
PA 5'
Prefeed 30'
Various minutes of delay
Assay 5'
Delay time (min)
C
PA 5'
Prefeed 30'
Starvation (0~2.5hrs)
Assay 5'
Solid food
0hr starvation
1hr starvation
2.5hr starvation
D
PA 5'
Prefeed 30'
Assay 5'
Sugar Conc.
E
PA 5'
Prefeed 30'
Starvation (0~2.5hrs)
Assay 5'
0hr starvation
1hr starvation
2hrs starvation
2.5hrs starvation
F
Fed
2.5hrs starvation
Absorbance of dyed food

Figure 2.2. 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 or83b1mutants. (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- shits1 larvae were normal in PA-stimulated feeding response. (C) At 31 C, expression of UAS- shits1 in GH146-Gal4, but not OK107-Gal4, neurons attenuated PA-stimulated feeding activity in fed larvae. Different letters indicate statistically significant differences; $p < 0.01$.

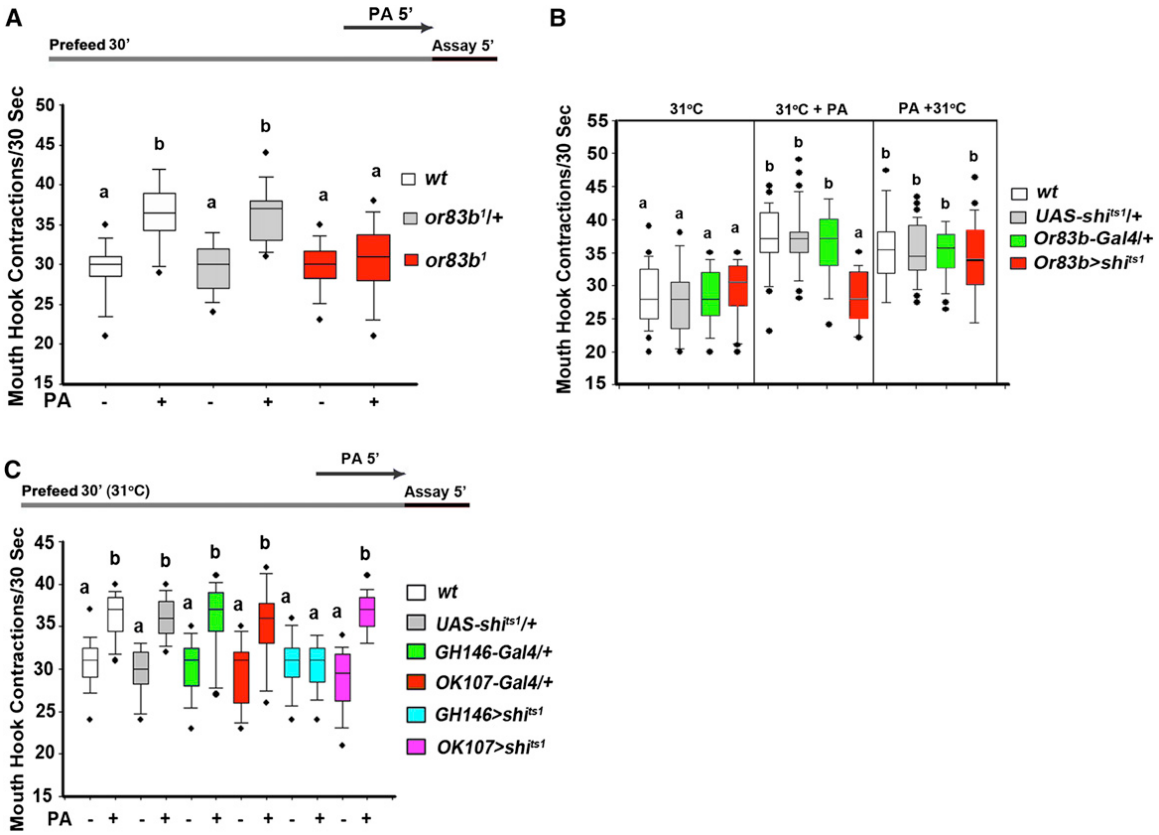

A
PA 5'
Prefeed 30'
Assay 5'
Mouth Hook Contractions/30 Sec
PA
wt
or83b1/+
or83b1
B
31°C
31°C + PA
PA +31°C
wt
UAS-shits1/+
Or83b-Gal4/+
Or83b>shits1
C
PA 5'
Prefeed 30' (31°C)
Assay 5'
Mouth Hook Contractions/30 Sec
wt
UAS-shits1/+
GH146-Gal4/+
OK107-Gal4/+
GH146>shits1
OK107>shits1

Figure 2.3. 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. A loss-of-function mutation ($DopR^{f02676}$) of the D1-like receptor gene attenuated the PA-stimulated feeding increase. (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. Different letters indicate statistically significant differences; $p < 0.01$.

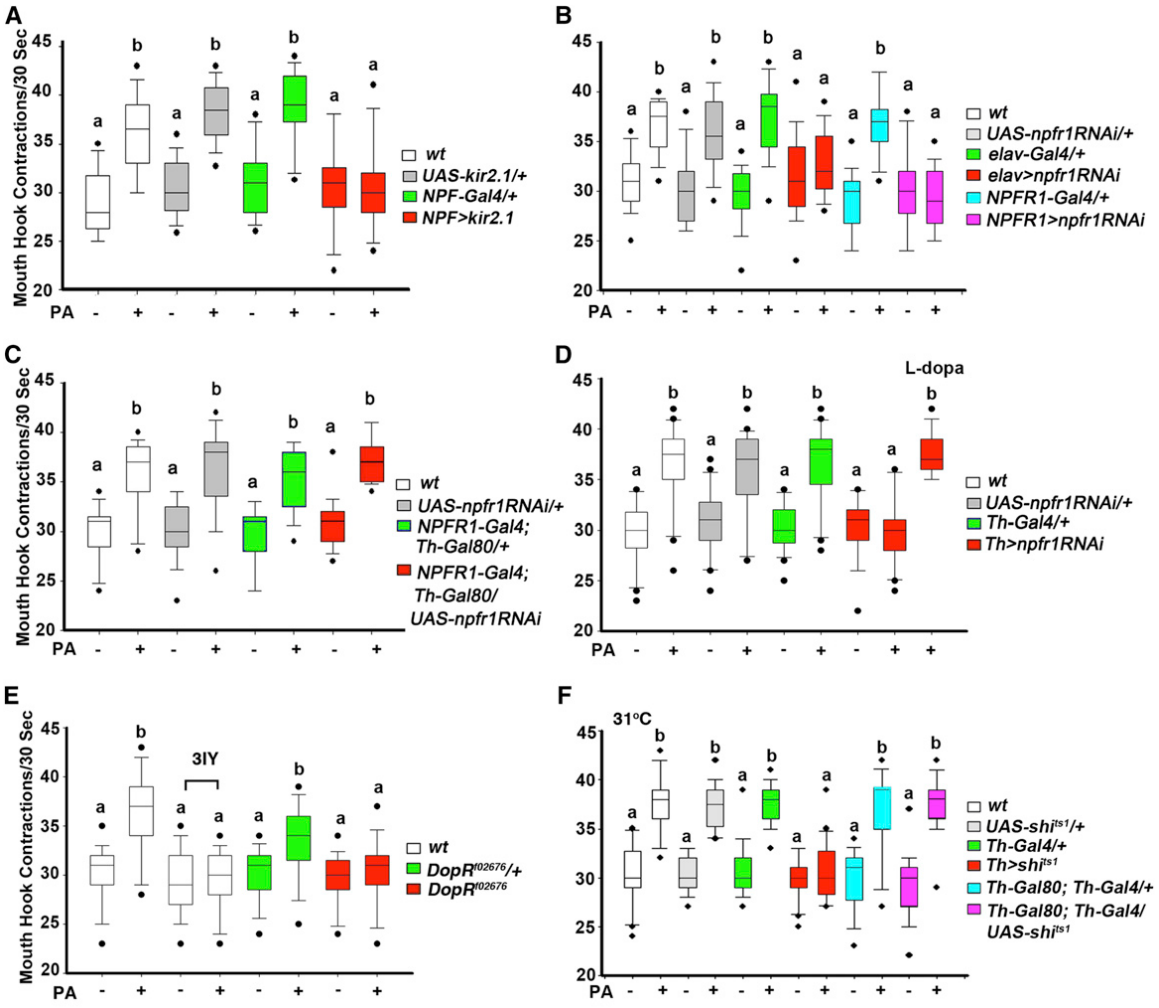

A
Mouth Hook Contractions/30 Sec
PA
wt
UAS-kir2.1/+
NPF-Gal4/+
NPF>kir2.1
B
wt
UAS-npfr1RNAi/+
elav-Gal4/+
elav>npfr1RNAi
NPFR1-Gal4/+
NPFR1>npfr1RNAi
C
wt
UAS-npfr1RNAi/+
NPFR1-Gal4; Th-Gal80/+
NPFR1-Gal4; Th-Gal80/ UAS-npfr1RNAi
D
L-dopa
wt
UAS-npfr1RNAi/+
Th-Gal4/+
Th>npfr1RNAi
E
3IY
wt
DopR f02676/+
DopR f02676
F
31°C
wt
UAS-shi ts1/+
Th-Gal4/+
Th>shi ts1
Th-Gal80; Th-Gal4/+
Th-Gal80; Th-Gal4/ UAS-shi ts1

Figure 2.4. 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) immunofluorescence 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. Scale bar, 20 mm. (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. The antenna lobe (AL) is marked by dotted circles. Scale bar, 20 mm. (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 mm.

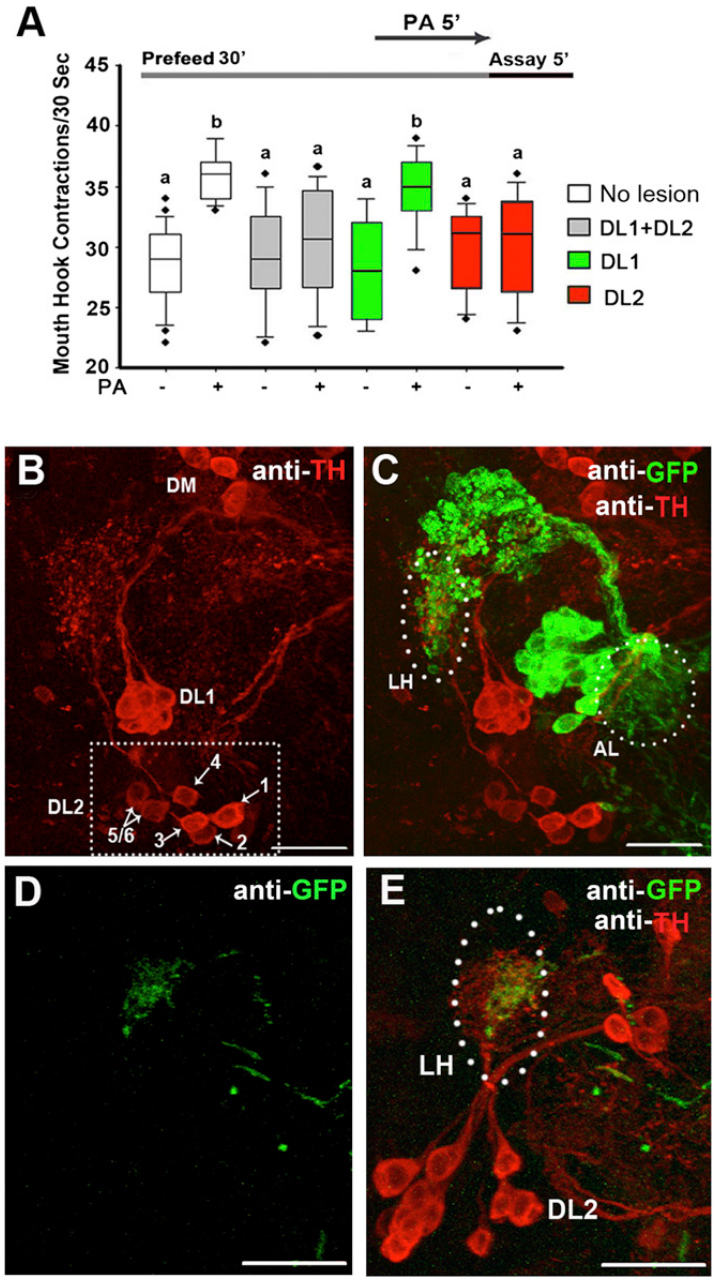
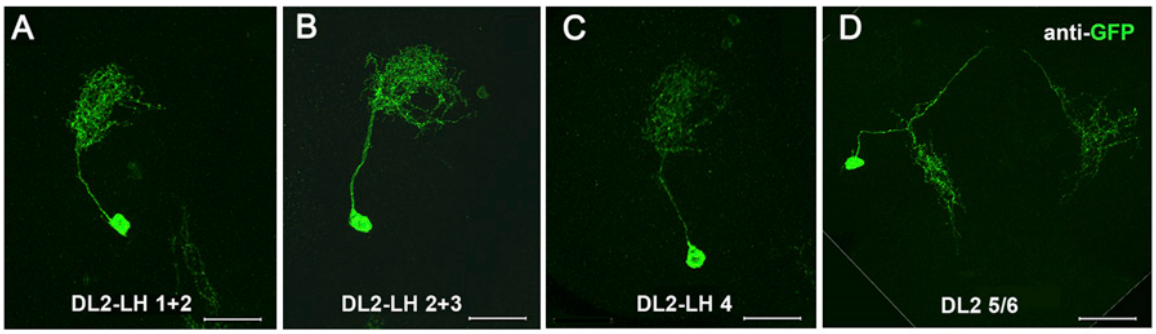

A
PA 5'
Prefeed 30'
Assay 5'
Mouth Hook Contractions/30 Sec
45
40
35
30
25
20
a
b
a
a
a
b
a
a
PA
-
+
-
+
-
+
-
+
No lesion
DL1+DL2
DL1
DL2
B
DM
anti-TH
DL1
DL2
4
1
5/6
3
2
C
anti-GFP
anti-TH
LH
AL
D
anti-GFP
E
anti-GFP
anti-TH
LH
DL2

Figure 2.5. 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). (B) An example of the projection of two DL2-LH neurons (DL2-LH2 and DL2-LH3). (C) An example of the projection of one DL2-LH neurons (DL2-LH4). Scale bars, 20 mm. (D) An example of the projection of two other DL2 neurons (DL2-5/6). (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 . 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. $p < 0.01$.


A
DL2-LH 1+2
B
DL2-LH 2+3
C
DL2-LH 4
D
anti-GFP
DL2 5/6

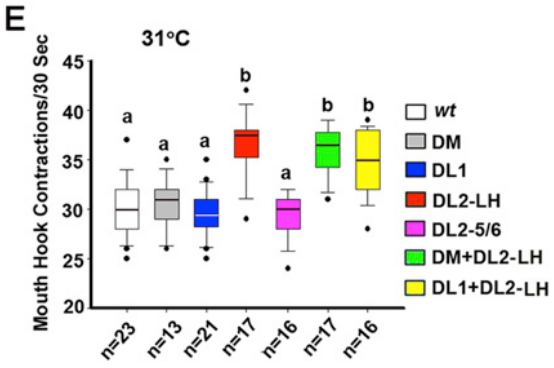

E
31°C
Mouth Hook Contractions/30 Sec
45
40
35
30
25
20
a
a
a
b
a
b
b
n=23
n=13
n=21
n=17
n=16
n=17
n=16
wt
DM
DL1
DL2-LH
DL2-5/6
DM+DL2-LH
DL1+DL2-LH

Figure 2.6. 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 mm. 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 mm. Genotype: *NPFR1-Gal4/UAS-mCD8GFP*.

