UNIVERSITY OF CALIFORNIA, SAN DIEGO

Hunger and smell: neuropeptidergic push-pull modulation in starvation dependent odor-driven food search

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by

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The thesis of Gang Ill Ko is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

Chair

University of California, San Diego

2011

EPIGRAPH

“Your efforts are budding – results will appear soon.”

Panda Express ▫ Panda Inn fortune cookie message

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ABSTRACT OF THE THESIS

Hunger and smell: neuropeptidergic push-pull modulation in starvation dependent odor-driven food search

by

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Professor Jing W. Wang, Chair

Internal physiology has a dramatic effect on animals' natural behavior. We therefore investigated the effect of starvation in shaping olfactory processing in *Drosophila.* Previous work from our lab has shown that glomeruli in the first olfactory relay are hardwired for attraction and aversion. DM1 glomerular activity signals for attraction to food odor, whereas DM5 glomerular activity triggers aversion. We observed that DM1 glomerular activity increases via presynaptic facilitation while DM5 activity decreases via presynaptic inhibition. DM1 modulation is mediated by short NPF (sNPF) signaling and DM5 modulation is mediated by tachykinin (DTK) signaling. Both of these

opposite modulatory mechanisms in DM1 and DM5 are required for the fly's food search behavior. Together, these two different neuropeptide signaling mechanism represents a push-pull mechanism, whereby starvation causes attraction to be enhanced and aversion to be suppressed. This leads to maximal attraction in starved flies and minimal attraction in fed flies.

Chapter 1: Introduction

1.1 Introduction

Animals face different challenges under different internal physiological states that influence their behaviors. For example, prolonged starvation leads to increased probability of finding food in the blow flies (Gelperin, 1971). Many animals rely on the olfactory cues for essential survival behaviors such as feeding, mating and predator avoidance. Does internal state change features of the olfactory system? A number of modulator systems has been identified in olfactory systems of many animals (Olsen and Wilson, 2008; Root et al., 2008; Ignell et al., 2009). Whether changes in an animal's internal state engage these modulatory systems has not been well investigated. For animals experiencing starvation, it makes sense to increase olfactory sensitivity to improve the food finding efficacy. This thesis investigates neuromodulation in the *Drosophila* antennal lobe and identifies a push-pull mechanism mediated by two neuropeptides, short NPF (sNPF) and tachykinin (DTK) in response to starvation, and its behavioral consequences of food search in *Drosophila*.

1.2 The olfactory system in *Drosophila*

The basic organization of the olfactory system is remarkably conserved from flies to humans (Hildebrand and Shepherd, 1997). The primary sensory neurons, olfactory receptor neurons (ORNs) have ciliary nerve endings exposed to the external world (Anholt et al., 1987). A chemical substance called odorant can bind to the odorant receptors that transform the chemical information into electrical impulses by way of G-protein signaling (Brunet et al., 1996; Belluscio et al., 1998; Wong et al., 2000). ORNs send their axons to the antennal lobe in flies (olfactory bulb in mice), where they synapse

onto projection neurons (PNs, mitral/tufted cells in the olfactory bulb) (Gao et al., 2000; Vosshall et al., 2000; Scott et al., 2001; Couto et al., 2005). This region where ORNs synapse onto PNs is called a glomerulus. In rats, 2-deoxyglucose autoradiography studies showed that an odor activates a distinct population of glomeruli, and that increased concentration of odor recruits more glomeruli (Stewart et al., 1979; Lancet et al., 1982). Thus, a glomerulus is believed to be the functional unit in the olfactory system and the pattern of glomeruli is thought to create the first internal representation of odor.

The discovery of a novel multigene family of ORNs in 1991 by Buck and Axel led to the molecular genetic manipulation of the olfactory system. This study showed that ORNs express different but distinct olfactory receptor genes (Buck and Axel, 1991). Buck hypothesized that hypervariable regions found in these genes interacted with the different odorants and conferred specificity. Furthermore, lacZ expression under a promoter of a specific receptor gene in mice demonstrated a convergence of axons of ORNs expressing the same olfactory receptor gene onto a single glomerulus (Mombaerts et al., 1996). Thus, an activated glomrulus is a direct representation of the activated ORNs expressing the specific receptor genes. In flies, nearly all olfactory receptor genes have been identified (Couto et al., 2005; Fishilevich and Vosshall, 2005), and a family of GPCRs and ionotrpic glutamate receptor currently account for nearly all odorant responsive glomeruli (Benton et al., 2009). *Gal-4* lines for each receptor gene are available in flies, which allows for a precise genetic dissection of distinct glomeruli and makes *Drosophila* an attractive model organism.

1.3 Dynamic modulation in the antennal lobe

A dense network of excitatory and inhibitory local interneurons (LNs) are found in the antennal lobe (Wilson et al., 2005; Shang et al., 2007; Olsen and Wilson, 2008; Root et al., 2008; Ignell et al., 2009). However, their role in the olfactory processing remains largely unknown. Electrode recording and two-photon imaging studies in *Drosophila* demonstrated that interglomerular pre-synaptic inhibition mediated by GABAergic LNs plays an important role in normalizing olfactory information (Olsen and Wilson, 2008; Root et al., 2008; Olsen et al., 2010). This normalization, rather than decreasing the maximal PN response, widened the range span of PN response as odor concentration increases. Interestingly, this shift in dynamic range is also seen in the visual system by a population of horizontal and amacrine cells, and it plays an indispensable role in light perception (Sakmann and Creutzfeldt, 1969). In flies, perturbing GABA signaling in the antennal lobe impairs mate-localizing behavior, suggesting that such modulation is important for behavior (Root et al., 2008). Thus, LNs in the antennal lobe have the capacity to critically impact olfactory processing and behavior.

Other neuromodulators such as serotonin (Dacks et al., 2009) and tachykinin (DTK) (Ignell et al., 2009) have been shown to alter olfactory representation in flies. In particular, DTK peptides are expressed in the local LNs and released onto ORN terminals that express the DTK receptor. Two-photon calcium imaging revealed that DTK signaling mediates presynaptic inhibition, and knocking down DTK receptors by expressing RNAi in ORNs alters behavioral response to high odor concentration (Ignell et al., 2009). Other studies showed that DTK signaling plays a role in nutrition-based

mechanism such as regulation of adipose-kinetic hormone (a homolog of mammalian glucagon) and stimulation of visceral muscle (Nassel and Winther, 2010 for review). A more precise understanding of the role DTK plays in odor-driven behavior remains to be determined.

1.4 Olfactory circuit and behavior

How does olfactory representation among glomeruli relate to an animal's behavior? Studies in the past have shown that CO_2 is an aversive odorant that activates the V glomerulus only. Blocking synaptic vesicle release in the ORNs projecting to the V glomerulus using a temperature sensitive dynamin gene, called *Shibire* (*Shi*^{ts}), was sufficient to eliminate avoidance behavior to CO_2 in flies (Suh et al., 2004). In addition, artificial activation of the V glomerulus using channelrhodopsin was sufficient to elicit avoidance behavior in flies (Suh et al., 2007). In the fly pheromone system, activation of Or67d neurons, which project to DA1 glomerulus, mediates male-male courtship behavior and aggression behavior (Kurtavic et al., 2007; van der Goes van Nater et al., 2007; Wang and Anderson, 2010). Thus, each glomerulus can be a functional unit both in terms of olfactory representation and behavioral output.

Most odors activate a pattern of glomeruli rather than a single glomerulus. Thus, it has been long speculated that the unique combinatorial activation of glomeruli is the key to understanding how odor identities are encoded. However, recent studies have shown that the activation of a single glomerulus is necessary and sufficient to elicit attraction and aversion behavior. Apple cider vinegar is an odorant that resembles natural food odorant. Using two-photon calcium imaging, Julia Semmelhack and Jing Wang

found that cider vinegar activates six glomeruli. Using *Shi*^{ts} to individually block each of these six glomeruli, they have shown that suppressing DM1 and VA2 eliminates attraction behavior in flies towards apple cider vinegar (Semmelhack and Wang, 2009). In addition, selective rescue of each glomerulus in Or83b mutant background showed that DM1 and VA2 are sufficient to restore attraction to cider vinegar. Interestingly, activation of DM5 glomerulus at high concentration of cider vinegar is necessary and sufficient to mediate aversion. Thus, particular glomeruli that make up odor-evoked patterns appear to be hard-wired for attraction and aversion.

In this thesis, we investigate how DM1 and DM5 glomeruli are modulated under nutritional stress. We hypothesize that starvation will lead to increased DM1 activation and decreased DM5 activation, and that there should be a different mechanism to mediate such opposite modulation. We show that sNPF mediates pre-synaptic facilitation in DM1 and DTK mediates pre-synaptic inhibition in DM5 to achieve such modulation.