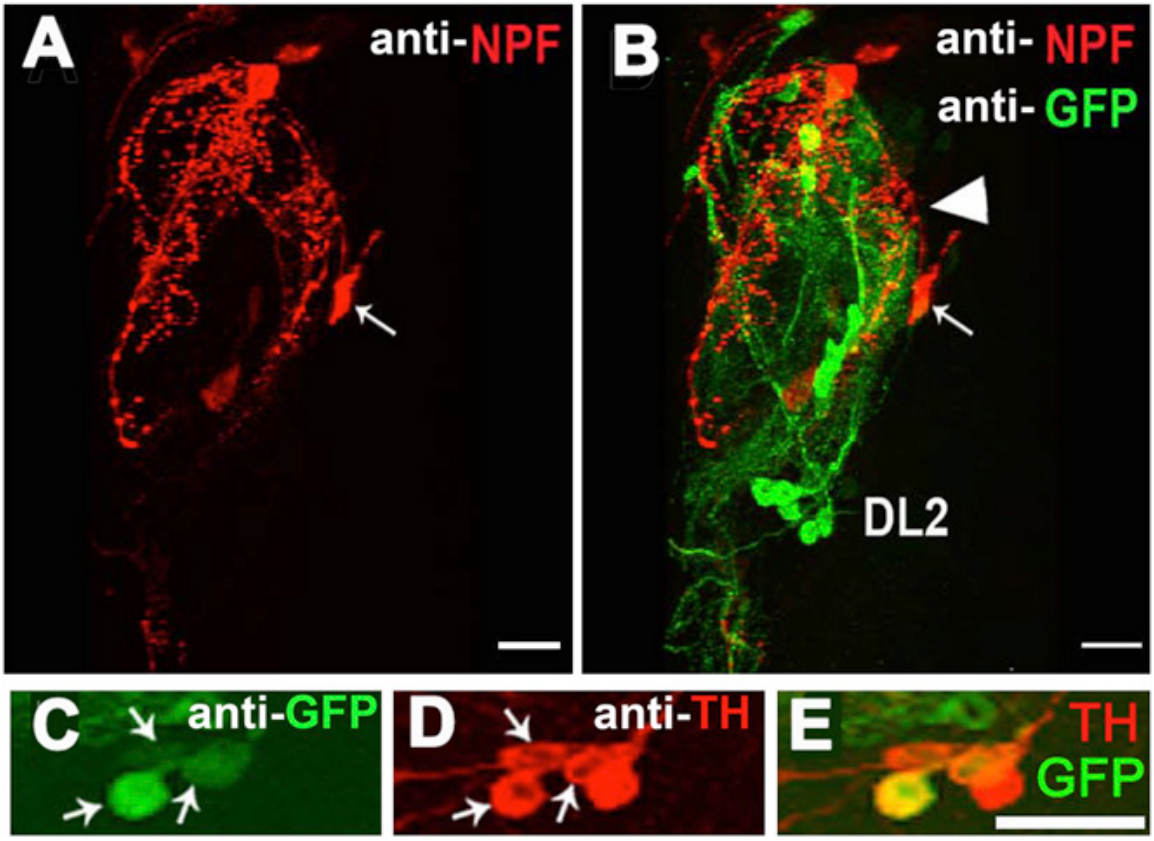
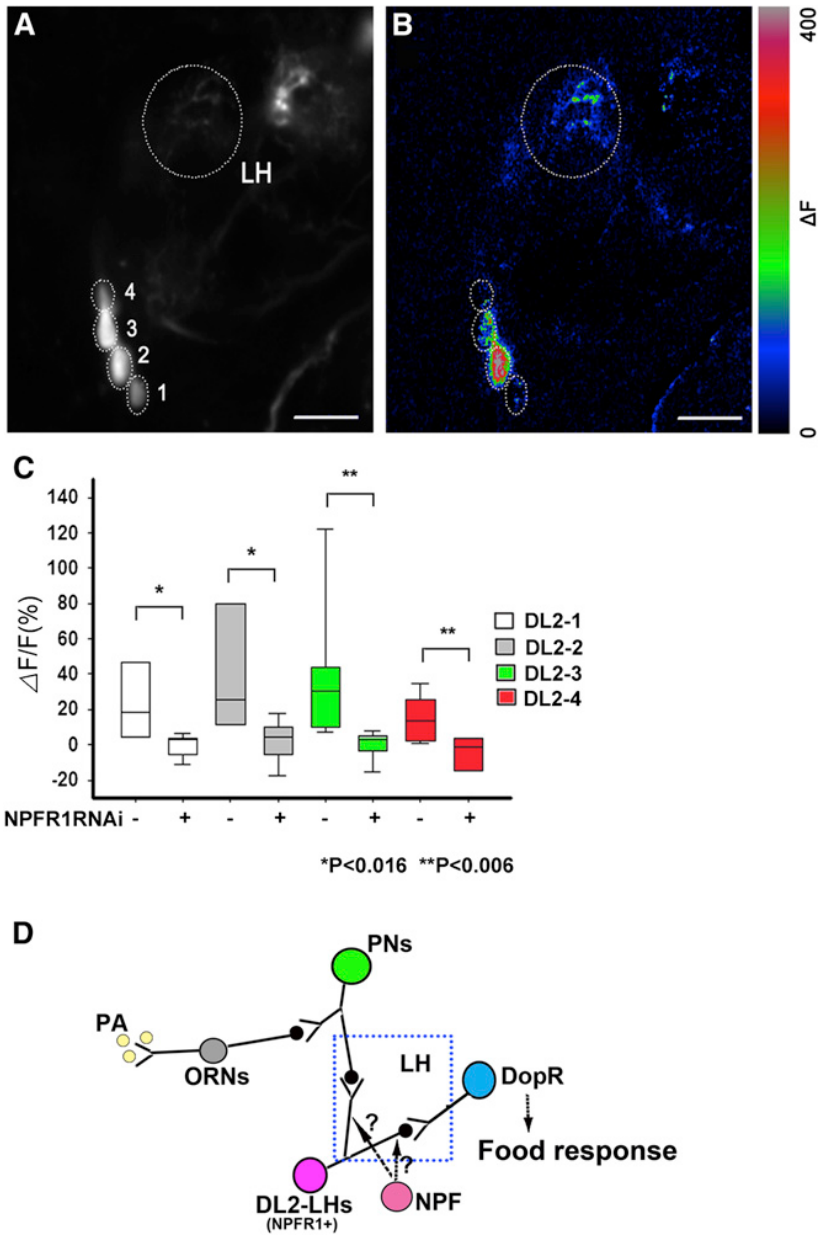

A
anti-NPF
B
anti- NPF
anti- GFP
DL2
C
anti-GFP
D
anti-TH
E
TH
GFP

Figure 2.7. 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 mm. (B) Ca^{2+} imaging analysis revealed PA-induced fluorescence increases in DL2- LH neurons (DF) (see Movie S9). Scale bar, 20 mm.

(C) Quantification of fluorescence changes (DF/F) in the soma of DL2-LH neurons with or without expressing NPFR1RNAi, n = 8. Statistical analysis was performed using the Mann-Whitney test. *$p < 0.016$; **$p < 0.006$. (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 un- characterized LH-projecting DopR neurons, thereby transforming processed food odor information to appetitive drive.


A
B
LH
4
3
2
1
400
ΔF
0
C
140
120
100
80
60
40
20
0
-20
△F/F(%)
*
*
**
**
DL2-1
DL2-2
DL2-3
DL2-4
NPFR1RNAi - + - + - + - +
*P<0.016 **P<0.006
D
PNs
PA
ORNs
LH
DopR
Food response
?
?
DL2-LHs
(NPFR1+)
NPF

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CHAPTER 3

ROLE OF THE *DROSOPHILA* DOPAMINE SYSTEM IN APPETITIVE ODOR PERCEPTION AND PRECISE CONTROL OF APPETITIVE AROUSAL[1]

[1]Pu, Y., Palombo, M.M., Zhang, Y., & Shen, P., submitted to *Neuron*, 05 / 2016

INTRODUCTION

Olfaction as an evolutionarily primitive sense, is functionally interconnected to other senses such as taste, and may significantly impact emotion and cognition (De Araujo et al., 2003; Johnson, 2011; Rolls, 2015). However, elucidation of brain mechanisms that underlie such higher-order neural functions remains challenging in traditional mammalian models. Nevertheless, *Drosophila* larvae have a highly evolved yet numerically simpler olfactory system, and the functional organization strikingly similar to mammals (Su et al., 2009). Our recent study shows that well-nourished *Drosophila* larvae display aroused appetite for anticipated food reward following a brief presentation of food-related odors (Wang et al., 2013). This finding suggests that like mammals, fly larvae may also have a neural capacity for integrating rewarding olfactory and gustatory inputs and enabling functional interactions between the olfactory and cognitive system.

In recent years, several studies suggest a strong connection between the dopamine system and olfactory processing in both human and flies (McGuire et al., 2005; Waddell, 2010). In human studies, olfactory dysfunction may be a prodrome of dopamine related neurodegenerative diseases such as Alzheimer's and Parkinson's disease (Hawkes, 1995; Ruan et al., 2012; Takeda et al., 2014; Thomas-Danguin et al., 2014). These impairments affect different aspects of olfactory function, for example, detection threshold, odor type identification, intensity discrimination, and pleasantness (Doty, 2012; Ruan et al., 2012; Thomas-Danguin et al., 2014). In addition, anatomical evidence also point out the partial over-lap between the brain structures involved in olfactory processing and dopamine pathway. For example, hippocampus and olfactory tubercle which are known as higher

olfactory centers, are both found to receive dopaminergic innervations from the midbrain (Ikemoto, 2010; Reymann et al., 1983). Moreover, dopamine neuron denervation in higher olfactory centers, may lead to selective hyposmia in olfactory identification (Bohnen et al., 2007; Valle-Leija and Drucker-Colín, 2014). Those results indicate the DA system may play a critical role in cognitive processing of olfactory sensory. In *Drosophila*, there are approximately 70 dopamine neurons (DANs) neurons in the *Drosophila* larval central nervous system (CNS) (Monastirioti, 1999; Selcho et al., 2009a). Three paired clusters of DA neurons named DM, DL1, and DL2 are well characterized in the brain of third-instar larvae and their neuritis innervate different region of the brain lobe (Friggi-Grelin et al., 2003; Selcho et al., 2009b). Due to the anatomical segregation of DA neurons, *Drosophila* supports a provocative model to study how dopaminergic neurons regulate odor processing and olfactory related behavior.

Same as mammal, dopamine system is implicated with reward perception and motivational control in *Drosophila* (Bainton et al., 2000; Bromberg-Martin et al., 2010; Rohwedder et al., 2016). Dopamine circuits are also implicated with olfactory conditioning and food type memory consolidation, through innervating segregated local circuits within the mushroom body (MBs) in adult flies (Burke et al., 2013; Das et al., 2008; Krashes et al., 2009; Liu et al., 2012; Mao and Davis, 2009; Rohwedder et al., 2016; Waddell, 2010). Despite DA neurons are involved in association olfactory related cues with internal state or past experience, it still unclear if dopamine neurons could directly encode olfactory input to drive innate behavior in fly.

In our previous studies, we have identified a cluster of four dopamine (DA) neurons in each brain hemisphere appears to form synaptic connections with at least a

subset of the projection neurons in an olfactory processing center known as the lateral horn (LH). Functional analyses of the DA neurons reveal that their activities are necessary and sufficient to induce odor-evoked appetitive arousal in fed larvae (Wang et al., 2013). Given that DA has been widely implicated in a variety of cognitive functions including reward perception, attention and working memory in diverse animals (Arnsten, 2011; Colombo, 2014), these DA neurons may provide a potential interface for the functional interaction between the olfactory and cognitive system.

In the prefrontal cortex of mammals, an optimum level of D1-type DA receptor activity is crucial for spatial working memory, while its signaling at levels that are too low or too high leads to impaired working memory (Vijayraghavan et al., 2007). In flies, D1-like receptor are found enrich in higher order olfactory center mushroom body (Han et al., 1996) and D1-like receptor is a key receptor that mediates both aversive and appetitive learning in olfactory conditioning (Kim et al., 2007). In fed larvae, a D1-type DA receptor Dop1R1 has also been shown to mediate odor-induced appetite for anticipated food reward (Wang et al., 2013). These findings suggest raise the possibility that a conserved D1-type DA receptor mechanism may have parallel functions for cognitive controls in both flies and mammals.

In this chapter, we provide evidence that various types of appetitive odors, including natural food odor balsamic vinegar, and monomolecular odor trigger feeding response in third-instar larvae and the appetizing effect of the different dose of odorant followed an inverted U function. Through further analysis we showed 4 DL2-dopaminergic neurons in each brain lobe receive and integrate olfactory input by providing cellular substrates to combinatorically process the odor information. We also

found that the excitatory response of DL2 positively regulated by intensity of odor input. In addition, the appetizing effect requires an optimum level of odor-evoked DA signaling, since too high or too low dopamine transaction in brain abolish the appetizing feeding. These findings suggest that; first, by integrating olfactory input, 4 DL2 neurons coordinately encode appetitive olfactory cues. Second, optimal level odor-evoked dopamine signaling is needed in appetizing effect. Third, Dop1R1 receptor level predetermine which range of DA signaling is appetitive value.

RESULTS

Attribution of anticipated sugar reward to discrete odor stimuli

Our previous study shows that even under well-nourished conditions, fly larvae display aroused appetite for palatable food following a brief exposure to appetitive odors, and this reward-driven feeding response appears to be controlled by a conserved neural mechanism involving DA and its D1-like receptor Dop1R1 (Wang et al., 2013). To test whether fly larvae have an innate cognitive ability to discriminatively attribute anticipated food rewards to selected sensory cues, we first examined how the appetite of fed larvae is aroused by various food-related odor stimuli. Fly larvae use their external mouth hooks to scoop liquid food into the oral cavity, and their appetite can be reliably quantified by measuring changes in the rate of mouth hook contraction under blind conditions (Wang et al., 2013; Zhang et al., 2013). Prior to the assays for feeding behavior, fed larvae were briefly presented with an odor stimulus for 5 minutes in a sealed chamber fumigated with a defined dose of an odorant(s). Subsequently, the treated larvae were transferred to a 10% glucose liquid medium for the feeding test in the absence of the odorant(s). By quantifying larval responses to increasing doses of each of

the three monomolecular odorants, pentyl acetate (PA), heptanal (Hep) or trans-2-hexenal (T2H), we have generated three dose-response curves (Figure3.1 A-C). In each case, the appetizing effect of the odorant followed an inverted U function. However, their effective dose ranges can differ significantly. Similarly, balsamic vinegar vapor, a chemically complex odor mixture, also exhibited inverted-U effects (Figure 3.1D). These results indicate that fed larvae are able to selectively extract salient features from a small fraction of odor stimuli that may vary in quality and quantity.

To better understand how larvae recognize appetitive olfactory cues, we analyzed the potential appetizing effects of binary odor mixtures containing various proportions of two odorants. Mixing PA and Hep or PA and T2H, each at a lower ineffective dose, triggered a significant appetitive response. In contrast, combining the two odorants, each at an effective dose, completely abolished their appetizing effects (Figure 3.1E,F). Therefore, the larval brain appears to perform a summation function in a processing center before assigning appetitive significance to mixed odor inputs. We also tested whether fed larvae aroused by an appetitive stimulus (e.g., 5µl PA) display motivational specificity to any particular type(s) of food rewards. Interestingly, in response to PA, fed larvae showed increased feeding response to palatable liquid media that are rich in glucose but not those rich in protein or fatty acids (Figure 3.1G; (Bjordal et al., 2014; Fougeron et al., 2011)). Together, our findings suggest that fly larvae are able to selectively attribute a specific type(s) of food reward to a given odor stimulus.

Combinatorial roles of clustered DA neurons in appetitive odor perception

We previously identified two clusters of four DA neurons, one in each brain hemisphere, that likely function as third-order olfactory neurons (labeled DL2-1 to 4 in

Figure 3.2A). These DL2 neurons form presumptive synaptic connections with second-order olfactory (projection) neurons ipsilaterally in the lateral horn region of the larval brain (Wang et al., 2013). We examined how these clustered DA neurons respond to each of the three monomolecular odorants using a larval preparation expressing an in vivo Ca^{2+} indicator GCaMP6.0 (Chen et al., 2013) in the DL2 neurons. Each of the four DA neurons showed an increased Ca^{2+} influx in response to PA, Hep or T2H. Furthermore, individual neurons within each cluster exhibited differential excitatory responses (Figure 3.2B). For example, DL2-2 neuron in the left cluster exhibited varied responses to each of the three odorants while the responses of DL2-1 neuron were largely similar. These results raise the possibility that a processing power for summation of olfactory inputs may be distributed among these DL2 neurons.

We also performed a functional analysis of the DL2 neurons by systematically generating lesions in these neurons using targeted laser beams. We found that fed larvae, with as few as two functional DA neurons in the left or right cluster, still displayed normal appetitive response to an effective dose of PA or Hep (Figure 3.2C). Consistent with this observation, a previous mosaic analysis shows that genetic activation of two of the four DL2 neurons from the same cluster is sufficient to increase the larval feeding response (Wang et al., 2013). However, as olfactory stimuli become more complex, the number of DL2 neurons required for appetitive arousal also increases. For instance, the appetitive response to a binary odor mixture requires at least four neurons in the right cluster (Figure. 3.2D), while the response to a more complex mixture (balsamic vinegar vapor) requires even more DL2 neurons in addition to the entire right cluster (Figure 3.2E). Therefore, our findings provide functional evidence that larval perception of

appetitive natural odors requires a network of two higher-order neural processors that involve the coordinated activities of the clustered DL2 neurons.

Over-stimulation of DA Neurons Is Detrimental to Larval Appetitive Arousal

At present, little is known about neural mechanisms underlying higher-order representations and processing of appetitive odors in any organism. To address these challenging issues, we tried to investigate how the DL2 neurons mediate appetitive odor perception in freely behaving fed larvae. This was achieved by measuring their responses to appetitive or non-appetitive odor stimuli using a fluorescent Ca^{2+} sensor named CaMPARI (Calcium Modulated Photoactivatable Ratiometric Integrator (Fosque et al., 2015). Since PA vapor elicited identical appetitive responses from fed larvae (Figures 3.1G), we chose to use PA in most of the following molecular and cellular studies. Fed larvae were presented with a PA stimulus under the conditions described for feeding behavioral assays. After odor treatment for various lengths of time, the larvae were irradiated with 405 nm light for 3 seconds. This light irradiation irreversibly turns Ca^{2+}-bound CaMPARI protein from green to red fluorescence, thereby capturing the excitatory state of the DL2 neurons in freely behaving larvae at the defined time point. Subsequently, the brain tissues were isolated for quantitative imaging analyses. We found that when stimulated by an effective dose of PA (7µl), the clustered DA neurons showed a gradual increase in intracellular Ca^{2+} level over a 5-min test period, as evidenced by increased ratios of red and green fluorescence signals (Figure 2F). This result suggests that stimulation by 7µl PA for two minutes or less triggers relatively low levels of DA release from the DL2 neurons, providing an explanation for why fed larvae stimulated by an effective dose of PA for 2 minutes failed to show a significant appetitive arouse

(Wang et al., 2013). We also found that stimulation of fed larvae with 20 µl PA caused a rapid surge of intracellular Ca^{2+} in the DL2 neurons within the first minute, but the intracellular Ca^{2+} level became gradually reduced as the PA stimulation is extended. In addition, a brief but not prolonged activation of DA neurons using dTrpA1, a temperature-sensitive TRP family cation channel(Hamada et al., 2008), show a strong stimulating effect on the larval feeding response (Figure 3.2G). These results suggest that over-stimulation of the DL2 neurons is detrimental to appetitive arousal.

A DA Mechanism That Functionally Links Olfactory and Cognitive Systems

The failure of 20µl PA to arouse appetite may be account for by two opposing explanations. One possibility is that it may cause silencing of DL2 neurons after a 5-minute stimulation, thereby preventing acutely required DA signaling to downstream targets. The other is that it may cause the release of excessive DA, which is ineffective to activate the targets. To distinguish these two possibilities, we first stimulated fed larvae with 20µl PA for 30 seconds instead of 5 minutes. Indeed, fed larvae showed a significant increase in their feeding response to the sugar medium (Figure 3.2H). Since the 30-second stimulation by 20µl PA is already sufficient to trigger DA signaling to an effective level, this finding suggests that the total amount of DA released by a 5-minute stimulation with 20µl PA is likely much higher than that with 7µl PA.

We then tried to examine how changes in the baseline DA level in fed larvae affect appetitive arousal. To reduce the DA level, we performed RNAi-mediated knockdown of Tyrosine Hydroxylase (TH), a rate-limiting enzyme for DA synthesis (Friggi-Grelin et al., 2003). Fed larvae (TH-GAL4/UAS-THRNAi) expressing a double-stranded RNA of TH in DA neurons exhibited no significant appetitive responses to

normally effective doses of odorants (e.g., 5 µl PA; Figure 3.3A). However, when presented with higher, normally ineffective doses such as 20µl PA or a binary mixture of 10µl PA and 30µl Hep, the same larvae exhibited appetitive responses. In parallel, we also pre-fed larvae with food containing a TH inhibitor 3IY and observed similar behavioral changes (Neckameyer, 1996). For example, after pre-feeding with 3IY for 4 hrs, the larvae required abnormally high doses of PA or odor mixtures to trigger the appetitive response (Figure 3.3B). We also increased the baseline level of DA in fed larvae by pre-feeding with L-dopa, a precursor of DA. Larvae pre-fed with L-dopa for 4hrs displayed opposite behavioral phenotypes (Neckameyer, 1996); these larvae only showed positive responses to odorants at doses lower than normally required (e.g., 2.5µl PA; Figure. 3.3C). Therefore, L-dopa can augment lower doses of odorants to arouse the appetitive response, and the minimal strength of an odor stimulus required to arouse appetite is inversely correlated with the baseline level of DA in fed larvae. Together, these findings lend further support to the notion that an excessively high level of DA, evoked by a strong odor stimulus, is not perceived as appetizing by fed larvae.

Our previous work showed that Dop1R1 is essential for the appetitive arousal of fed larvae (Wang et al., 2013). To further understand how the larval brain discriminatively attribute incentive salience to discrete DA-coded olfactory cues, we tested appetitive responses to various odors in heterozygous Dop1R1 fed larvae, which have a 50% reduction in Dop1R1 activity (Lebestky et al., 2009). When stimulated by various doses of monomolecular or mixed odorants, Dop1R1/+ larvae displayed a right shift in each of the dose-response curves (Figures 3.3D, E). In addition, pre-feeding of L-dopa restored their appetitive responses to a normally effective dose (Figure 3.3F).

Therefore, by using multiple experimental approaches, our studies suggest that a DA system, residing in two clusters of DL2 and their Dop1R1 target neurons, provides a functional interface between the olfactory and cognitive system. Our evidence also suggests that the DL2 neurons perform sequentially two key functions: integration of olfactory signals encoding odor mixtures such as natural food odors and enabling a measured release of DA that is positively controlled by the total intensity of odor inputs. Another important insight is that DA-coded outputs from the DL2 neurons have no intrinsic appetitive values, and the inverted U effects of odor stimuli largely reflect the function of a Dop1R1-mediated cognitive mechanism that selectively attributes appetitive significance to a narrow range of odor-evoked DA signals that are not too low or too high.

DISCUSSION

Olfaction is known to influence other senses as well as emotion and cognition in humans and animals alike (De Araujo et al., 2003; Johnson, 2011; Rolls, 2015). However, to date, little is known about neural processes in higher cognitive processing centers the brain that mediate odor representations, processing of complex olfactory cues, and how such processed olfactory cues interacts with neural systems for cognitive controls. Through an in-depth analysis of the odor-induced appetitive behavior of *Drosophila* larvae, we have demonstrated that these animals have an innate cognitive ability to selectively attribute incentive salience to discrete food-related odors to control appetitive motivation specific for anticipated sugar reward. Moreover, we have identified an array of conserved neural substrates including dopamine and D1-like receptor Dop1R1, and characterized their roles in cognitive controls in the context of defined

neurons and neural circuits of freely behaving animals. Our findings suggest that *Drosophila* larvae may offer a useful complementary animal model for investigating many fundamental issues in cognitive neuroscience.

The sensory representation of food-related odor is complex and has no intrinsic behavioral meaning. Here, we provide behavioral evidence that fed fly larvae selectively assign appetitive significance to a small fraction of food-related odor stimuli. Through an in-depth analysis, we have identified an array of conserved neural substrates including dopamine and D1 like receptor Dop1R1 underlying odor-dependent feeding motivation specific to sugar-rich food. First, we showed that subset of dopaminergic neurons combinatorially represent and integrate odor input and convert it into one-dimensional dopamine signaling. Second, we reveal that it is required for an optimum level of odor-evoked DA signaling by two clustered DL2 neurons for appetitive odor perception. Thus, we provide a cellular evidence which those DL2 DA neurons as a functional interface between the olfactory system and reward system. In addition, we found that this DA encoded appetitive cues may further relay on Dop1R1 target neurons, the activities of which determine the appetitive value of the odor. Our findings suggest that *Drosophila* larvae may offer a useful complementary animal model for investigating many fundamental issues in cognitive neuroscience.

Among a broad array of monomolecular odorants that are chemotactically attractive to fly larvae (Mathewa et al., 2013), some of them are capable of arousing appetite for sugar food. Our previous study showed that a functional deficiency in the DL2 neurons blocked the appetizing effect of PA but not its stimulation of larval chemotactic attraction (Wang et al., 2013). In this work, we also find that the appetizing

effects of all tested odorants, whether monomolecular or complex, follow an inverted U function. Another important feature is that these odorants display a narrow effective dosage range (~2 fold), which is in stark contrast to the extremely broad effective dosage ranges (>103 fold) reported for the chemotactic response (Mathewa et al., 2013). The functional imaging analysis reveals that all four DL2 neurons in each cluster are responsive, at various degrees, to diverse monomolecular odorants. Those findings suggest that DL2 neurons may be involved a conserved and hardwired circuit mechanism underlying integration various odor input and further association with sugar food expectation.

Lateral horn neurons (LHNs) are implicated with odor discrimination as their responses are highly selective to certain types of odor (Luo et al., 2010). Moreover, it reports that LH may be involved in regulating innate olfactory behavior as the connective pattern from PNs to LHNs are spatially stereotyped (Marin et al., 2002; Wong et al., 2002). Our previous result showed a cluster of DL2 DA neurons in each brain hemisphere appears to form synaptic connections with at least a subset of the PNs in lateral horn region, implying that DL2 neurons are a subset of lateral horn neurons (LHNs). Here, we further analysis 4 DL2 neurons correlatively represent the distribution of appetitive odors at sensory coding layer. The distinct excitatory pattern among DL2 neurons under different types of odors indicates those DL2 neurons are likely to use a combinatorial coding scheme to encode odor value.

Another interesting finding is that, as the chemical composition of odor stimuli becomes more complex, increasingly higher numbers of DL2 neurons from both clusters are required to properly process such olfactory information for its potential appetizing

effect. These findings suggest that the appetitive perception of natural food odors involves a signal integrator that is powered by the distributed activities of individual DA neurons. Furthermore, by taking multiple approaches, we provide evidence that the DL2 neurons perform a summation function to mixed odor inputs by additively contribute to a common pool of odor-evoked DA signals. It has also been reported that simultaneous excitation of a large number of projection neurons and associated interneurons by a mixture of multiple odorants triggers a signal processing operation known as divisive normalization (Olsen et al., 2010). Such divisive normalization reduces and equalizes the strengths of output signals. Therefore, to trigger appetitive arousal, complex odor stimuli may undergo two sequential rounds of signal processing at the levels of second- and third-order olfactory (DL2) neurons.

The Dop1R1 gating mechanism in shaping the inverted-U dose response

The dose-response analysis shows that the appetizing effects of both monomolecular and mixed odor stimuli follow an inverted U function. We have provided evidence that such dose-response relationships reflect the requirement of an optimum level of odor-evoked DA released from the DL2 neurons. First, in freely behaving larvae, stimulation by an appetizing dose of PA (7µl) caused gradually increased excitatory responses from these DA neurons, while a higher non-appetizing dose of PA(20µl) triggered a rapid surge of DA release (Figure 3.2F). This finding suggests that the amount of DA released from the DL2 neurons is positively correlated with the strength of odor stimulation. Second, reduction of DA synthesis, either by knocking down the TH activity or pre-feeding with a TH inhibitor 3IY, rendered higher doses of PA to be appetizing but blocked the appetitive responses to odor stimuli that are

normally appetizing (Figure 3.3A, B). Conversely, oral administration of L-Dopa augmented a lower ineffective dose of odor to trigger appetitive arousal while the appetizing effects of normally effective odor stimuli were inhibited (Figure 3.3C). Finally, a 50% reduction of Dop1R1 activity led to a right-shift of the dose-response curve regardless whether the odor stimulus is monomolecular or chemically complex; in other words, DA-coded olfactory information has no intrinsic appetitive values, and it is the Dop1R1 mechanism that determines which range of the DA signals will be assigned with appetitive significance. Together, these results suggest that appetitive response of fed larvae is gated by Dop1R1 activity, and, to be perceived as appetizing cues, odor-evoked DA signals must be within a narrow range that matches the pre-existing level of Dop1R1 activity. Therefore, our findings may provide a general understanding of the neurobiological basis of dopamine neurons modulation of sensory coding and D1-receptor regulated cognitive control.

METHODS

Fly Stocks and Larval Growth

All flies are in the w1118 background. Larvae were reared at 25°C, and early third instars (~74 hr after egg laying, AEL) were fed before behavioral experiments as previously described (Wang et al., 2013). The transgenic flies include TH-GAL4 (Friggi-Grelin et al., 2003), UAS-dTrpA1(Hamada et al., 2008), UAS-GCaMP6.0(BL42749), UAS-THRNAi (BL25796), UAS-CaMPARI (BL58761), were obtained from Bloomington stock center. $DopR^{f02676}$ flies were described previously (Lebestky et al., 2009).