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Chapter 2: Presynaptic Facilitation by Neuropeptide Signaling Mediates Odor-Driven Food Search

2.1 Abstract

Internal physiological states influence behavioral decisions. We have investigated the underlying cellular and molecular mechanisms at the first olfactory synapse for hunger modulation of food search behavior in *Drosophila*. We found that a local signal by short neuropeptide F (sNPF) and a global metabolic cue by insulin are integrated at specific odorant receptor neurons (ORNs) to enable hunger modulation of olfactory sensitivity. Results from two-photon calcium imaging show that starvation increases presynaptic activity via intraglomerular sNPF signaling. Expression of sNPF and its receptor (sNPFR1) in ORNs is necessary for starvation-induced food search behavior. Furthermore, this presynaptic facilitation specifically in Or42b neurons is necessary and sufficient for food finding. Quantitative RT-PCR experiments demonstrate that starvation increases the transcription level of *sNPFR1* but not that of *sNPF*, and insulin signaling suppresses *sNPFR1* expression. Thus, starvation increases expression of *sNPFR1* to change the odor map, resulting in more robust food search behavior.

2.2 Introduction

The modulation of behavior by basic physiological need is essential for animal survival. Physiological modulation is often accomplished by release of neuromodulators that alter neuronal excitability or network properties (Destexhe and Marder, 2004). In particular, appetite and satiety modulate feeding behavior in most animals through the actions of neuropeptides. In mammals, the hypothalamus, an important brain region controlling appetite (Berthoud, 2002), integrates hormonal signals such as ghrelin, insulin and leptin from the gut, pancreas and adipose tissues, respectively. Activation of neurons

containing neuropeptide Y (NPY) and AgRP in the arcuate nucleus of the hypothalamus augment food intake (for review see (Barsh and Schwartz, 2002)). In insects, two independent homologs of NPY, neuropeptide F (NPF) and short neuropeptide F (sNPF) (Brown et al., 1999; Hewes and Taghert, 2001), promote feeding behavior (Lee et al., 2004; Wu et al., 2003) when broadly overexpressed in neurons. Although much is known about the central control of feeding behavior, little is known about hunger modulation of sensory representation in any animal.

For most animals in their natural environment, feeding begins with a search for the appropriate food source in which the sense of smell plays an indispensible role (Dethier, 1976). While important inroads have been made in identifying neuropeptides that regulate feeding behavior, little is understood about whether or how these hormones/neuropeptides alter olfaction and how that leads to behavioral changes. In rodents, central projections of olfactory fibers from the olfactory bulb largely bypass the thalamus and project directly to the olfactory cortex (Mori et al., 1999; Shepherd, 2004). Nonetheless, internal state does influence olfactory response in the olfactory cortex (Murakami et al., 2005). However, it is not clear whether these metabolic hormones act directly on the olfactory cortex or whether they play a modulatory role in the olfactory bulb where a variety of different neuromodulators influence neural activity (Shepherd, 2004).

Insulin is a global metabolic cue that promotes glucose uptake in both vertebrates and invertebrates (Rulifson et al., 2002). In addition to the regulation of blood glucose, insulin signaling is implicated in the modulation of behaviors relating to feeding,

reproduction and memory (Gerozissis, 2003) and insulin injection into the hypothalamus reduces food intake in rodents (Bruning et al., 2000; Woods et al., 1998). However, how insulin signaling fine-tunes defined neural circuits to alter behavior is not well understood. Studies of hunger modulation in the *Drosophila* nervous system affords an opportunity to investigate an evolutionarily conserved mechanism for energy homeostasis and establish a causal link between neuropeptide modulation and feeding behavior.

We have investigated whether hunger modulates olfactory processing that mediates food-search behavior. We report that starvation alters olfactory representation of food odor at the first olfactory synapse. The neuropeptide, sNPF, which is implicated in feeding behavior (Lee et al., 2004) and expressed in *Drosophila* olfactory receptor neurons (ORNs) (Carlsson et al., 2010; Nassel et al., 2008), mediates this change by facilitating synaptic transmission from select ORNs. Intraglomerular signaling by sNPF is necessary for starvation-dependent enhancement of odor-driven food search behavior. Furthermore, starvation increases the expression level of the sNPF receptor (sNPFR1) by a reduction in insulin signaling. Thus, neuropeptide signaling causes starvation-dependent presynaptic facilitation of sensory transmission, which optimizes olfactory representation for food finding.

2.3 Hunger alters olfactory representation and food search behavior

The antennal lobe is the center for early olfactory processing and is a target for many neuromodulators. Within the antennal lobe, ORNs expressing the same odorant receptor genes (Clyne et al., 1999; Vosshall et al., 1999) converge onto a single glomerulus (Vosshall et al., 2000). ORNs make synapses with many local interneurons

and the cognate projection neurons (PNs) (Distler and Boeckh, 1997). Output PNs of the antennal lobe transmit olfactory information from glomeruli to higher brain centers such as the lateral horn and mushroom body (Stocker et al., 1990; Vosshall and Stocker, 2007). Although ORNs are the main drivers of PN output (Olsen et al., 2007; Root et al., 2007), interneurons have been shown to control olfactory sensitivity by presynaptic inhibition (Ignell et al., 2009; Olsen and Wilson, 2008; Root et al., 2008) and lateral excitation (Olsen et al., 2007; Root et al., 2007; Shang et al., 2007). Two neuromodulators, serotonin (Dacks et al., 2009) and tachykinin (Ignell et al., 2009), have been shown to alter antennal lobe activity. If hunger modulates antennal lobe neurons, we should observe a change in odor-evoked activity in PNs.

We performed two-photon imaging (Denk et al., 1990) to measure PN dendritic calcium responses to odor stimulation in fed and starved flies. Flies bearing *GH146-Gal4* and *UAS-GCaMP* transgenes express the calcium sensor GCaMP in many PNs allowing the select measurement of calcium response in PN dendrites (Wang et al., 2003). We investigated calcium response of PNs to apple cider vinegar, which is highly attractive for *Drosophila* and is a complex odor that resembles a natural food source (Semmelhack and Wang, 2009). We imaged PN dendritic activity in flies that were fed and flies that were starved overnight (Figure 2.1a). Cider vinegar excites five glomeruli at the tested concentrations. Starvation significantly enhances odor response in three glomeruli (DM1, DM4 and DM2) but decreases odor response in two glomeruli (VM2 and VA3; Figure 2.1b, c). It is interesting to note that starvation alters the amplitude of calcium activity without changing the temporal kinetics (Figure 2.1b). In sharp contrast, our previous

study shows that activation of $GABA_B$ receptors causes presynaptic inhibition and alters the temporal kinetics of PN calcium activity (Root et al., 2008). Therefore, a change in $GABA_B$ receptor signaling is unlikely to account for the starvation dependent change in olfactory response. Rather, our results are more consistent with an excitability change in antennal lobe neurons.

The apparent starvation-dependent change of olfactory response in the DM1, DM2, DM4, VM2 and VA3 glomeruli could be due to intra- or inter-glomerular mechanisms. We therefore investigated hunger modulation of individual glomeruli with reduced lateral activity. To do this, we imaged PN responses to a panel of five different odorants, each of which excites one or a few glomeruli at low concentrations (Figure 2.1d, e). The responses in DM1 and DM4 to ethyl acetate were significantly enhanced by starvation; the response of DM2 but not VM2 to ethyl hexanoate is enhanced by starvation; and the responses in DM1, DM4 and DP1M to 1-octen-3-ol are also significantly enhanced. In contrast, the responses of VA3 and VM2 to 2-phenylethanol and 3-heptanol, respectively, are not modulated by starvation. Therefore, DM1, DM4 and DM2 are more sensitive to odor stimulation in starved animals. However, VA3 and VM2 are not subject to starvation modulation. This result suggests that the apparent suppression of VA3 and VM2 in response to cider vinegar is due to lateral inhibition. We conclude that some antennal lobe neurons are subject to hunger modulation resulting in an alteration of the odor map.

Hunger as an internal state affects feeding behavior (Gelperin, 1971), which begins with an olfaction-dependent search for an appropriate food source. Therefore, we

expect that the starvation-dependent change in olfactory representation should be matched by an alteration in behavior. We developed a single fly assay that allows the assessment of hunger modulation on odor driven food search behavior. We reasoned that latency to find food is a metric of food search. We employed an automatic computer system to monitor the position of individual flies from which we measured the latency required for individuals to reach an odor target. Individual flies were introduced into small arenas that contained a food odor, apple cider vinegar, at the center. During the 10 minute observation period, starved flies spend most of the time walking near the food source, whereas fed flies wander in the entire arena with a preference for the perimeter (Figure 2.2a). The latency of food finding is significantly decreased upon starvation (Figure 2.2b) and is independent of fly speed (data not shown). Furthermore, surgical removal of the antennae impairs this behavior (data not shown). Thus, the sense of smell, mediated by the antennae, is required for food search behavior, and hunger enhances food finding in *Drosophila*.