Behavioral Experiments

Quantification of mouth hook contraction rate in liquid food was performed as previously described (Wu et al., 2005). A published protocol for fly larvae odor stimulation was used with slight modifications (Wang et al., 2013). Briefly, synchronized early third instars, fed on yeast paste, were stimulated for 5 minutes with specified doses of single or mixed odors including pentyl acetate (PA) (Sigma-Aldrich, 628-63-7), heptanal (Hep) (Sigma-Aldrich, 117-71-7), trans-2-Hexen-1-al (T2H) (Sigma-Aldrich, 6728-26-3), and the vapor of balsamic vinegar. After rinsing with water, larvae were tested for their feeding responses. Feeding media include agar paste (US Biological, A0940) containing 10% glucose, 0.5% Tryptone (Becton-Dickinson, 628200) or 3% oleic acid (Sigma-Aldrich, 112-80-1). UAS-dTrpA1 was expressed by allowing larvae to feed in pre-warmed yeast paste in a 31°C incubator for defined periods, followed by rinsing with 31°C water prior to feeding assays.

3IY and L-dopa Feeding

3-iodo-L-tyrosine (3IY) (Sigma-Aldrich, 70-78-0) and L-DOPA-ring-d3 (L-dopa) (Sigma-Aldrich, 53587-29-4) were used. The protocols for administration of 3IY and L-dopa were described previously (Wang et al., 2013). The concentrations of 3IY and L-dopa in yeast paste were 10mg/ml and 0.5mg/ml, respectively.

Immunostaining

Tissue dissection and fixation, Antibodies used and dilution conditions were described previously (Wang et al., 2013).

Targeted Laser Lesion

Protocols for calibration of 337 nm nitrogen laser unit and laser lesion experiments have been described (Xu et al., 2008).

Calcium and CaMPARI Imaging

Processing of intact tissues of the larval nervous system and imaging of DA neurons that project to the lateral horns was performed as previously described (Wang et al., 2013). UAS-GCaMP6.0 was used in this work. The conditions of larval feeding and odor treatment for CaMPARI imaging are identical to those for odor-aroused feeding behavioral assays. After odor stimulation, larvae were irradiated with 405 nm light for 3s using 500mw 405nm 5V blue-violet laser module. The treated larval CNS was dissected and individually scanned using a Zeiss LSM 510 confocal microscope.

Arclight-based Analysis of the membrane potential of neurons

The experimental protocol for Arclight imaging is described in the Supplemental Experimental Procedure.

Statistical Analysis

One-way ANOVA followed by Dunnett's or Dunn's test was used for all behavioral, GCaMP and CaMPARI imaging analyses.

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Figure 3.1 The Appetizing Effects of Monomolecular and Mixed odorants.

(A-C) Third-instar fed larvae (74h after egg laying, AEL) were exposed to an odor stimulus for 5 minutes in a sealed chamber, fumigated with defined concentrations of monomolecular odorants PA, Hep, T2H, prior to the feeding test in 10% glucose liquid media. The odor effects on larval feeding rate were quantified by counting mouth hook contractions of each larva over a 30s period. The rate of mouth hook contraction is positively correlated to the amount of dyed food ingested (Wang et al., 2013). (D-F) The test conditions are identical except odor mixtures were used instead: balsamic vinegar (BV) (D), PA plus Hep (E) and PA plus T2H (F). (G) Fed larvae, stimulated by PA at an effective dose (5 μl), displayed aroused feeding response to 10% glucose but not protein and fatty acid-rich palatable diets. All behavioral quantifications for this and other figures were performed under blind conditions. *$P<0.05$; **$P<0.001$; $n>14$.

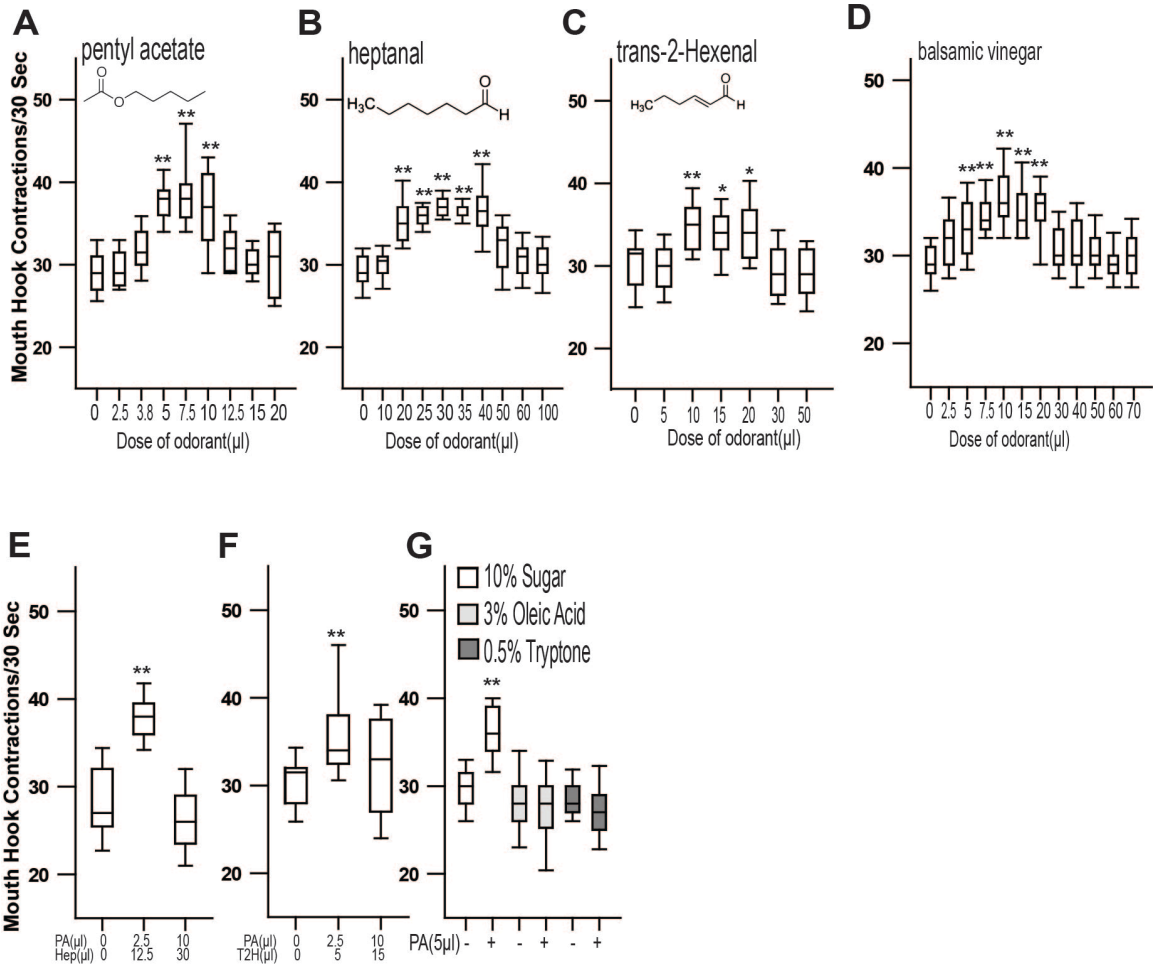

A
pentyl acetate
B
heptanal
C
trans-2-Hexenal
D
balsamic vinegar
Mouth Hook Contractions/30 Sec
Dose of odorant(µl)
E
F
G
10% Sugar
3% Oleic Acid
0.5% Tryptone
PA(µl)
Hep(µl)
T2H(µl)
PA(5µl)

Figure 3.2 The Combinatorial Role of DA Neurons in Higher-order Representations and Processing of Food Odor Stimuli in Freely Behaving Fed Larvae.

(A) A schematic drawing of the larval olfactory circuit shows two clusters of four DA neurons (DL2-1 to 4) in the left and right brain hemisphere (Wang, 2013). The DL2 and projection neurons form synaptic connections in the lateral horn (LH) of each brain lobe. AL: antenna lobe; MB: mushroom bodies; PNs: projection neurons. (B) Calcium imaging of the four DA neurons in intact CNS tissues from fed larvae. The left cluster displayed a larger excitatory response to PA than the right cluster ($P=0.026$). Within the left cluster, for example, the three odorants (PA, Hep and T2H) triggered significantly different responses from DL2-2 ($P< 0.001$), while responses of DL2-1 were similar ($p=0.109$). $n \geq 8$. (C) DA neurons in second-instar *TH*-GAL4/UAS-*nls*GFP larvae were lesioned by focused laser beams. After one day of recovery, third-instar (74h AEL) larvae were tested for the odor effects. The mock group was handled in the same way as that for experimental groups except for the laser treatment. Three groups of experimental larvae, each containing one, two or three DA neurons within the same cluster were tested for the feeding response to an appetitive dose of PA or Hep ($n\geq11$). (D) Experimental larvae containing three or four DA neurons in the right cluster were tested for the feeding response to PA, Hep or a binary mixture of PA and Hep ($n\geq10$). (E) Experimental larvae containing four DA neurons in the right cluster failed to respond to balsamic vinegar (BV) vapor, a complex odor mixture ($n\geq17$). (F) In freely behaving larvae, DL2 neurons display dynamic responses to two different doses of PA after a defined time period of stimulation, as revealed by CaMPARI-based fluorescence imaging. Data are presented as ±SEM. $n>24$; (G) Genetic activation of fed larvae by dTrpA1 at 31^{o}C for 10 minutes led

to increased feeding response in the absence of odor stimulation (n≥21). (H) Fed larvae display appetitive responses to 30-sec stimulation by 20µl PA. n≥11; **P< 0.001.

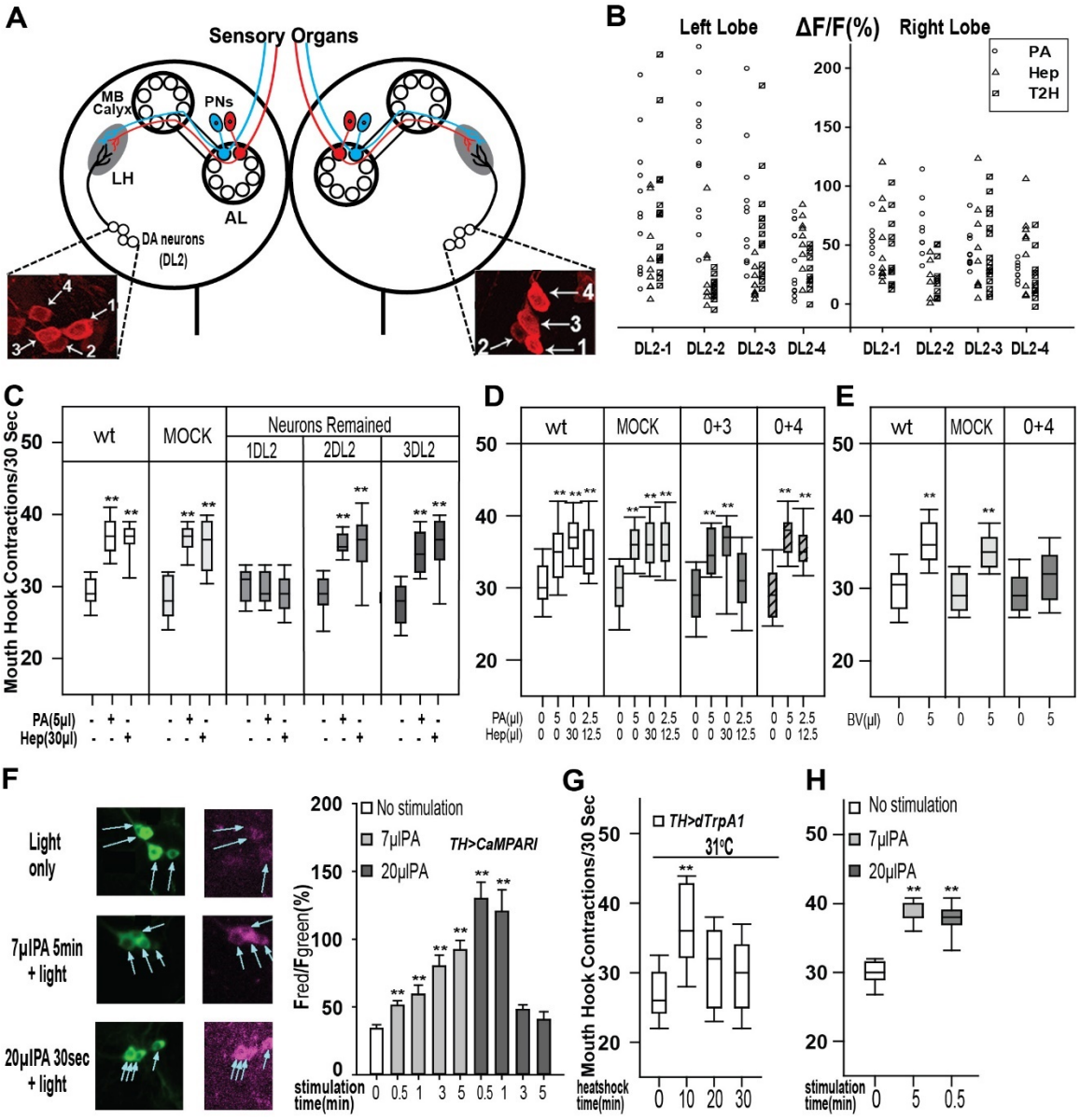
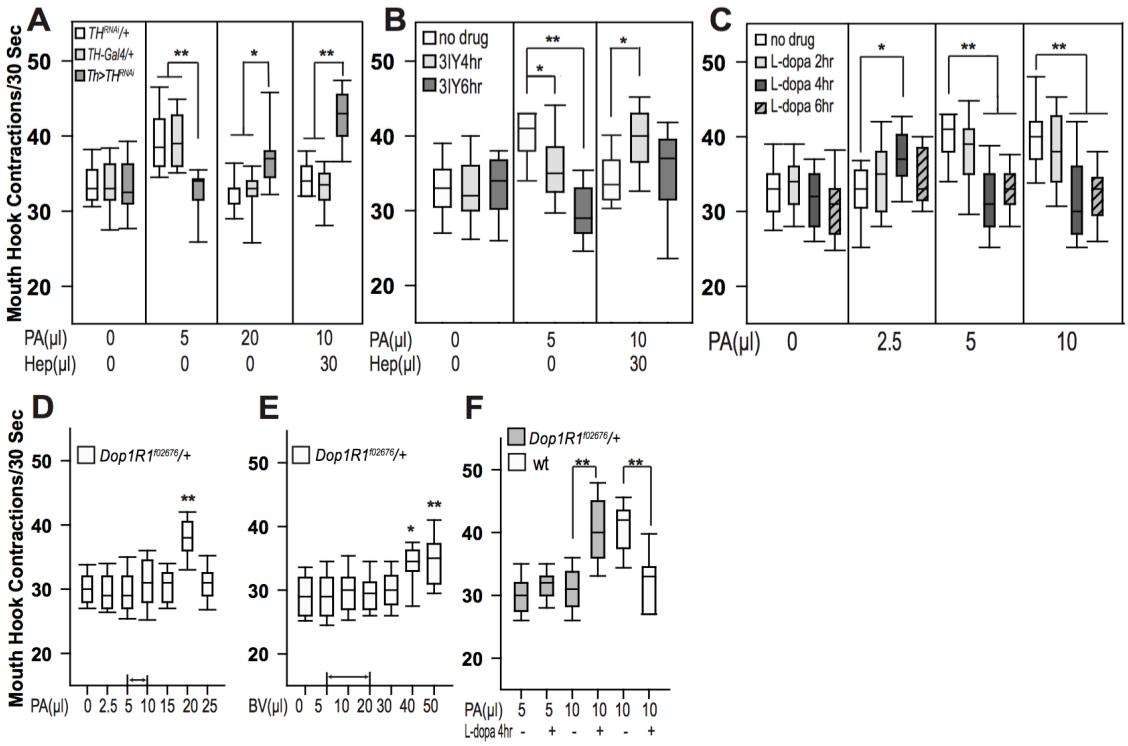