What is the time-course of the starvation-dependent change in olfactory activity and food-search behavior? We first varied starvation time and measured calcium imaging of PN dendrites in response to precise electrical stimulation of the olfactory nerve in flies bearing the *GH146-Gal4* and *UAS-GCaMP* transgenes. Imaging calcium activity in PN dendrites of the DM1 glomerulus, we found that calcium activity increased with starvation duration up to four hours. Longer starvation duration for twelve hours did not result in more neuronal response (Figure 2.2c, d). We next varied starvation time and examined the latency of food search behavior. Similar to the starvation-dependent effect

on calcium activity, food search behavior increases up to four hours and is not further increased with 12 hours of starvation (Figure 2.2e). Thus, the change in antennal lobe activity and food search behavior occurs within four hours of starvation.

2.4 sNPF signaling in ORNs mediates hunger modulation of food search

What is the mechanism by which starvation affects odor-guided behavior? The neuropeptide sNPF promotes feeding behavior (Lee et al., 2004) and is expressed in some ORNs (Carlsson et al., 2010; Nassel et al., 2008). We therefore hypothesized that sNPF signaling in ORNs is responsible for the starvation-dependent enhancement of food search behavior. We expressed RNAi to knockdown ORN sNPF expression in flies bearing the *Or83b-Gal4* and *UAS-sNPF-RNAi* transgenes, and as a control we expressed sNPF-RNAi in PNs of flies bearing *GH146-Gal4* and *UAS-sNPF-RNAi* transgenes. If sNPF signaling in ORNs is important for food search behavior, we expect that knockdown of sNPF in ORNs would eliminate the effect of starvation and expression of the RNAi in PNs should not. We measured the latency of food finding in our behavioral assay and found that indeed starved flies lacking sNPF in ORNs exhibit a significantly longer latency in food finding (Figure 2.3a, b). Within 10 minutes, about 22% of control flies reach the odor source while only 9% of sNPF knockdown flies do so. Interestingly, sNPF knockdown flies behave similarly to fed flies (data not shown), suggesting that low sNPF signaling mimics the fed state in the antennal lobe. The difference in latency between sNPF knockdown flies and control flies however cannot be attributed to a change in locomotor activity (data not shown). Furthermore, flies with a P-element disruption of the first exon of the sNPF gene ($sNPF^{c00448}$) are similarly impaired in food

finding (data not shown). Thus, sNPF expression in olfactory receptor neurons mediates the starvation-dependent enhancement of food search behavior.

While our findings are in accord with previous work indicating that ORNs express the sNPF peptide (Carlsson et al., 2010; Nassel et al., 2008), the population of neurons that express sNPFR1 (Feng et al., 2003), the receptor for sNPF, is not known. In salamanders, the NPY receptor localizes to sensory neurons of the olfactory epithelium (Mousley et al., 2006), and is thus poised for a feedback modulation. In the mammalian hypothalamus, NPY neurons project from the arcuate nucleus to the lateral hypothalamus (Barsh and Schwartz, 2002; Cowley et al., 1999) and are poised for a feedforward modulation. Thus, two possible mechanisms may account for the observed modulatory effects of the neuropeptide: 1) if sNPFR1 localizes to ORNs, its peptide may modulate starvation-induced behavior through ORN-ORN feedback modulation, or 2) If sNPFR1 localizes to PNs, its peptide may modulate starvation-induced behavior through ORN-PN feedforward modulation. To discriminate between these two possibilities, we expressed RNAi to knockdown sNFPR1 in either the ORNs or PNs in flies bearing either *Or83b-Gal4* or *GH146-Gal4*, respectively, and *UAS-sNPFR1-RNAi*. We found that expression of *sNPFR1-RNAi* in ORNs mimics the effect of the neuropeptide knockdown (Figure 2.3c, d). In contrast, expression of *sNPFR1-RNAi* in the PNs has no effect on food search behavior. The difference in latency between sNPFR1 knockdown and control flies cannot be attributed to a change in locomotor activity (data not shown). Furthermore, disruption of sNPFR1 by expression of a dominant negative gene (Lee et al., 2008) in ORNs results

in a similar decrease in food finding (data not shown). Thus, feedback modulation by sNPFR1 expressed in ORNs is necessary for starvation-dependent food search.

2.5 Presynaptic activity in ORNs is modulated by sNPF signaling

Given that knockdown of sNPF and its receptor in ORNs has a profound effect on starvation-dependent food search behavior, we reasoned that hunger should alter activity in ORN axon terminals. To investigate this, we imaged odor-evoked activity in ORNs in flies that were fed and flies that were starved overnight. Flies bearing the *Or83b-Gal4* and *UAS-GCaMP* transgenes allow the select measurement of calcium activity in ORN axon terminals. We observed that cider vinegar activates the same five glomeruli when comparing ORNs (Figure 2.4a) to PNs (Figure 2.1a). Three glomeruli (DM1, DM4 and DM2) exhibit significant increases in calcium activity upon starvation, while the VM2 glomerulus exhibits significant suppression of response to low odor concentration, and the VA3 glomerulus is not affected (Figure 2.4b,c). Thus, starvation alters olfactory representation in sensory neurons, which is largely consistent with the changes observed in the antennal lobe output PNs.

We next asked if sNPF signaling in ORNs causes the hunger-induced changes in olfactory representation. To investigate this, we imaged ORN response to cider vinegar in starved and fed flies with perturbed sNPF signaling. We found that expression of *sNPF-RNAi* in the ORNs eliminates the effect of starvation such that the olfactory representation in starved flies lacking sNPF mirrors that of fed control flies (Figure 2.4c). The overlapping curves between control fed flies and starved RNAi flies suggest that the effect of RNAi is specific to sNPF signaling rather than a potential non-specific effect on

neuronal properties. Furthermore, there is no difference between starved and fed sNPF knockdown flies, indicating that sNPF mediates the hunger modulation of ORN activity. In addition, expression of RNAi to knockdown expression of the sNPFR1 in ORNs similarly eliminates the effect of starvation (data not shown). We further investigated whether abolishing sNPF signaling presynaptically in ORNs eliminates the starvation-dependent enhancement in postsynaptic PNs. To do this, we used flies bearing the *GH146-LexA*, *LexAop-GCaMP*, *Or83b-Gal4* and *UAS-sNPF-RNAi* transgenes. Imaging PN calcium activity in the DM1 glomerulus in the absence of presynaptic sNPF, we found that the effect of starvation is abolished such that PN response in starved flies matches that of fed flies (data not shown). The data suggest that the effect of *sNPF-RNAi* is not due to a non-specific disruption of synaptic transmission from ORNs. Thus, we conclude that sNPF signaling causes the change in olfactory representation upon starvation.

2.6 sNPF signaling mediates presynaptic facilitation

The above results indicate that hunger enhances activity in ORNs by sNPF signaling, suggesting that the neuropeptide could act to facilitate presynaptic activity. To directly test this hypothesis we asked if exogenous application of sNPF affects presynaptic calcium activity in ORN terminals. In order to eliminate the contribution of any potential modulation at ORN cell bodies, we removed the antennae and delivered precise electrical stimulation to one olfactory nerve while imaging calcium activity in the ipsilateral antennal lobe. We expressed *sNPF-RNAi* in ORNs to eliminate endogenous sNPF, which may occlude the effect of exogenously applied sNPF. Flies bearing the

Or83b-Gal4, *UAS-GCaMP* and *UAS-sNPF-RNAi* transgenes lack sNPF expression and express GCaMP in ORNs. Electrical stimulation of the olfactory nerve elicits a calcium transient that is increased upon sNPF application (Figure 2.5a-c). Interestingly, this increase occurs only in starved flies but not in fed flies, suggesting that sNPFR1 signaling is upregulated upon starvation. We compared the sensitivity to sNPF between the five glomeruli that respond to cider vinegar and found that the DM1, DM2 and DM4 glomeruli exhibit enhanced activity by the neuropeptide, whereas the VM2 and VA3 glomeruli do not (Figure 2.5d). This result reveals that ORNs terminating in VM2 and VA3 are not modulated by sNPF, which is consistent with the results we obtained with odor stimulation (Figure 2.1b-e). Therefore, the suppression of calcium activity in VM2 ORNs (Figure 2.4b) could be a result of lateral presynaptic inhibition (Olsen and Wilson, 2008; Root et al., 2008). Furthermore, the suppression of VA3 PN calcium activity (Figure 2.1b) could be due to lateral feedforward inhibition (Sachse and Galizia, 2002). Thus, the sNPF peptide and its receptor mediate presynaptic facilitation in starved flies at select glomeruli.