A
Sensory Organs
MB Calyx
PNs
LH
AL
DA neurons (DL2)
B
Left Lobe
ΔF/F(%)
Right Lobe
PA
Hep
T2H
DL2-1
DL2-2
DL2-3
DL2-4
C
Mouth Hook Contractions/30 Sec
wt
MOCK
Neurons Remained
1DL2
2DL2
3DL2
PA(5μl)
Hep(30μl)
D
wt
MOCK
0+3
0+4
PA(μl)
Hep(μl)
E
wt
MOCK
0+4
BV(μl)
F
Light only
7μlPA 5min + light
20μlPA 30sec + light
No stimulation
7μlPA
20μlPA
TH>CaMPARI
Fred/Fgreen(%)
stimulation time(min)
G
TH>dTrpA1
31°C
Mouth Hook Contractions/30 Sec
heatshock time(min)
H
No stimulation
7μlPA
20μlPA
stimulation time(min)

Figure 3.3 The DA/Dop1R1 Pathway Functionally Couples Olfactory and Cognitive Systems for Appetitive Odor Perception

(A) Reduced baseline level of DA by Expressing UAS-TH^{RNAi} in *TH*-GAL4 neurons led to an increase in the minimal effective dose of odorants required for appetitive arousal (n >13). (B) Wild type larvae fed with 3IY-containing media for 4 hours before feeding tests also led to an increase in the minimal effective dose of odorants required for appetitive arousal (n >11). (C) Wild type larvae fed with L-dopa-containing media for 4 hours before feeding tests caused a decrease in the minimal effective dose of odorants required for appetitive arousal (n >10). (D, E) Fed larvae heterozygous for $Dop1R1^{f02676}$, a loss-of-function mutation in Dop1R1, showed a right shift in the inverted-U dose response. The range of normally effective doses is indicated by arrows (n≥10). (F) Feeding heterozygous $DopR^{f02676}$ larvae with L-dopa restored the appetitive response to a normally effective dose of PA (n≥13). *P<0.05; **P<0.001


A
B
C
D
E
F
Mouth Hook Contractions/30 Sec
THRNAi/+
TH-Gal4/+
Th>THRNAi
no drug
3IY4hr
3IY6hr
L-dopa 2hr
L-dopa 4hr
L-dopa 6hr
PA(µl) 0 5 20 10
Hep(µl) 0 0 0 30
PA(µl) 0 5 10
Hep(µl) 0 0 30
PA(µl) 0 2.5 5 10
Dop1R1f02676/+
PA(µl) 0 2.5 5 10 15 20 25
BV(µl) 0 5 10 20 30 40 50
wt
PA(µl) 5 5 10 10 10 10
L-dopa 4hr - + - + - +

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CHAPTER 4

DISSECTION OF MOLECULAR AND NEURONAL PATHWAYS OF DOPAMINE-RESPONSIVE NEUROPEPTIDE F NEURONS IN APPETITIVE AROUSAL

INTRODUCTION

Drosophila expresses neuropeptide F, which is related evolutionarily to mammalian orexigenic NPY. In recent years, several studies have emphasized on the functional role of NPY system in encoding motivational states for a variety of goal-directed behaviors (Krashes et al., 2009; Shohat-Ophir et al., 2012; Wu et al., 2003; 2005). In *Drosophila*, NPF has several distinct putative functions such as hunger-driven feeding, representing reward status, and regulate innate attraction. First, it was shown that activation of the NPF circuit prolongs the feeding state or the motivation to consume noxious or cold food mimicking the hunger-state fly (Wu et al., 2005). Those findings indicate NPF system may encode internal hunger-satiety states for promoting hunger-driven feeding activities. In addition, NPF neurons are also found to regulate hunger-driven memory retrieval by encoding internal motivational states (Krashes et al., 2009). Furthermore, NPF promotes innate attraction to appetitive odors in food-deprived flies (Beshel and Zhong, 2013). Inhibition of NPF neurons decreases food odor attractions in starved flies, and conversely, activation of NPF neurons promotes robust food odor attractions in fed flies. Moreover, it suggests that activation of NPF neurons is rewarding itself to fly. It has been reported that NPF regulate reward ethanol consumption after sexual deprivation and artificial activation of NPF neurons interferes with the ability to

find ethanol consumption as a reward(Shohat-Ophir et al., 2012). Collectively, those findings support that NPF system may signal the state of reward system through encoding both internal state and external reward cues in *Drosophila*.

In fly studies, the current data indicate NPF system interact with dopamine system in both homeostatic or hedonic feeding regulation. In our previous studies, NPF receptors gate subset of dopamine neurons to promote appetitive behavior in larvae. The single neuropeptide F receptor (NPFR) is expressed in many dopaminergic neurons in larvae, including DL2 neurons, and knockdown NPFR in dopamine neurons blocks both appetitive odor enhancement of DL2 neuronal activity and feeding behavior (Wang et al., 2013). In addition, NPF neurons modulate the effect of satiety on sugar reward memory through its signaling acting on dopamine PPL1 neurons, suggesting NPF neurons together with dopamine neurons encode internal motivational states for hunger-driven memory retrieval (Krashes et al., 2009). More recently discovered in adult flies, NPF family promotes sugar sensitivity and may be upstream of the TH-VUM neuron located in the SOG (Marella et al., 2012). Together, those finding all indicate that to NPF system may intact with dopamine system to encode motivational state regulate rewarding signaling.

In the prefrontal cortex of mammals, an optimum level of D1-type DA receptor activity is crucial for several cognitive functions such as spatial working memory, since its signaling at levels that are too low or too high leads to impaired working memory (Arnsten, 2009; Floresco, 2013; Vijayraghavan et al., 2007). In fed larvae, a D1-type DA receptor Dop1R1 has also been shown to mediate odor-induced appetite for anticipated food reward. Another preeminent behavioral feature of fed larvae is that even after

appetitive odor stimulation is terminated, the larvae remain in the aroused state for at least 10 minutes (Wang et al., 2013). These findings raise the possibility that a conserved D1-type DA receptor mechanism may service parallel functions for cognitive controls in both flies and mammals.

In this chapter, we investigate the functional role of the NPF signaling and NPF neurons in promoting feeding under brief food related odor stimulation. Our findings suggest DM NPF neurons activation and NPF signal toward SOG region is required for feeding rate increase. We showed odor-induced signaling modulate NPF neurons activities through D1-like receptor Dop1R1, which has been identified previously as its crucial role in shaping the inverted-U dose-response. We also identify that the Dop1R1 precisely regulate NPF neurons activities through two layered precision tuning strategy which comprise a Dop1R1/Gβ13F/IRK2 -mediated inhibitory and a Dop1R1-Gαs-mediated excitatory mechanism, for shaping NPF neurons excitation in response to optimal odor-evoked DA signaling.

RESULTS

A pair of NPF neurons in the brain is essential for larval appetitive arousal

Based on our previous notion about NPF signaling and NPF neuron activities is required by odor-induced feeding, we began to investigate the requirement of NPF signaling under various dose of feeding assay. We test npf-deficient fed larvae, which express UAS-npfRNAi under the direction of npf-gal4. We found that NPF-deficient fly larvae failed to display appetitive response to odorants (e.g., PA) at all doses tested (Figure 4.1A). The npf-gal4 driver predominantly labels six NPF neurons in the larval central nervous system (CNS; Figure 4.1C; (Brown et al., 1999; Wu et al., 2003)). We

also show that the dendrites of a pair of npf-gal4 neurons in the dorsomedial region of the larval brain are extensively distributed in the lateral horn region (Laura J J Nicolaia et al., 2010) (Figure 4.1 C). The imaging analysis using a split GFP technique suggests that these NPF neurons form presumptive synaptic connections with the DL2 neurons in the lateral horn(Figure 4.1 D; (Feinberg et al., 2008)).

To test their potential function in larval appetitive response, we genetically activated NPF neurons using a fly TRP family channel, dTrpA1 (Hamada et al., 2008). The npf-GAL4/UAS-dTrpA1 fed larvae, heat shocked at 31^{o}C for 15 or 30 min, showed increased feeding activity even in the absence of appetitive odor stimuli (Figures 4E). We also performed a laser lesioning analysis to determine which pair(s) of NPF neurons in the brain mediates larval appetitive arousal. When two dorsomedial NPF neurons in fed larvae were lesioned, their odor-induced feeding response was abolished. However, selective lesioning of the two dorsolateral NPF neurons did not affect the feeding response (Figure 4.1 F). In parallel, we used dTrpA1 to genetically activate the NPF neurons. At 31^{o}C, npf-Gal4/UAS-dTrpA1 fed larvae showed increased feeding activity even in the absence of appetitive odor stimuli. However, the npf-Gal4/UAS-dTrpA1 larvae with lesions in two dorsomedial but not dorsolateral NPF neurons failed to increased feeding response (Figure. 4.1G). The two dorsomedial NPF neurons project caudally towards the subesophageal ganglia, which is implicated in gustatory sensation and feeding control (Bader et al., 2007; Flood et al., 2013; Vosshall and Stocker, 2007; Wang et al., 2004). Severing the two axons of these NPF neurons above, but not below, the subesophageal ganglia abolished the appetitive response (Figure 4.1H). Together, these results suggest that activation of the dorsomedial NPF neurons is necessary and

sufficient to induce appetitive arousal in fed larvae. Given the extensive presence of the dendrites of the dorsomedial NPF neurons is present in the lateral horns, these findings also support the notion that these NPF neuron is the postsynaptic target of odor-evoked DA signals from the ipsilateral cluster of the DL2 neurons.

Our previous study suggests that the arousal state of fed larvae is diminished within 17 minutes after odor stimulation (Wang et al., 2013). Since the activation of the dorsomedial NPF neurons is necessary and sufficient for the appetitive response, we decided to test whether these NPF neurons are responsible for the temporal control of the state of appetitive arousal in fed larvae. We found that after the PA stimulation, the excitatory state of the dorsomedial NPF neurons in freely behaving larvae gradually decayed, and returned to the pre-stimulation level within15 minutes (Figure 4.1 I). Furthermore, we performed a more precise measurement of the decay of the arousal state in fed larvae under the same conditions of PA stimulation. As expected, the appetitive response of fed larvae also returned to the baseline level within 15 minutes after the removal of PA (Figure 4.1 J). Therefore, these findings suggest that the timed decay of the excitatory state of the dorsomedial NPF neurons may determine the attention span of PA-stimulated larvae for anticipated sugar reward.

To further characterize the cellular basis for NPF neurons activities under various dose of odor stimulation. First we tried to determine how odor stimuli influence the membrane potentials of the dorsomedial NPF neurons in a larval preparation using a fluorescent indicator of membrane potential (Arclight). The dorsomedial NPF neurons showed a gradual increase in excitatory response over a 10-minute continuous exposure to PA vapor at an effective dose. In contrast, when a higher non-appetizing dose of PA

was applied, no excitatory responses were observed, except for a transient depolarization immediately following the odor application (Figure 4.2 A-D). In parallel, we also examined in freely behaving fed larvae how the dorsomedial NPF neurons respond to appetizing odors using CaMPARI-based imaging (Figure 4.2 E). Similar to the Arclight imaging results, these neurons showed an excitatory response to an appetizing dose of PA (7µl) , but failed to respond to non-appetizing PA doses that are either higher or lower (e.g., 20µl or 3.5µl). Therefore, similar to its behavioral effects, PA also exerts inverted-U effects on the excitatory response of the dorsomedial NPF neurons in freely behaving fed larvae.

DopR1-gated NPF neuronal response to odor-evoked DA signals

Based on these findings, we decided to test whether functional knockdown of Dop1R1 activity in NPF neurons might affect odor-evoked appetitive arousal as well as NPF neuronal response in fed larvae. Indeed, when stimulated by PA or Hep, npf-Gal4/UAS-Dop1R1dsRNA fed larvae displayed a right shift of the dose-response curve, phenocopying Dop1R1/+ fed larvae (Figure 4.3 A). Furthermore, the dorsomedial NPF neurons expressing both UAS-Dop1R1RNAi and UAS-CaMPARI also displayed a right shift in their dose-response profile when stimulated by PA. For example, the Dop1R1-deficient NPF neurons became excitable by PA at 20µl but not at 7µl (Figure 4.3B). In addition, expression of dTrpA1 in Dop1R1-deficient NPF neurons can bypass the requirement of Dop1R1 activity to trigger appetitive arousal in an odor-independent manner (Figure 4.3C), suggesting that Dop1R1 is involved in modulation of DA-mediated excitation of NPF neuronal rather than NPF signaling per se. In combination, these observations suggest that the Dop1R1 activity in the NPF neurons modulates the

inverted U effects of food odor stimuli on larval appetitive arousal by tuning their responses to an optimum range of odor-evoked DA signals.

A key remaining question is how Dop1R1 precisely tunes the two NPF neurons in response to odor-evoked DA signals. D1-type DA receptor is known to be associated with the heterotrimeric G protein complex consisting of Gαs, Gβ, Gγ subunits (Rogan and Roth, 2011). Upon its activation by DA, the dissociated Gαs subunit and Gβγ complex each defines a separate effector pathway. The *Drosophila* genome contains genes encoding three Gβ subunits (Boto et al., 2014; Yarfitz et al., 1988; 1991). We performed RNAi-mediated knockdown of Gβ genes in the NPF neurons. Functional knockdown of Gβ13F activity in the NPF neurons of npf-GAL4/UAS-Gβ13FRNAi fed larvae led to an expansion of appetitive response to PA at all doses tested (from 5 to 20 µl), while the appetitive responses of control larvae (npf-GAL4/UAS-Gβ76CRNAi) remained normal at the PA doses of 5 and 20µl (Figures 4.3D).