2.7 sNPF signaling in DM1 is necessary and sufficient for starvation-dependent food search behavior

The ORNs of the DM1, DM2 and DM4 glomeruli have the ability to respond to exogenous sNPF, however the endogenous source of the neuropeptide is unclear. The peptide could come from receptor neurons of the same glomerulus or alternatively from neighboring glomeruli. We therefore investigated the inter- vs. intraglomerular source of sNPF by knocking down sNPF expression in specific ORNs and imaging ORN activity in

all glomeruli. Flies bearing the *Or83b-LexA*, *LexAop-GCaMP*, *UAS-sNPF-RNAi* and *Or-specific-Gal4* transgenes permit the measurement of calcium activity in the axonal termini of many glomeruli, while knockdown of sNPF expression is targeted to one specific glomerulus. We found that knockdown of sNPF expression in Or42b ORNs eliminates hunger modulation in only the cognate DM1 glomerulus without any impact on the ORNs of DM2 or DM4 glomeruli (Figure 2.6a). Similarly, knockdown of sNPF in Or22a and Or59b ORNs abolished hunger modulation in ORNs of DM2 and DM4 glomeruli, respectively, without any impact on the other glomeruli (Figure 2.6a). These results suggest that intraglomerular sNPF peptide is necessary while interglomerular sNPF is not sufficient for hunger modulation of olfactory sensitivity.

The above results indicate that intraglomerular sNPF signaling selectively increases activity in only three of the five glomeruli activated by cider vinegar. Given that a previous study has found that not all glomeruli contribute equally to odor-guided behavior (Semmelhack and Wang, 2009), we next asked if sNPF signaling in individual glomeruli is necessary for food search behavior. We expressed RNAi to knockdown the peptide or the receptor in the DM1, DM2 and DM4 ORNs, which are modulated by sNPF. We found that knockdown of the neuropeptide or its receptor in DM1 ORNs results in significantly decreased food finding in starved flies (Figure 2.6b). Within 10 minutes, only about 10% of the RNAi expressing flies reach the odor target, whereas about 24% of the control flies do so. This difference cannot be attributed to a difference in locomotor activity (data not shown). Strikingly, knockdown of the neuropeptide or its receptor in the DM2 or DM4 ORNs has no effect on the starvation-dependent food search

behavior (Figure 2.6b). Expression of the sNPF-RNAi in the VM2 and VA3 ORNs that are not sensitive to sNPF signaling does not affect food search behavior (data not shown). These results indicate that sNPF signaling in a single ORN channel is necessary for the starvation-dependent food search behavior.

It has been observed that sNPF is also expressed in the mushroom body (Nassel et al., 2008), which suggests that hunger modulation in the central nervous system could be important for food search behavior. We therefore evaluated the contribution of the peripheral modulation by performing gain of function experiments in fed flies to determine if peripheral modulation alone is sufficient to induce starvation-like food search behavior. We first performed imaging experiments to determine if overexpression of *sNPFR1* increases odor-evoked calcium activity. We imaged calcium activity in Or42b ORNs in control flies bearing the *Or83b-LexA* and *LexAop-GCaMP* transgenes and overexpression flies that also contained the *Or42b-Gal4* and *UAS-sNPFR1* transgenes. Ectopic expression of *sNPFR1* significantly increases $\Delta F/F$ in the DM1 glomerulus in fed flies (Figure 2.7a). Furthermore, this enhanced activity is translated into a shorter latency in food finding behavior in fed flies. Within 10 minutes, about 20% of fed flies with ectopic expression of *sNPFR1* in Or42b neurons have found the odor source, whereas only 7% of control flies have done so (Figure 2.7b). Thus, modulation of activity in the Or42b ORNs is both necessary for, and sufficient to mimic, state-dependent food search behavior. This result also suggests that sNPF is released even in the fed state. Furthermore, the data suggest that modulation of peripheral olfactory activity makes an important contribution to food search behavior.

2.8 Insulin functions as a satiety signal to suppress sNPFR1 expression.

What is the molecular mechanism to increase ORN sensitivity in starved flies to gate appetitive behavior? We first investigated whether this physiological switch involves gene transcription by performing quantitative RT-PCR. We measured the level of *sNPF* and *sNPFR1* transcripts in isolated antennae of fed and starved flies relative to a control gene, *rp49* (a ribosomal protein). Interestingly, we found that the level of *sNPFR1* mRNA is increased by approximately four-fold upon starvation, while the level of *sNPF* mRNA does not change (Figure 2.8b). Although we do not detect a change in *sNPF* mRNA, we cannot rule out the possibilities of starvation-dependent changes in neuropeptide translation or release. Nevertheless, ectopic expression of *sNPFR1* expression is sufficient to induce presynaptic facilitation in fed flies (Figure 2.7a). Therefore, starvation leads to increased expression of *sNPFR1*, which is sufficient to cause presynaptic facilitation even in the absence of any starvation-dependent change in sNPF.

We next asked what is the metabolic sensor for ORNs to induce expression of *sNPFR1*? It has been well established that the levels of circulating *Drosophila* insulin-like peptide plummets in the hunger state (Geminard et al., 2009), and that the downstream signaling from the insulin receptor (InR) has the capacity to control gene expression (Edgar, 2006). Furthermore, expression of an insulin receptor has been observed in ORNs of *C. elegans* (Chalasani et al., 2010). We therefore asked if ORNs express the insulin receptor, by assaying immunoreactivity with InR antiserum in flies that express GFP in Or83b ORNs. Indeed there is a large overlap between Or83b neurons

and InR immunoreactivity indicating that some ORNs express InR (Figure 2.8a) and therefore could be subject to insulin modulation.

Does InR activity alter the expression of sNPFR1 signaling? We reasoned that ectopic expression of a constitutively active InR (InR-CA) in ORNs should mimic the fed state. We first looked at the starvation-dependent expression of *sNPFR1* transcripts and found that starved flies bearing *Or83b-Gal4* and *UAS-InR-CA* do not exhibit an increase in *sNPFR1* transcripts measured by qRT-PCR (Figure 2.8b). Similarly, calcium imaging experiments reveal that expression of InR-CA in ORNs eliminates the sensitivity to exogenous sNPF application in the DM1 glomerulus of starved flies (Figure 2.8c). This experiments was carried out in the same way as those in Figure 2.5. In control starved flies, bath application of sNPF enhances the axonal calcium transient evoked by electrical stimulation of the olfactory nerve. These results predict that starvation should not sensitize Or42b ORNs in these flies with the constitutively active InR. Indeed, calcium imaging experiments show that starvation does not increase olfactory response to cider vinegar in DM1 (Figure 2.8d). Constitutive activation of InR specifically eliminates the starvation-dependent sensitization because the odor response in fed InR-CA flies is not different from fed controls, indicating that the manipulation does not impair these neurons. Measurement of food search behavior indicates that the constitutively active InR reduces food finding (Figure 2.8e). Therefore, activation of InR prevents starvation-dependent presynaptic facilitation and food search behavior.

We next asked if blockade of InR could mimic the effect of starvation in ORNs. Phophatidylinositol 3-kinase (PI3K) is a crucial downstream molecule for insulin control

of gene transcription and translation to promote cell growth (Leevers et al., 1996; Weinkove et al., 1999). We hypothesized that pharmacological inhibition of PI3K should mimic the hunger state by preventing InR signaling. Two commonly used anti-tumor drugs, wortmannin and LY294002, have been shown as effective inhibitors of PI3K (Arcaro and Wymann, 1993; Vlahos et al., 1994). Indeed feeding flies overnight with 4% sucrose plus 25 nM wortmannin or 30 μM LY294002 sensitizes olfactory response in the DM1 glomerulus to the same level as that of starved flies and significantly greater than that of flies fed only 4% sucrose (Figure 2.8f). Do these PI3K antagonists alter ORN *sNPFR1* mRNA levels? Indeed qRT-PCR experiments from isolated antennae revealed that feeding flies with wortmannin or LY294002 causes a significant increase in *sNPFR1* expression relative to flies fed with 4% sucrose (Figure 2.8b). Thus, either of these PI3K antagonists causes increased expression of *sNPFR1* in ORNs in addition to sensitized olfactory response. However, these two PI3K inhibitors appear to increase peptide mRNA level that is not observed in starved flies. Therefore, we further investigated the link between the drug-induced increase in *sNPFR1* and the drug-induced olfactory sensitization with epistatasis experiments. Expression of *sNPFR1-RNAi* in ORNs eliminates the drug-induced sensitization (Figure 2.8f), indicating that the sensitization resulting from blocking insulin signaling depends on *sNPFR1* expression in ORNs. Lastly, we asked if blocking PI3K induces starvation-like behavior in fed flies. Feeding flies either wortmannin or LY294002 led to significantly increased food finding in comparison to those expressing *sNPFR1-RNAi* in Or83b neurons and control flies fed only 4% sucrose (Figure 2.8g). These results demonstrate that insulin signaling is necessary and sufficient for starvation-dependent up-regulation of *sNPFR1* and the

induction of presynaptic facilitation, indicating that InR in ORNs is the nutrient sensor to trigger appetitive behavior (Figure 2.8h).

2.9 Discussion

We report here that a state of hunger modulates olfactory sensitivity at the first synapse in a form of presynaptic facilitation. Starvation increases *sNPFR1* transcription in ORNs, which is both necessary and sufficient for presynaptic facilitation and to mediate a starvation-dependent food search behavior. It has been well established that fluctuation of insulin is a key metabolic cue to maintain energy homeostasis. This study implicates that a low insulin signal via the PI3K pathway increases *sNPFR1* expression. Interestingly, a subset of glomeruli exhibit starvation-dependent presynaptic facilitation that depends on intraglomerular sNPF signaling, while selective knockdown of sNPF or sNPFR1 in only the DM1 glomerulus affects food search behavior. This finding corroborates our previous work revealing that the DM1 glomerulus is hardwired for innate odor attraction (Semmelhack and Wang, 2009). Thus, an internal state of hunger, with insulin as a global satiety signal acting on sensory neurons through a local sNPF signal, shifts the odor map to increase the saliency of glomerular activity to match the changing physiological needs of an organism.

Our results and a number of other reports reveal that modulation of early sensory processing can have profound effects on stimulus detection. For instance, serotonin mediates presynaptic facilitation of mechanosensory neurons in *Aplysia* to sensitize the siphon and gill withdrawal reflex (Brunelli et al., 1976). Serotonin mediates presynaptic inhibition of mechanosensory transmission in the leech to establish a behavioral

hierarchy in which feeding suppresses tactile sensation (Gaudry and Kristan, 2009). In the olfactory system, serotonin modulates activity at the first olfactory synapse in mammals (Petzold et al., 2009) and insects (Dacks et al., 2009; Kloppenburg and Hildebrand, 1995), and GABA-mediated synaptic inhibition serves as a mechanism to modulate sensitivity (Aroniadou-Anderjaska et al., 2000; Murphy et al., 2005; Olsen and Wilson, 2008; Root et al., 2008; Sachse et al., 2007; Wachowiak et al., 2005) and olfactory behavior (Root et al., 2008; Sachse et al., 2007). This study reveals modulation of the first olfactory synapse by internal physiological state. Furthermore, the Or42b sensory neurons may be considered as a neural substrate for appetitive choices, because they integrate internal and external cues to influence an important innate behavior.

The present results indicate that a highly conserved neuropeptide (Hewes and Taghert, 2001) plays an important role in the early olfactory system to mediate starvation-dependent neuromodulation. A similar presynaptic facilitation mechanism may exist in vertebrates as well. In an aquatic salamander, NPY has been shown to enhance electrical responses of cells in the olfactory epithelium to a food related odorant in hungry animals (Mousley et al., 2006). In addition, NPY immunoreactivity has been observed in the olfactory epithelium of mouse (Hansel et al., 2001) and zebrafish (Mathieu et al., 2002). In the nematode *C. elegans*, elevated activity levels of an NPY-like receptor cause a change in foraging pattern (Macosko et al., 2009). Our study demonstrates that a fluctuating metabolic cue controls *sNPFR1* levels, which in turn modulate the peripheral sensory system to alter appetitive behavior. Given the ubiquitous

use of insulin as a metabolic cue, modulation by NPY/sNPF receptors in the early olfactory system could be a conserved mechanism between different animal species.

Central mechanisms to control appetitive behavior, similar to the well-documented modulation of the hypothalamus by NPY, also appear to be important in *Drosophila*. A recent study demonstrates that appetitive memory requires the NPF receptor in the dopaminergic neurons that innervate specific mushroom body lobes (Krashes et al., 2009). This poses the question: what functions are subserved by hunger modulation of multiple neural substrates? It is interesting to note that sensitization of Or42b ORNs is sufficient to enhance food search behavior in fed flies. Perhaps central modulation by hunger is not necessary for food search behavior. Modulation in the periphery may serve to gate an animals' sensitivity to specific food odorants, while central modulation may serve to enhance an animal's ability to remember the relevant cues in finding a particular food source. As olfaction plays an important role in our flavor perception (Shepherd, 2006), peripheral modulation of the olfactory system by hunger may thus be a potential therapeutic target to control appetite.

2.10 Methods

Two-photon calcium imaging. GCaMP imaging was performed as previously described (Root et al., 2008; Wang et al., 2003). In odor experiments, a constant airflow of 1 l/min was applied to the antennae via a pipe of 12 mm diameter. Odor onset was controlled by mixing a defined percentage of carrier air with air redirected through odor bottles as previously described (Root et al., 2008; Semmelhack and Wang, 2009). Nerve stimulation was performed with a glass suction electrode and an S48 stimulator (Grass,

Warwick, RI) as previously described (Root et al., 2008; Wang et al., 2003). Starved flies were starved overnight with water for 17-24 hr.

Behavior assay. Female flies that were 2-5 days old and presumed non-virgin were used for all experiments. Single flies were introduced into chambers that were 60 mm in diameter and 6 mm in height. The chamber was illuminated by 660 nm LEDs. Flies were tracked at 2 Hz with custom software written in Labview (V.8.5, National Instruments), and analysis was performed with Igor Pro (V.6, Wavemetrics, Inc) using a custom macro. Latency is defined as the elapsed time before an individual fly spends more than 5 seconds within a 5 mm distance from the odor source, which minimizes false positives due to random entry into the odor zone. Apple cider vinegar was diluted 1:100 in 1% low melting temperature agarose and 5 μl were placed in the center of the chamber. We observed that 17-24 hr starvation and 4-6 hr starvation produced similar results, consistent with the starvation effect measured by calcium imaging (Figure 2e). Therefore, some experiments were carried out with 4-6 hr starvation and others overnight; controls and experimentals were always treated the same.

Pharmacology. sNPF peptide, AQRSPSLRLRF-NH_2, 98% purity (Celtek Peptides) was dissolved in saline to a final concentration of 10 μM. Wortmannin and LY294002 (LC Laboratories, Woburn, MA) were dissolved in DMSO at stock concentrations of 10 mM and 50 mM, respectively. Flies were fed overnight in vials containing 4% sucrose solution alone, or that plus 25nM wortmannin or 30μM LY294002.

Transgenic flies. The following fly stocks were used: *Or83b-Gal4* (Kreher et al., 2005); *Or83b-LexAVP16* (Lai and Lee, 2006); *Or42b-Gal4*, *Or43b-Gal4*, *Or22a-Gal4* and *Or67d-Gal4* (Fishilevich and Vosshall, 2005); *Or59b-Gal4* (Couto et al., 2005); *GH146-Gal4* (Stocker et al., 1997); *GH146-LexAGAD* (Lai et al., 2008); *UAS-GCaMP (Wang et al., 2003)*; *LexAop-GCaMP* (Root et al., 2008)*; UAS-sNPF-RNAi* (Lee et al., 2004); *UAS-sNPFR1-DN, UAS-sNPFR1*, and *UAS-sNPFR1-RNAi* (Lee et al., 2008)*; sNPF*^{c00448} (obtained from the Exelixis stock collection at Harvard Medical School), *UAS-InR-CA* (obtained from the Bloomington stock center #8263).

Quantitative RT-PCR. RNA was isolated from antennae of 50 female flies for each sample. The RNeasy kit (Qiagen) was used to isolate RNA and the reverse transcription was performed using the Retroscript kit (Ambion) with random decamers. This cDNA was subjected to PCR analysis using SYBR green detection on an iCycler thermocycler (Biorad). All values are normalized against *rp49* as a control gene.

Immunostaining. Antennal sections were obtained by mounting live fly heads in OCT, freezing at -20°C on the stage of a cryostat, and 12 mm thick section were cut. Slides were immediately fixed with ice-cold 4% paraformaldehyde in 0.1X PBS for 10 min. Staining was performed using standard techniques with chick-anti-GFP (Ab13970, Abcam, Cambridge, MA), rabbit-anti-InR (3021, Cell Signaling Technology, Danvers, MA), at 1:1000, and 1:200 respectively.