One of potential molecular targets of the Gβγ complex is a G protein-dependent inward-rectify potassium channel (Karschin et al., 1996; Tipps and Buck, 2015). Since fly larvae have three such channels (IRK1-3; (Döring et al., 2002; Hibino et al., 2010)) we functionally knocked down each of them. Indeed, fed larvae that are deficient in IRK2 but not IRK1 or IRK3 activity photocopied npf-GAL4/UAS-Gβ13FRNAi fed larvae (Figures 4.3D). In parallel, we also tested how the two dorsomedial NPF neurons in freely behaving npf-GAL4/UAS-Gβ13FRNAi and npf-GAL4/UAS-IRK2RNAi fed larvae respond to stimulation by 7 or 20 µl PA. We found that the dorsomedial NPF neurons expressing UAS-CaMPARI, along with UAS-Gβ13FRNAi or UAS-IRK2RNAi, displayed an abnormally high level of excitatory responses to the stimulation by 20µl PA,

while their responses to 7µl PA remained similar. Therefore, these findings indicate that Dop1R1, together with its effectors Gβ13F and IRK2, define an inhibitory mechanism in the NPF neurons. When this pathway is deficient, the effective range of odor-induced DA signals is greatly expanded, converting the inverted U dose-response curve to the sigmoidal shape in these larvae.

We have also tested the effect of Gαs activity in the NPF neurons on the appetitive response of fed larvae. RNAi-mediated knockdown of Gαs activity blocked appetitive response to PA at all doses tested (5 to 20 µl; Figures 4.4A). Gαs is known to positively regulate an adenylyl cyclase and protein kinase A (PKA; (Nishi et al., 2011)). As expected, functional knockdown of rutabaga, which encodes an adenylyl cyclase(AC) activity, or PKA also led to a reduction of appetitive response of these fed larvae when stimulated by an effective dose of PA (5µl). Furthermore, in freely behaving fed larvae, a deficiency in Gαs or rutabaga abolished the excitatory response of the dorsomedial NPF neurons to stimulation by an effective dose of PA (Figure 4.4B). Therefore, these results suggest that the Dop1R1-mediated precision tuning of the NPF neurons also involves a Gαs/cAMP/PKA-mediated excitatory mechanism; This Dop1R1/Gαs pathway appears to have two functions: setting up a minimal level of odor-evoked DA signals required for NPF neuronal excitation and mediating potential NPF neuronal responses to any odor stimuli above a threshold level. Taken together, the Dop1R1/Gβ13F/IRK2-mediated inhibitory and Dop1R1/Gαs-mediated excitatory mechanisms define a two layered precision tuning strategy that restricts NPF neuronal response to an optimum level of odor-evoked DA signals and thereby shapes the inverted-U dose response of fed larvae (Figure 4.4C).

DISCUSSION

In chapter II, our results demonstrate that the olfactory-driven feeding is abolished with inhibiting NPF neurons or inactivating NPF receptor function. In this chapter, through functional test we identify that a pair of DM NPF is crucial for odor induced feeding. Anatomical evidence shows that DM NPF neurons are putative postsynaptic targets of odor-evoked dopamine signaling from DL2 neurons. In addition, we investigate the cellular and molecular basis of DM NPF neurons activities under various dose of odor stimulation. The inverted U shape of DM NPF neurons activities under odor stimuli further indicates the appetitive arousal may positive correspond with NPF neurons activities. Thus, we further map the D1-like receptor Dop1R1 activities reside in NPF neurons. Dop1R1-mediated precise tuning mechanism to restrict NPF neurons activities to an optimum level of odor-evoked DA signals and thereby shapes the inverted-U dose response of fed larvae. Our findings provide novel mechanistic insights into how precise DA and D1-like receptor action enable superior cognitive control insects and possibly mammals.

There are debates about the involvement of NPF neurons in olfactory coding. Although few pieces of evidence argues that NPF neurons activation is independent with olfactory processing pathway (Rohwedder et al., 2016), two-photon calcium imaging shows the amplitude of food odor-evoked activity in NPF neurons, strongly correlates with food-odor attractiveness in adult flies (Beshel and Zhong, 2013). Another interesting piece of finding is that NPF neuron activation is only observed under the natural food odor or food-related monomolecular odor stimuli. However, interestingly those synthetic non-food odors failed to activate NFP neurons. The selectivity of the odor-evoked NPF

response indicates food odor information may get through specific food related odor processing pathways to trigger NPF activation. In our studies, GRASP analysis indicate that NPF neurons form putative synaptic connections with DL2 neurons in LH. Also in freely behaving fed larvae, we showed the NPF neurons display excitatory responses to stimulation by appetizing doses of PA (Figure 4.3A-E). Those results indicate NPF neurons function as the downstream targets of food odor processing neurons which specifically encode food odors values. To sum up, it suggests that NPF neurons selectively response to food related odor, due to food odors activate specific zone in LH where DL2 neurons connect with PNs. Thus, it is also interesting to know if DL2 or NPF neurons could also be activated by non-appetitive odor.

NPF has been known as a primary neuromodulator for encoding the motivational state of the fly. It remains unclear if activation of NPF directly promote sugar reward expectation in a context-independent manner. Here, we have provided functional evidence that two NPF neurons, located in the dorsomedial region of the brain lobes, are necessary and sufficient to elicit appetitive arousal in fed larvae. In freely behaving fed larvae, the NPF neurons display an inverted U like excitatory responses to odor stimulation (Figure 4.3A-E), and these responses are also gated by the Dop1R1 activity in the dorsomedial NPF neurons (Figure 4.4). Thus, these results point to a critical role of the NPF neurons in selective attribution of incentive salience to discrete DA-coded odor cues. We also provide evidence that the NPF neurons are centrally responsible for maintaining larval attention to anticipated sugar reward. This is achieved through the timed decay of the excitatory state of the two dorsomedial NPF neurons. Therefore, the

NPF neurons may provide a useful model to investigate the molecular basis of such memory trace, attention span and working memory

With the notion that NPF neurons are precisely regulated by odor-evoke DA signaling and Dop1R1 activities, we further provided molecular and cellular evidence for how Dop1R1 activity in the NPF neurons shapes the inverted U effects of appetitive odor stimuli. Using both behavioral and functional imaging assays, we show that Dop1R1 determines which range of odor-evoked DA signals may acquire appetitive significance through precision tuning of NPF neurons. Two separate molecular signaling modules have been identified for this process. One of them, involving a Dop1R1/-Gβ13F/Irk2-mediated pathway, sets an upper limit of the optimum effective range of odor-induced DA signals by silencing the NPF neurons when Dop1R1is hyper-activated. Attenuation of the Gβ13F or IRK2 activity greatly broadens the width of the effective dose range of DA signals, as evidenced by the change of the dose-response curve from an inverted U shape to a sigmoidal shape. Again, this result also strongly supports the notion that the ineffectiveness of stronger odor stimuli is caused by an excessive release of odor-evoked DA, which leads to a high level of dissociated Gβγ complex that activates IRK2 channels and subsequently silences the NPF neurons. The second module, involving a Dop1R1/Gαs-mediated pathway, provides a default excitatory mechanism that mediates NPF neuronal response to any odor stimuli that are at or above a minimal threshold strength. Therefore, they together define a two-layered precision tuning strategy (Figure 4.4C).

METHODS

Fly Stocks and Larval Growth

All flies are in the w^{1118} background. Larvae were reared at 25°C, and early third instars (~74 hr *a*fter *e*gg *l*aying, AEL) were fed before behavioral experiments as previously described (Wang et al., 2013). The transgenic flies include *TH*-GAL4 (Friggi-Grelin et al., 2003), UAS-*dTrpA1* (Hamada et al., 2008), UAS-*nsyb*GFP (Feinberg et al., 2008), UAS-*syt*GFP (Nicolaï et al., 2010), *UAS-Denmark* (Nicolaï et al., 2010), UAS-GCaMP6.0(BL42749), UAS-TH^{RNAi} (BL25796), UAS-$G\alpha s^{RNAi}$(BL29576), UAS-$G\beta 76C^{RNAi}$(BL28507)，UAS-$G\beta 13F^{RNAi}$(BL35041), UAS-AC^{RNAi}(BL27035), UAS-npf^{RNAi} (BL27237), UAS-PKAi (BL35550), UAS-Arclight (BL51056), UAS-CaMPARI (BL58761), UAS-$IRK2^{RNAi}$ (BL41981), UAS-$IRK1^{RNAi}$ (BL42644), UAS-$IRK3^{RNAi}$ (BL26720), and *npf*-GAL4 were obtained from Bloomington stock center. UAS-$Dop1R1^{RNAi}$ (V107058) was obtained from the Vienna *Drosophila* RNAi Center. UAS-*CD4::sp*GFP^{1-10}, LexAop-*CD4::sp*GFP^{11} were kindly provided by K. Scott (Gordon and Scott, 2009).

Behavioral Experiments

Quantification of mouth hook contraction rate in liquid food was performed as previously described (Wu et al., 2005). A published protocol for fly larvae odor stimulation was used with slight modifications (Wang et al., 2013). Briefly, synchronized early third instars, fed on yeast paste, were stimulated for 5 minutes with specified doses of single odor, pentyl acetate (PA) (Sigma-Aldrich, 628-63-7). UAS-*dTrpA1* was expressed by allowing larvae to feed in pre-warmed yeast paste in a 31°C

incubator for defined periods, followed by rinsing with 31°C water prior to feeding assays.

Molecular Cloning

To construct the *npf*-LexA driver, a DNA fragment of ~1-kb containing a region spanning from the 5' regulatory sequence to the beginning of the second axon was amplified by genomic PCR. This fragment was subsequently cloned into the KpnI site in the pBPnlsLexA::GADflUw vector. Forward Primer: cagggagagagaacggagac; Reverse primer: gtgtcacaatgcaattgttcg.

Immunostaining

Tissue dissection and fixation, Antibodies used and dilution conditions were described previously (Wang et al., 2013).

Targeted Laser Lesion

Protocols for calibration of 337 nm nitrogen laser unit and laser lesion experiments have been described (Xu et al., 2008). The sites of axons from the dorsomedial NPF neurons for laser lesioning were selected based on their unique morphological features that are anterior or posterior to the subesophageal ganglia.

Calcium and CaMPARI Imaging

Processing of intact tissues of the larval nervous system and imaging of DA neurons that project to the lateral horns was performed as previously described (Wang et al., 2013). UAS-GCaMP6.0 was used in this work. The conditions of larval feeding and odor treatment for CaMPARI imaging are identical to those for odor-aroused feeding behavioral assays. After odor stimulati, larvae were irradiated with 405 nm light for 3s

using 500mw 405nm 5V blue-violet laser module. The treated larval CNS was dissected and individually scanned using a Zeiss LSM 510 confocal microscope.

Arclight-based Analysis of the membrane potential of neurons

The method for making larval CNS preparation is the same as previously described for calcium imaging preparation (Wang et al., 2013). The preparation was incubated in *Drosophila* PBS. Effective and ineffective odor vapors were prepared by fumigating a sealed 24L foam box with 150 or 800µl PA for 2 hours, respectively. Odor was continuously delivered to larval head region by pumping at the rate of 0.36L/min.

The protocol for ArcLight Imaging (Cao et al., 2013) was followed with minor modifications. Briefly, larval CNS was imaged under 40X water immersion lens using Zeiss Axio Examiner. NeuroCCD-SM camera and Turbo-SM software (RedShirt Imaging) were used for recording and data processing. Images were captured at a frame rate of 100 Hz, and exposure time is 10ms. 2000 frames were collected for each of the seven 20s periods.

All the time series curves were low pass filtered with a Kaiser-Bessel 30 filter (200 Hz cut off). Then, each curve was fitted with a single exponential equation, I=Ae(-at). Bleaching of each curve was corrected based on the formula: It,corrected=It+(A¬¬¬-Ae(-at)). Normalization of each trace was achieved by dividing It,corrected with the average value. Standard deviation and fast fourier transform were obtained using normalized data.

Statistical Analysis

One-way ANOVA followed by Dunnett's or Dunn's test was used for all behavioral, GCaMP and CaMPARI imaging analyses. Two-way ANOVA was used for the ArcLight imaging analysis.

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Figure 4.1 Anatomical and Functional Analyses of Two dorsomedial NPF neurons for Appetitive Arousal

(A) Functional knockdown of *npf* expression in *NPF*-GAL4/UAS-npf^{RNAi} fed larvae led to failed appetitive responses to PA at all doses tested;(n>15.) (B) Four NPF neurons and the presumptive axons revealed by expression of UAS-*nsyb*GFP (Feinberg et al., 2008) directed by *NPF*-GAL4. DM: two dorsomedial NPF neurons; DL: two dorsolateral NPF neurons. Two NPF neurons in the subesophageal ganglia are out of the focal plane (Brown et al., 1999). (C) The presumptive axons (green) and dendrites (red) of NPF neurons are labeled by sytGFP and Denmark (Estes et al., 2000). The dotted box shows the dendrites from the dorsomedial NPF neuron at the lateral horn (LH; also see panel G). (D) Presumptive synaptic connections between NPF and DL2 neurons at the LH (the dotted box) are revealed using a split GFP technique(Feinberg et al., 2008), which involves *TH*-GAL4, *NPF*-LexA, UAS-$CD4::spGFP^{1-10}$ and LexAop-$CD4::spGFP^{11}$. DL2-LH: four DL2 neurons projecting to the LH. Green: immunofluorescence of split GFP; Red: anti-TH. Scale bar=20μm. (E) Genetic activation of NPF neurons in *NPF*-GAL4/ UAS-*dTrpA1* fed larvae at 31°C for 15 or 30 min led to increased appetitive response (n>17). (F) Targeted laser lesioning of two dorsomedial NPF neurons (NPF-DM) but not dorsolateral NPF neurons (NPF-DL) in *NPF*-GAL4/UAS-*nlsGFP* fed larvae abolished the appetitive response (n>10). (G) After a brief heat shock at 31°C, *NPF*-GAL4/ UAS-*nlsGFP*/UAS-*dTrpA1* fed larvae with lesions in two dorsolateral NPF neurons remained normal in appetitive response, while the control larvae with lesions in two dorsomedial NPF neurons failed to display appetitive response (n>10). (H) The axons of the two dorsomedial NPF neurons projecting towards the suboesophageal ganglia (see dotted

circle), a feeding control center of fly larvae. Arrows indicate sites where laser beams were focused on. Inset: an example of an axon of the NPF neuron before and after the laser treatment. Lesioning of two axons above but not below the suboesophageal ganglia abolished the appetitive response. (I) In freely behaving fed larvae, the excitatory state of two dorsomedial NPF neurons largely returned to the baseline 15 minutes after removal of PA ($n \geq 22$). (J) The state of appetitive arousal (attention span) of fed larvae returned to the pre-stimulation level within 15 minutes after removal of PA($n > 19$). **$P < 0.001$.

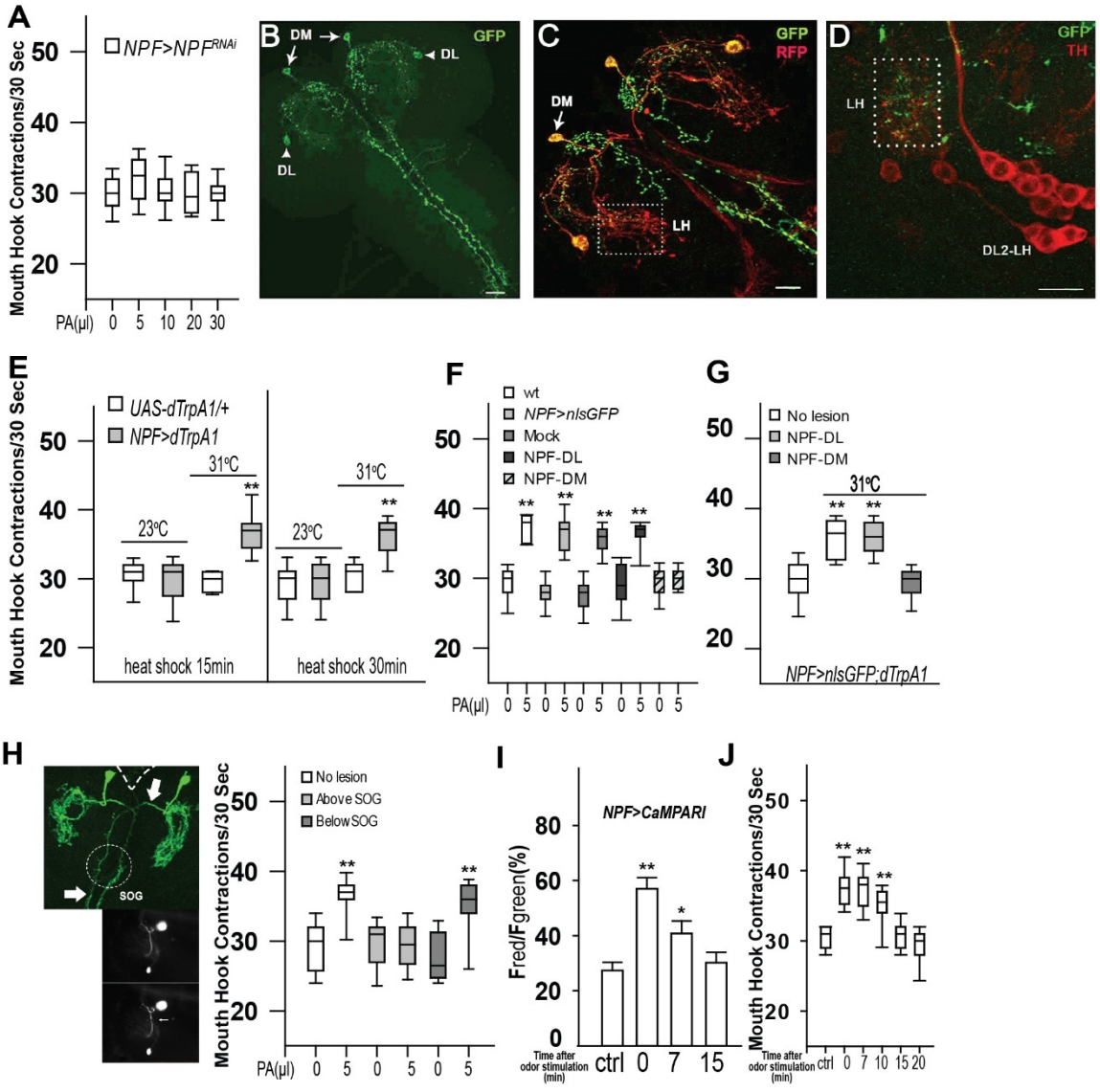

A
Mouth Hook Contractions/30 Sec
NPF>NPF^RNAi
PA(μl) 0 5 10 20 30
B
DM
DL
GFP
C
GFP
RFP
DM
LH
D
GFP
TH
LH
DL2-LH
E
UAS-dTrpA1/+
NPF>dTrpA1
23°C
31°C
heat shock 15min
heat shock 30min
F
wt
NPF>nlsGFP
Mock
NPF-DL
NPF-DM
PA(μl) 0 5 0 5 0 5 0 5 0 5
G
No lesion
NPF-DL
NPF-DM
31°C
NPF>nlsGFP;dTrpA1
H
SOG
No lesion
Above SOG
Below SOG
PA(μl) 0 5 0 5 0 5
I
NPF>CaMPARI
Fred/Fgreen(%)
Time after odor stimulation (min)
ctrl 0 7 15
J
Mouth Hook Contractions/30 Sec
Time after odor stimulation (min)
ctrl 0 7 10 15 20

Figure 4.2 Gated Cellular Responses of Two Dorsomedial NPF Neurons to Discrete Odor Stimuli.

(A) An imaging of a dorsomedial NPF neuron (circled) expressing ArcLight. Two sample recordings of normalized membrane activities of dorsomedial NPF neurons of *NPF*-GAL4/ UAS-*ArcLight* fed larvae at different time points: one is obtained during the stimulation by an effective dose of PA and the other using a higher ineffective dose. (B) Standard deviations (SDs) for membrane activity were calculated for both effective (n=6) and ineffective (n=9) treatments at 5 minutes of PA application. The higher SDs associated with the effective odor treatments indicate stronger excitatory responses by NPF neurons (t-test, $p<0.01$). (C,D) Power spectrums were calculated for baseline membrane activity and membrane activity after 5min odor treatment using fast Fourier transform with 0.05 Hz bin width. At the effective dose, amplitudes within the low frequency range (<3 Hz) showed a significant increase above the baseline (two-way ANOVA, $p<10^{-12}$). No significant differences were observed at the ineffective dose ($p=0.07$). (E) CaMPARI-based imaging of the dorsomedial NPF neurons in freely behaving larvae reveals that the excitatory response of NPF neurons is restricted to PA stimulation at the dose (7µl) that is effective to arouse appetite for anticipated sugar food. $n\geq16$; **$P<0.001$.

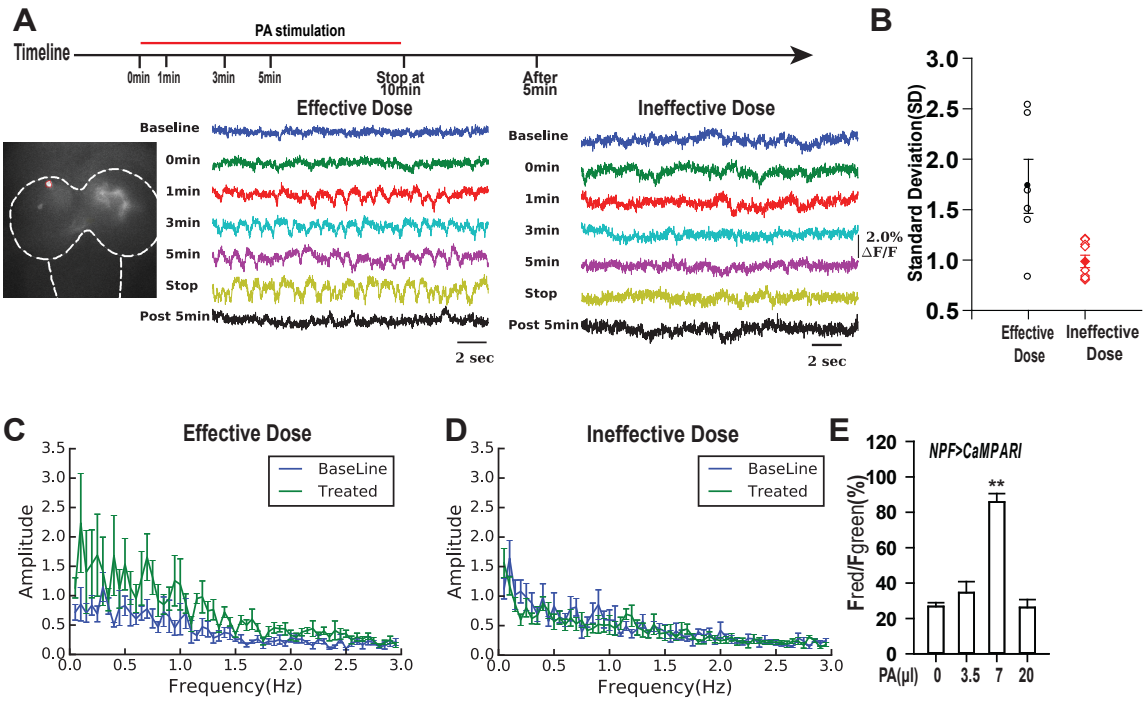

A
Timeline
PA stimulation
0min 1min 3min 5min
Stop at 10min
After 5min
Effective Dose
Ineffective Dose
Baseline
0min
1min
3min
5min
Stop
Post 5min
2 sec
2.0% ΔF/F
B
Standard Deviation(SD)
3.0
2.5
2.0
1.5
1.0
0.5
Effective Dose
Ineffective Dose
C
Effective Dose
BaseLine
Treated
Amplitude
Frequency(Hz)
D
Ineffective Dose
BaseLine
Treated
Amplitude
Frequency(Hz)
E
NPF>CaMPARI
Fred/Fgreen(%)
120
100
80
60
40
20
0
**
PA(μl) 0 3.5 7 20

Figure 4.3 A Dop1R1/Gβ13F/IRK2-mediated Inhibitory Pathway is Essential for Precision Tuning of NPF Signaling and Prevents Larval Appetitive Response to Higher Doses of Odorants

(A) Reduced *Dop1R1* activity in NPF neurons caused a right shift in the dose-response curve of *NPF*-GAL4/*UAS-Dop1R1*^{RNAi} fed larvae to PA and other odorants (n≥18). (B) CaMPARI-based imaging analysis also shows a right shift in the dose response at the level of the dorsomedial NPF neurons in freely behaving larvae (n≥6). (C) Fed larvae expressing dTrpA1 in normal or *Dop1R1*-deficient NPF neurons showed similar feeding responses (n>16). (D) The effects of various doses of PA on the appetitive responses of NPF-GAL4/UAS-*Gβ13F*^{RNAi}, NPF-GAL4/UAS-*Gβ76C*^{RNAi} fed larvae. In the presence of PA at 20µl or higher, NPF-GAL4/UAS-*Gβ13F*^{RNAi} and NPF-GAL4/ UAS-*IRK2*^{RNAi} larvae, but not others, showed increased feeding responses (n≥14). (E) CaMPARI-based imaging analysis also shows a right shift in the dose response at the level of in freely behaving larvae, the dorsomedial NPF neurons deficient for *Gβ13F*^{RNAi} or *IRK2*^{RNAi} activity showed excitatory responses to a broad dosage range including both 7 and 20µl PA(n≥8). *P<0.05; **P<0.001.

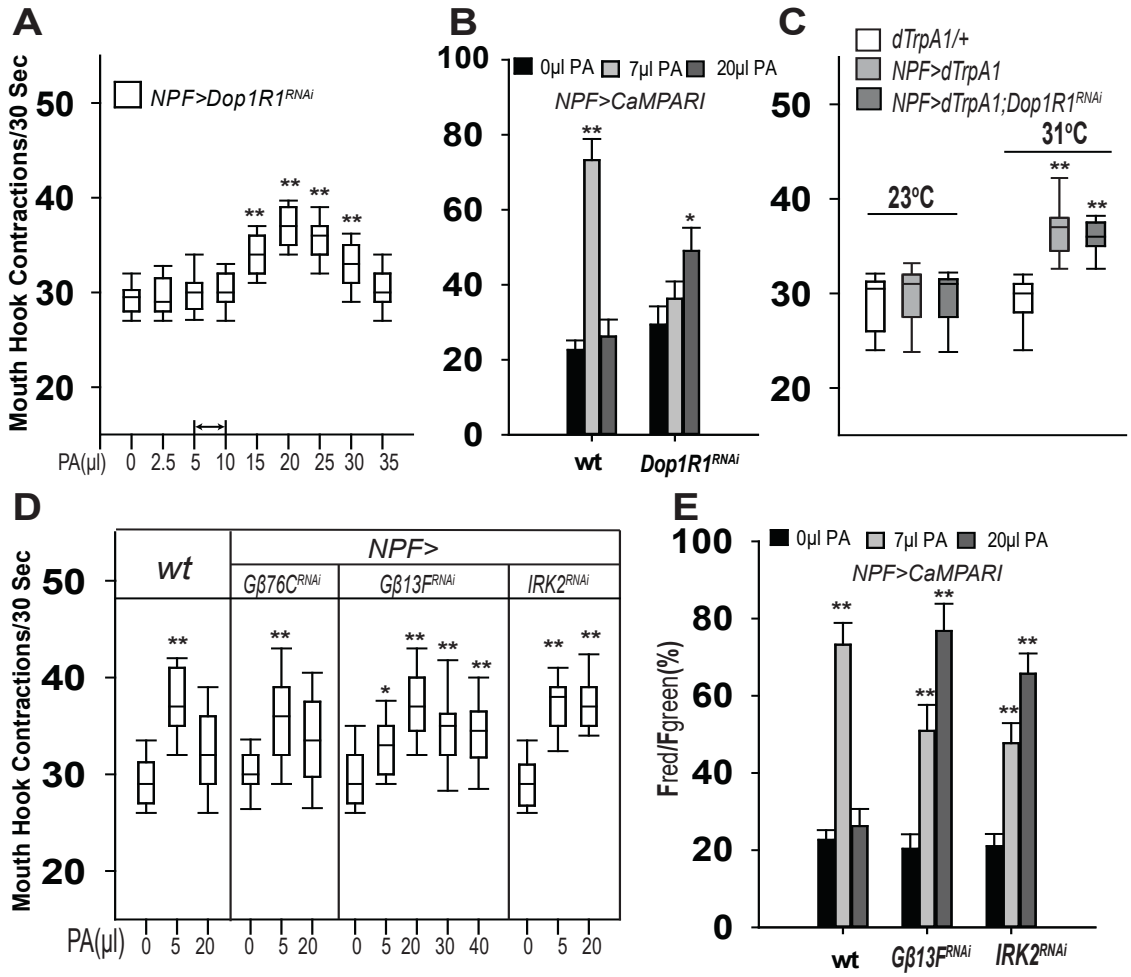
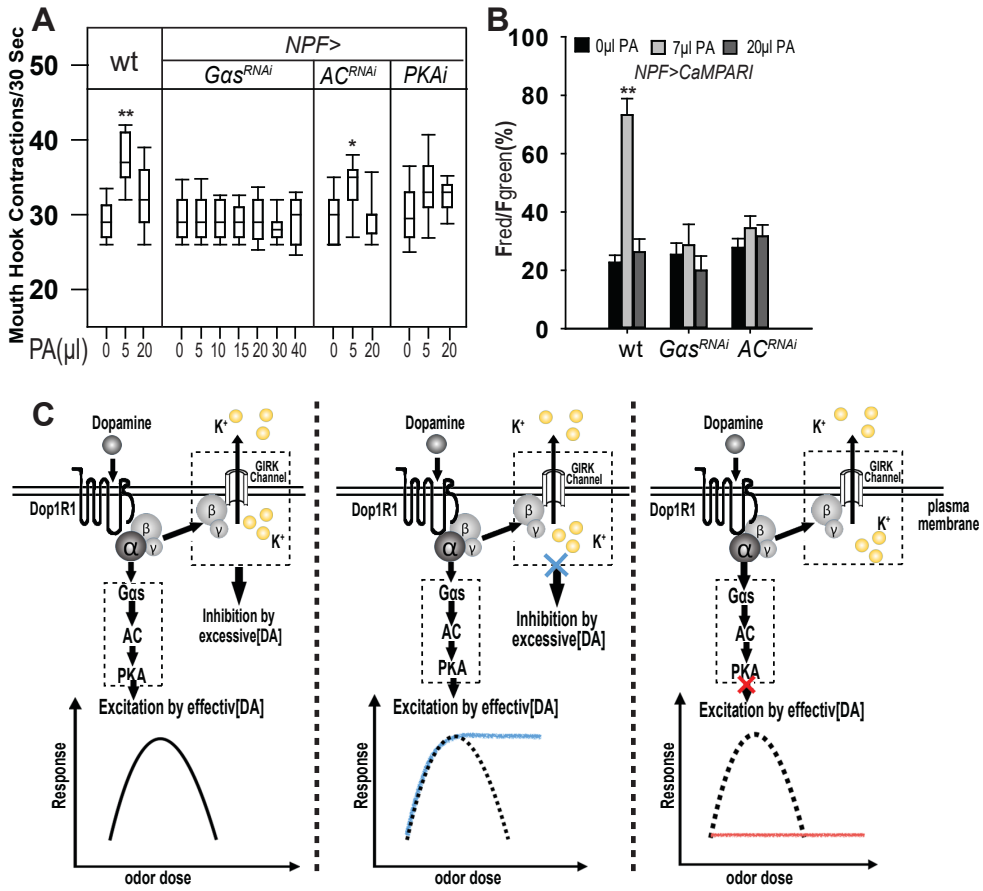