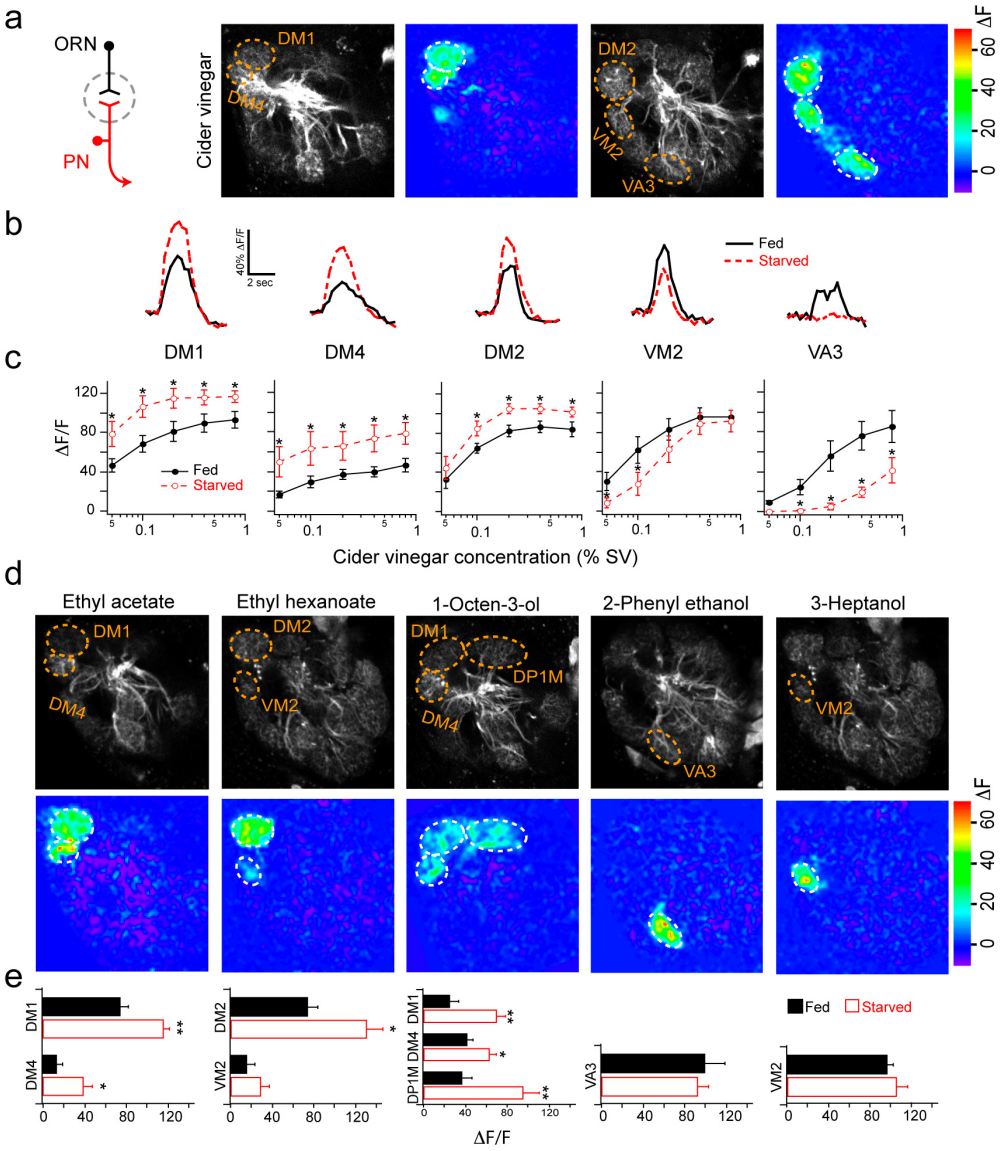

Figure 2.1. Olfactory representation in projection neurons is altered by starvation. **a,** Two-photon imaging of PN calcium activity in response to cider vinegar stimulation on two optical planes of the antennal lobe in fed flies. Gray-scale images show antennal lobe structure while pseudocolored images reveal odor-evoked activity at 0.4% SV. **b**, Representative traces of fluorescence change over time for the five glomeruli excited by cider vinegar at 0.1% SV. **c**, Peak ΔF/F across a range of cider vinegar concentrations for each glomerulus. **d**, PN activity of fed and starved flies in response to pure odorants. **e**, Peak ΔF/F for each glomerulus. **d**,**e**, Odors were applied at the following concentrations (% SV): 1% ethyl acetate, 0.1% ethyl hexanoate 1:10,000 in mineral oil, 1% 1-octen-3-ol, 0.5% 2-phenyl ethanol, and 0.1% 3-heptanol. **c**,**e**, n=5-10 for each condition; error bars show SEM. *P<0.05, **P<0.01; t-test

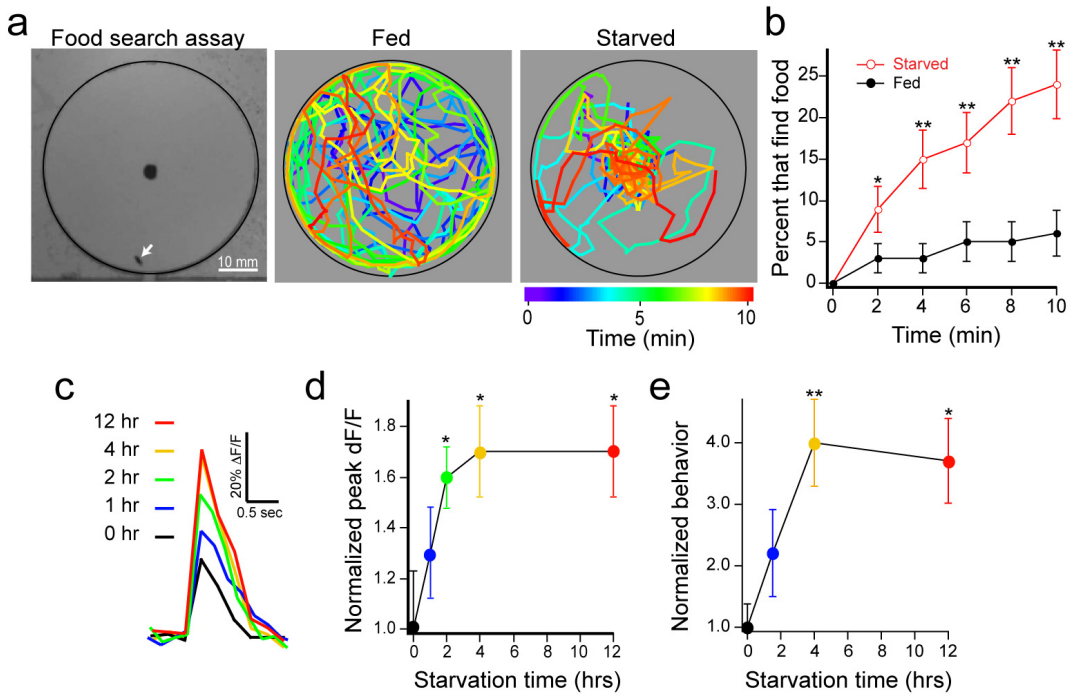

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Figure 2.2. Food search behavior and olfactory sensitivity are modulated within four hours of starvation.

a, A food search assay was used to measure the latency of odor-guided food finding. Grayscale image (left) shows an arena with a food odor, cider vinegar, in the center and a single fly (white arrow). The coordinates of single flies are plotted as a function of time in pseudocolor for a representative fed and starved fly. **b**, The latency of food search is quantified as the cumulative percentage of flies that find the odor source as a function of time. **c,d,** Two-photon imaging of PN calcium activity in the DM1 glomerulus in response to electrical stimulation of the olfactory nerve. **c**, Representative traces of fluorescence change over time from the DM1 glomerulus in flies with varied starvation durations. **d**, Peak ΔF/F normalized to the average response without starvation. Stimulation was 1 ms in duration, 10 V in amplitude and 4 pulses at 100 Hz. n=5-8 for each starvation condition. Error bars show SEM. *P≤0.05, t-test **e**, Data from behavioral experiments with varied starvation durations shown as the food finding percentage normalized to that of the fed state. **b**,**e** n=53-102 flies for each condition. Error bars show SEM. *P≤0.05, **P≤0.01, z-test for proportions.