A
Mouth Hook Contractions/30 Sec
NPF>Dop1R1RNAi
PA(µl) 0 2.5 5 10 15 20 25 30 35
B
0µl PA 7µl PA 20µl PA
NPF>CaMPARI
wt
Dop1R1RNAi
C
dTrpA1/+
NPF>dTrpA1
NPF>dTrpA1;Dop1R1RNAi
23°C
31°C
D
wt
NPF>
Gβ76CRNAi
Gβ13FRNAi
IRK2RNAi
Mouth Hook Contractions/30 Sec
PA(µl) 0 5 20 0 5 20 0 5 20 30 40 0 5 20
E
0µl PA 7µl PA 20µl PA
NPF>CaMPARI
Fred/Fgreen(%)
wt
Gβ13FRNAi
IRK2RNAi

Figure 4.4. A Dop1R1/*Gαs*-mediated Excitatory Pathway Mediates Precision Tuning of NPF Signaling by Setting a Minimum Threshold Dose of Odorants for Appetitive Response

(A) Functional knockdown of *Gαs* activity in NPF neurons abolished larval appetitive responses to PA at all doses tested. Reduction of AC or PKA activity also led to attenuated appetitive response to a normally effective dose of PA (e.g., 5 μl) compared with that of wild type controls (n≥12). (B) CaMPARI- based imaging also shows attenuated responses to a normally effective dose of PA (7μl) by the dorsomedial NPF neurons in freely behaving NPF-GAL4/UAS-$G\alpha s^{RNAi}$ and NPF-GAL4/ UAS-AC^{RNAi} fed larvae (n≥6) (C) A schematic summary of a two-layered precision tuning strategy for Dop1R1-mediated restriction of NPF neuronal response to an optimum level of DA-coded odor inputs. **P < 0.001.


A
Mouth Hook Contractions/30 Sec
wt
NPF>
Gαs^RNAi
AC^RNAi
PKAi
PA(μl)
B
0μl PA
7μl PA
20μl PA
NPF>CaMPARI
Fred/Fgreen(%)
wt
Gαs^RNAi
AC^RNAi
C
Dopamine
Dop1R1
GIRK Channel
plasma membrane
Gαs
AC
PKA
Inhibition by excessive[DA]
Excitation by effectiv[DA]
Response
odor dose

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CHAPTER 5

CONCLUSIONS AND DISCUSSION

Olfaction is a primary sensory modality for animals as it provides food related olfactory cues. Olfactory processing requires the involvement of the interaction between higher order olfactory centers and cognitive systems to precisely represent and encode odor information to direct behavior outputs. In addition, olfaction is functionally interconnected to other senses such as taste, and it can significantly impact emotion and cognition (De Araujo et al., 2003; Ehrlichman and Halpern, 1988; Johnson, 2011; Rolls, 2015). Imaging analyses also have shown that food odors can activate the brain circuits associated with reward and motivation processing (Bragulat et al., 2010). In the past 30 years, the peripheral olfactory system has been extensively studied, providing great insights about odor information are detected and transmitted to the higher order olfactory center (Vosshall and Stocker, 2007). Nevertheless, little is understood about the anatomical and functional properties of higher-order olfactory centers and the downstream reward system which translate partially processed olfactory information to motivationally salient signals.

Mapping the higher order olfactory neural circuitry and investigating the basic molecular and cellular mechanism for olfactory processing remain difficult using mammalian models, partly due to the complex network of the nervous system, limited techniques, as well as long duration of generating transgenic animals. *Drosophila* larvae has a highly evolved olfactory system that shows anatomical organizations similar to

adult flies and mammals, but is numerically simpler. In addition, molecular genetic techniques for selective visualization and perturbation of specific neurons and recent advances in recording neural activity makes *Drosophila* a powerful system for analyzing the neural circuit basis of behavior (Olsen and Wilson, 2008). Thus, we have developed a novel behavioral paradigm using *Drosophila* larvae to investigate reception and processing of appetitive olfactory inputs in higher-order olfactory centers, as well as how food related olfactory cues are perceived in downstream reward systems. We demonstrate well-nourished fly larvae have an innate cognitive ability to selectively attribute a specific type of anticipated food reward to discrete olfactory cues. Using this behavioral paradigm, we have identified a circuit mechanism, mediated by conserved DA and NPF systems including a pair of neural processors- four DL2 DA neurons and their downstream two DM NPF neurons. Our findings suggest that fly larvae constitute a useful model for elucidating the molecular, circuit and neural mechanisms underlying the perception of olfactory reward and behavioral organization. Besides, this invertebrate behavior paradigm for studying appetitive olfactory-driven feeding also could be further applied to studies other relevant questions in sensory biology and cognitive neuroscience, such as the control of hedonic feeding, attention span and multisensory integration.

In mammals, dopamine(DA) has been classically implicated in pleasure, addiction, learning and motivation (Colombo, 2014). Several lines of evidence support that phasic DA neurons responses could be triggered by many types of rewards and reward-related sensory cues (Schultz, 1998) and it could be used as teaching signal in reinforcement learning (Ikemoto, 2010; Petrovich, 2011; Schultz et al., 1997) or as an incentive signal that promotes immediate reward seeking (Berridge and Robinson, 1998).

Likewise, fly studies indicate that particular dopamine clusters and even individual DA neurons likely form valence-specific circuit motifs that are engaged by conditioned or innate values of external stimulus, and whose function can be modified by the internal state and past experience (Azanchi et al., 2013; Waddell, 2013; Lin et al., 2000; Cohn et al., 2015; Marella et al., 2012). Consistent with this notion, we provide direct cellular and functional evidence that DL2 DA neurons, as third order olfactory neurons, combinatorially respond to appetitive olfactory cues and are necessary and sufficient to regulate innate appetitive feeding activities in fed larvae. Using GRASP analysis, we showed that four DL2 DA neurons are postsynaptic to projection neurons in the lateral horns which are involved in mediating innate olfactory behaviors. Calcium imaging results displayed all four DL2 DA neurons respond towards various appetitive odor stimuli. Other major findings are that larval perception of a food odor, which varies based on its chemical composition and the intensity of stimulation, is encoded by the distributed and coordinated activity of the two clusters of DL2 DA neurons. In addition, specific activation of those DL2 DA neurons effectively elicits appetitive feeding, indicating that the sensory-driven feeding motivational circuit is hardwired. In summary, our results indicate four DL2 DA neurons in each brain lobe engage as a functional interface between the olfactory system and NPF-mediated cognitive systems as they integrate appetitive sensory cues and convert them into food anticipatory cues. In light of other evidence that DA signal also could be regulated by past experience of reward or punishment, internal state, and hormone levels, as well as the spatial segregation of DA neurons in fly CNS. Together, our results further suggest the DA system is a central

player in the regulation of feeding through the integration of appetitive cues, nutritional state, and memory expression.

Another important insight from this study is that DA-coded olfactory information has no intrinsic appetitive value, rather it is a Dop1R gating mechanism that determines which range of the DA signals will be assigned with appetitive significance. For example, a 50% reduction of Dop1R1 activity led to a right-shift of the dose-response curve regardless whether the odor stimulus is monomolecular or chemically complex (BV). Therefore, to be perceived as appetizing cues, odor-evoked DA signals must be within a narrow range that matches the pre-existing level of Dop1R1 activity.

Drosophila expresses NPF, which is related evolutionarily to mammalian orexigenic neuropeptide Y(Brown et al., 1999). Similar to dopamine, NPF is implicated in a variety of goal-directed behaviors through encoding three distinct putative functions in hunger, reward status, and innate attraction (Shohat-Ophir et al., 2012; Waddell, 2010; Wu et al., 2005a; 2005b; Krashes et al., 2009). First NPF could act as a natural reward which is same as ethanol consumption and mating (Shohat-Ophir et al., 2012). Second, NPF also is found as a motivational switch in food deprived fly to promote appetitive memory retrieval (Krashes et al., 2009). Third, NPF neurons respond to reward sensory cues and activation of NPF neurons promotes innately approaching to food-related odors in both hungry and fed fly (Beshel and Zhong, 2013). Nevertheless, it is still unknown if activation of NPF neurons could directly induce feeding behavior in context-independent manner. Here, we provid both cellular and functional evidence, at single-cell resolution, that two DM NPF neurons are necessary and sufficient to elicit appetitive arousal in fed larvae. Our findings support the proposal that activation of NPF neurons is rewarding to

fly and NPF molecule release acts as a motivational signal to directly promote feeding in a context-independent manner.

In freely behaving fed larvae, the NPF neurons display excitatory responses to stimulation by appetizing doses of PA but not the non-appetizing doses, and these responses are also gated by the Dop1R1 activity in the DM NPF neurons. Thus, our results point to a critical role of the NPF neurons in the selective attribution of incentive salience to discrete DA-coded odor cues. It remains to be determined whether the NPF neurons are responsive to sugar stimulation. If so, this would provide evidence for a third important role of these NPF neurons in integrating DA-coded olfactory and sugar-evoked gustatory signals.

Another important finding is that we provide molecular and cellular evidence for how the Dop1R1 activity in the NPF neurons shapes the inverted-U effects of appetitive odor stimuli. Using both behavioral and imaging assays, we show that Dop1R1 determines which range of odor-evoked DA signals may acquire appetitive significance through precision tuning of NPF neurons. Two separate molecular signaling modules have been identified for this process. One of them, involving a Dop1R1/Gβ13F/IRK2-mediated pathway, sets an upper limit of the optimum effective range of odor-induced DA signals by silencing the NPF neurons when Dop1R1 is hyper-activated. Attenuation of the Gβ13F or IRK2 activity greatly broadens the width of the effective dose range of DA signals, as evidenced by the change of the dose-response curve from an inverted U shape to a sigmoidal shape. Again, this result also strongly supports the notion that the ineffectiveness of stronger odor stimuli is caused by an excessive release of odor-evoked DA, which leads to a high level of dissociated Gβγ complex that activates IRK2 channels

and subsequently silences the NPF neurons. The second module, involving a Dop1R1/Gαs-mediated pathway, provides a default excitatory mechanism that mediates NPF neuronal response to any odor stimuli that are at or above the minimal threshold strength. Therefore, they together define a two-layered precision tuning strategy.

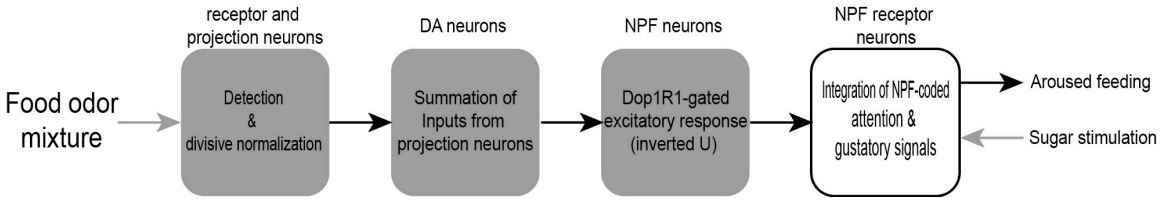
The inverted-U effects of dopamine level or D1-like receptor activation level on cognitive performance have been widely observed in both humans and other animals (Cools and D'Esposito, 2011; Takahashi et al., 2012). Imbalanced dopamine systems also underlie many psychiatric disorders such as schizophrenia (Abi-Dargham et al., 2002; Okubo et al., 1997). Here, we propose that a homologous D1-type receptor-mediated precision tuning strategy may be employed in different types of DA-responsive neurons to mediate the inverted-U effects of DA (Vijayraghavan et al., 2007). Moreover, our findings also suggest that fly larvae are a useful model for the neurobiological study of cognitive controls mediated by dopamine and other neural systems and associated disorders.

My work also contributed to the technical aspects of *Drosophila* neuroscience. Currently, most of the vivo calcium imaging is under a head-fixed condition with exposed brain under odor stimulation and scanning. Moreover, the field view of a microscope is restricted during real-time calcium imaging. However, in our studies, CaMPARI imaging allowed us to test neuronal activities in freely behaving larvae. In addition, the conditions of larval feeding and odor treatment for CaMPARI imaging are identical to those for odor-aroused feeding behavioral assays. Also, ratiometric CaMPARI signal eliminates the expression level difference among the target neurons. Thus, using CaMPARI enables us to measure the DM NPF and DL2 DA neurons

activities more precisely in freely behavior larvae. The CaMPARI protocol that we developed will be beneficial to our future work in recording neurons activities under various sensory stimulation such as gustatory stimuli. Gao et al. developed Arclight that enable optical electrophysiology olfactory sensory neurons in adult flies (Cao et al., 2013). ArcLight imaging is a novel optical measurement in intact neural circuits of membrane potential, enabling us to monitor multiple target neurons activities in animals simultaneously. For the first time, we use ArcLight imaging to monitor fly larvae peptidergic neuron's activities under odor stimulation. The protocol that we developed for analyzing results of Arclight imaging will support the future work on monitoring cellular response in larvae sensory and cognitive circuit.

Figure 5 A Working Model for a Neural Network Comprising DA and NPF Neurons and Its Regulation of Appetitive Arousal in Fed Larvae.

This working model includes following key points. Complex food odors are detected by multiple olfactory receptor neurons, which relay the stimuli to corresponding projection neurons where such inputs are subject to a signal processing operation known as divisive normalization (Olsen et al., 2010, Vosshall and Stocker, 2007). The projection neurons in the primary olfactory processing center convey the olfactory information to two clusters of four postsynaptic DA neurons (Figure 2; Wang, 2013), which together perform a summation function. In addition to their integrative function, the DA neurons also provide an interface that functionally links the olfactory system to the NPF-mediated cognitive system. The NPF neurons, gated by a Dop1R1-mediated precision tuning mechanism, display an inverted U excitatory response to various levels of presynaptic DA signals, and selectively attribute incentive salience to an optimum level of odor-evoked DA. It is proposed that NPF-coded attention signals are conveyed to uncharacterized NPF receptor neurons, which are part of a commanding circuit in the subesophageal ganglia that integrates NPF, gustatory and possibly other signals and control aroused feeding.


receptor and
projection neurons
DA neurons
NPF neurons
NPF receptor
neurons
Food odor
mixture
Detection
&
divisive normalization
Summation of
Inputs from
projection neurons
Dop1R1-gated
excitatory response
(inverted U)
Integration of NPF-coded
attention &
gustatory signals
Aroused feeding
Sugar stimulation

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