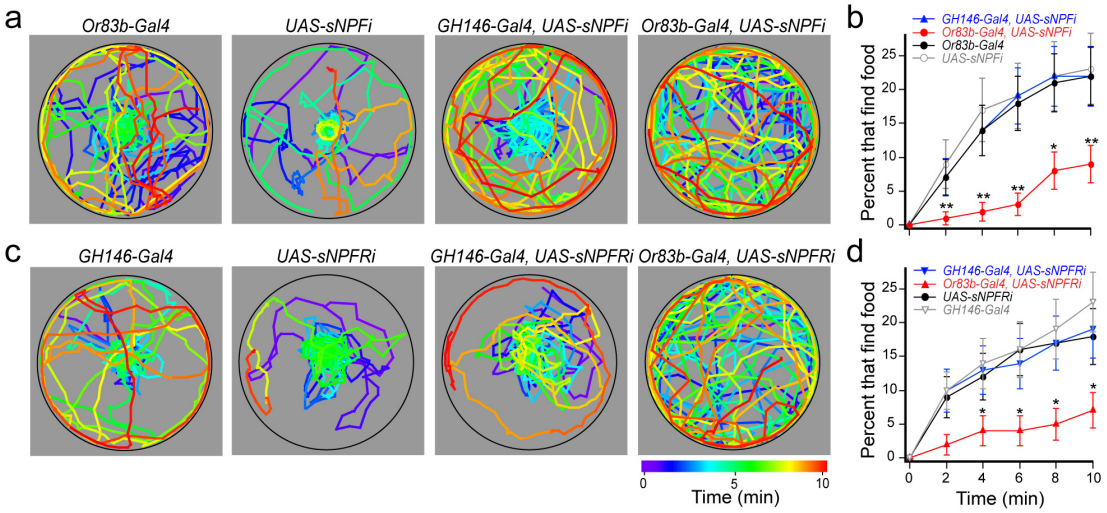

Figure 2.3. Starvation-dependent food search requires sNPF signaling in ORNs.
a, A behavioral assay was used to measure the latency of odor-guided food finding. Flies were introduced into a dark arena containing a food odor, cider vinegar, in the center. The coordinates of single flies for representative control flies (left two plots) and those expressing *sNPF-RNAi* (*sNPFi*) in PNs (third from left) or ORNs (right). **b**, The latency of food search is quantified as the cumulative percentage of flies that find the odor source as a function of time. **c,** The coordinates of two representative control flies (left two plots) and those expressing *sNPFR1-RNAi* (*sNPFRi*) in PNs (third from left) or ORNs (right). **d**, The latency of food finding as a function of time. n=64-103 flies for each condition. Error bars show SEM. *P<0.05, **P<0.01; z-test for proportions comparing the top three curves to the bottom curve in **b,d**.

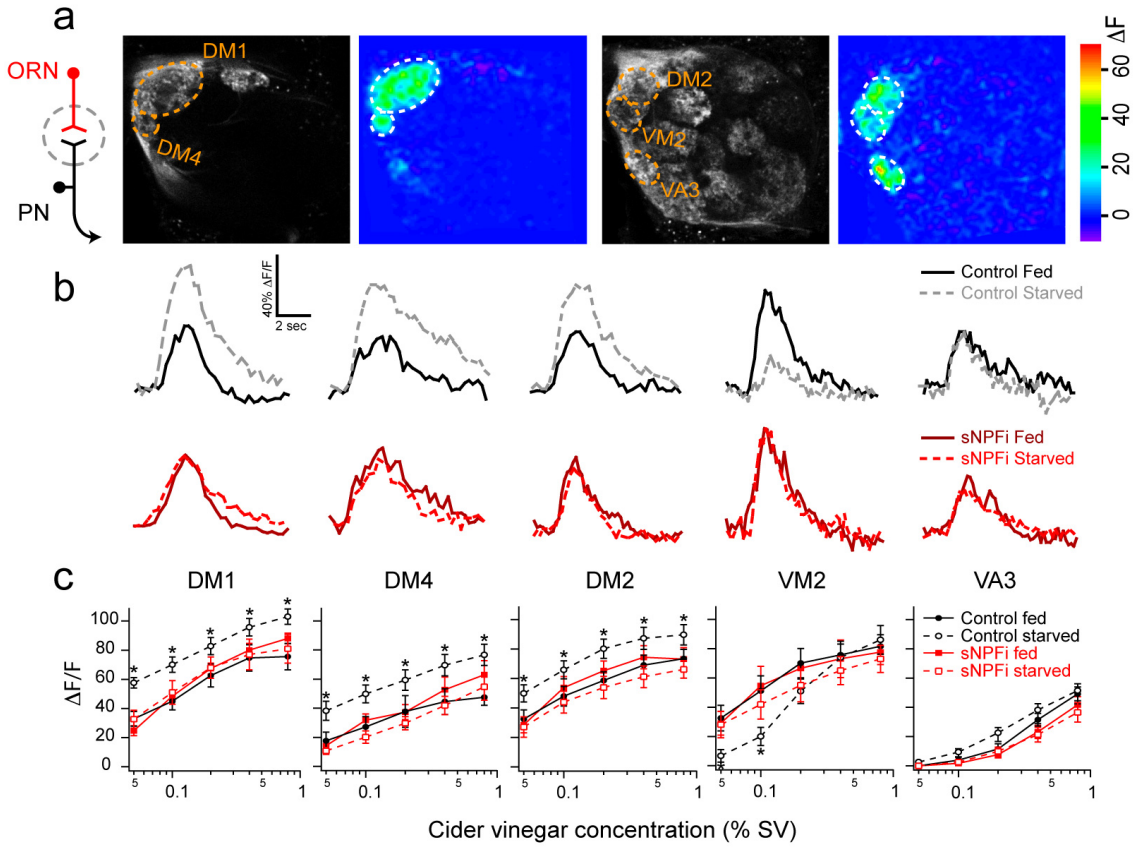

Figure 2.4. sNPF signaling alters presynaptic calcium activity in sensory neurons. **a**, Two-photon imaging of ORN axon terminal calcium activity in response to cider vinegar stimulation at 0.4% SV. **b**, Representative traces of fluorescence change over time for the five glomeruli excited by 0.1% cider vinegar in control flies (top) and those expressing *sNPF-RNAi* in ORNs (*sNPFi*) (bottom), in fed (solid line) and starved (dashed line) flies. **c**, Peak ΔF/F across a range of cider vinegar concentrations for each glomerulus. **c**, Starved and fed Control flies and those expressing *sNPFi*. n=10-12 each condition; error bars show SEM. *P<0.05; t-test comparing starved control to fed control.

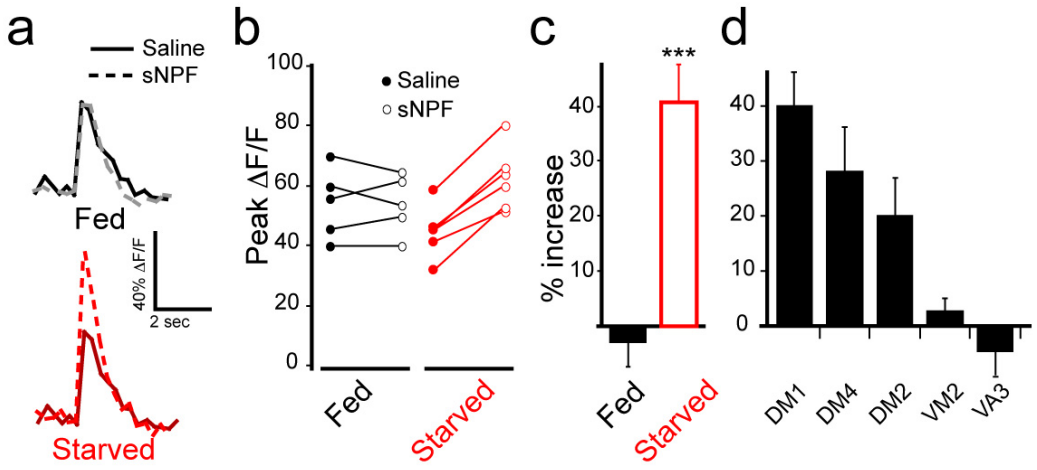

Figure 2.5. The sNPF receptor is upregulated upon starvation and mediates presynaptic facilitation.

a-b, Two-photon imaging of ORNs axon terminal calcium activity in response to electrical stimulation of the olfactory nerve before and after application of sNPF. Stimulation was 1 ms in duration, 10 V in amplitude and 16 pulses at 100 Hz. **a**, Representative traces of fluorescence change over time from the DM1 glomerulus in fed (top, black) and starved flies (bottom, red), in saline (solid line) and after addition of 10μM sNPF (dashed line). **b**, Peak ΔF/F before and after sNPF in fed and starved flies. **c**, Percent increase in peak ΔF/F after exogenous sNPF addition before and after sNPF in fed and starved flies. **d**, Percent increase in peak ΔF/F after sNPF addition for the five glomeruli that respond to cider vinegar in starved flies. n=5-6. Error bars indicate SEM *P<0.05, ***P<0.001, t-test.

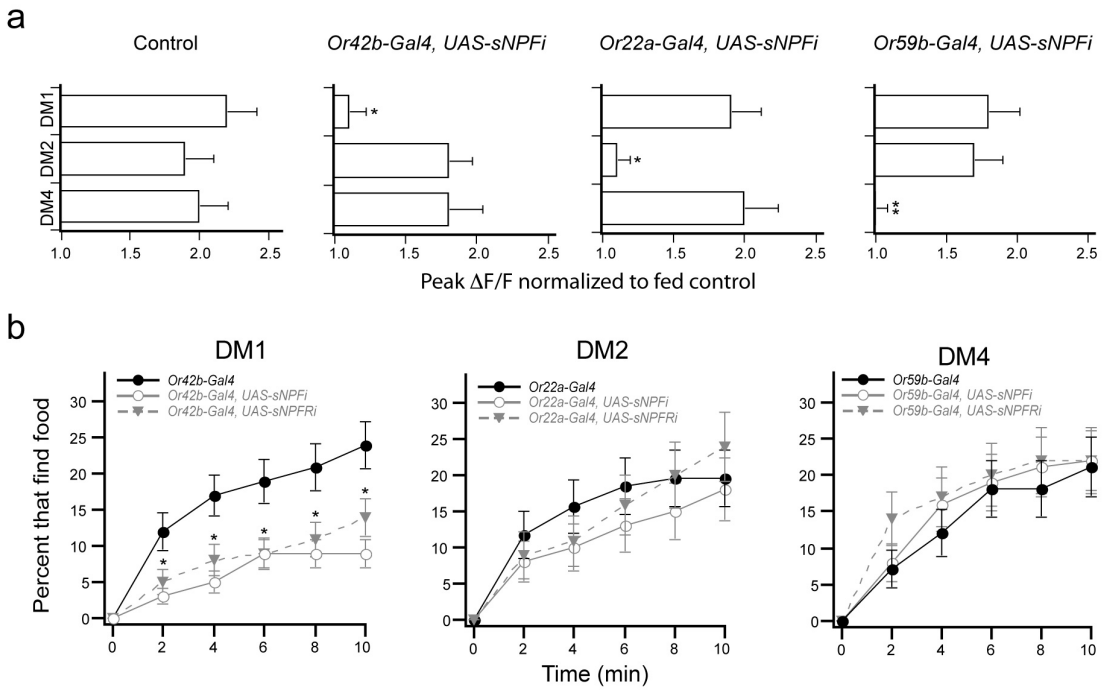

Figure 2.6. sNPF signaling in a single glomerulus is necessary for starvation-dependent food search.

a, Two-photon imaging of ORN axon terminal in flies expressing RNAi to knockdown sNPF expression in the ORNs of individual glomeruli. Peak ΔF/F normalized to the average response from fed control flies to 0.2% SV cider vinegar. n=5-6. *P<0.05, t-test. **b,** The latency of food search behavior for starved flies expressing RNAi to knockdown sNPF or sNPFR1 in individual glomeruli. RNAi expression in only the DM1 glomerulus significantly decreases food finding. n=80-195 flies for each condition. *P<0.05, z-test for proportions comparing *control to sNPFi* and to *sNPFRi*. Error bars show SEM.

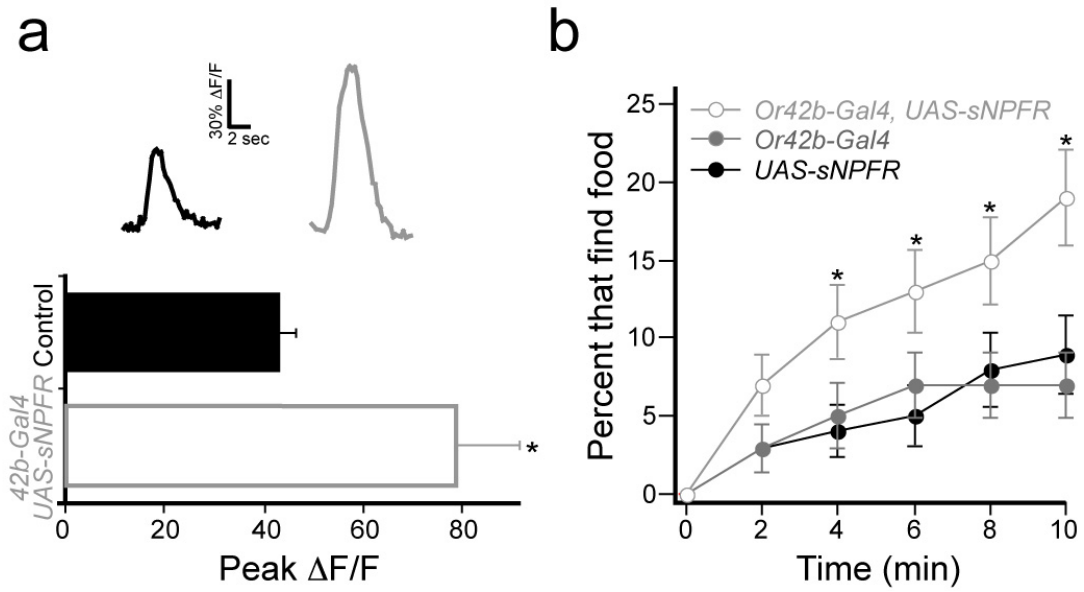

Figure 2.7. Overexpression of sNPFR1 is sufficient to enhance activity and food search behavior.

a, Two-photon imaging of ORN axon terminals in the DM1 glomerulus of fed flies in response to 0.2% SV cider vinegar. Control flies have the *Or83b-LexA* and *LexAop-GCaMP* transgenes, and experimental flies also bear the *Or42b-Gal4* and *UAS-sNPFR1* transgenes. n=5-6, *P<0.05, t-test. **b**, The latency of food search behavior in fed flies. n=134-168, *P<0.05, z-test for proportions comparing overexpression flies to two controls. Error bars show SEM.

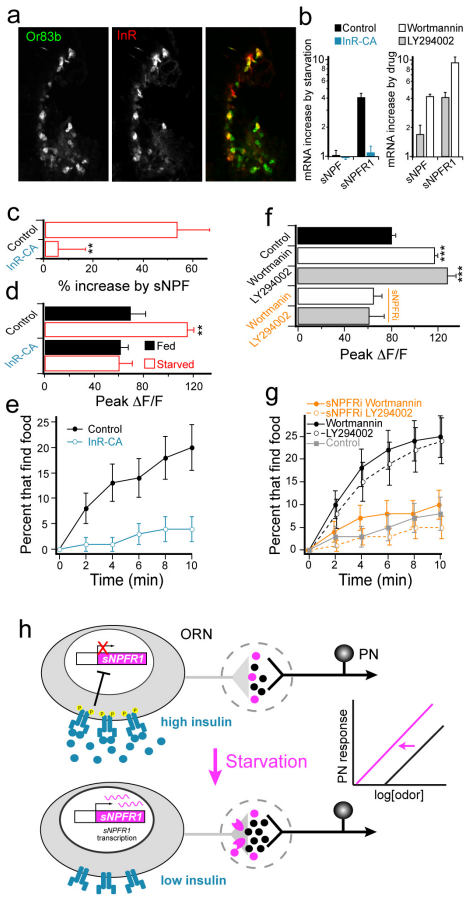

Figure 2.8. Insulin signaling modulates expression of *sNPFR1* and olfactory sensitivity.

a, Antennal tissue with immunoreactivity for the InR and GFP expression under the Or83b promoter. Tissue was stained with anti-GFP (green) and anti-InR (red) antibodies. **b**, Quantitative RT-PCR analysis of starvation-induced changes in mRNA expression in the antennae of control flies and flies expressing constitutively active InR (InR-CA) in ORNs (left), and that of flies fed PI3K antagonists relative to those fed only sucrose (right). Results are the average of four replicates, each of which was measured in triplicate and normalized to a control gene (rp49). **c**, Response to electrical stimulation of the olfactory nerve before and after application of 10 μM sNPF is plotted as the percent increase in peak ΔF/F after peptide addition in starved flies. Stimulus was 1ms duration, 10V in amplitude and 4 pulses at 100 Hz. n=6-9. **d**, PN dendritic response to 0.2% SV cider vinegar in the DM1 glomerulus for control flies and those expressing InR-CA in ORNs. n=5-9. **e**, The latency of food search behavior in starved control flies and those expressing InR-CA in ORNs. n=70-90 flies. **f**, PN dendritic response to 0.2% SV cider vinegar in the DM1 glomerulus for control flies fed sucrose overnight and those fed sucrose plus 25nM wortmannin or 30 μM LY294002. n=5 each. **g**, The latency of food search behavior in flies fed wortmannin and LY294002, and control flies fed sucrose only. n=60-92 flies. **h**, Model for hunger modulation of olfactory sensitivity. Error bars indicate SEM. *P<0.05, **P<0.01, ***P<0.001, t-test for **b, c, d, f**, and z-test for **e,g**.

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Chapter 3: Neuropeptidergic Presynaptic Inhibition Mediates Starvation Dependent Odor-Driven Food Search

3.1 Abstract

Internal physiology has a dramatic effect on animals' natural behavior. Here we investigate starvation-induced modulation in DM5—a glomerulus that mediates innate aversion behavior. We found that starvation suppresses DM5's sensitivity to odor stimulation, and that this suppression is mediated by *Drosophila* tachykinin (DTK) signaling. Experiments described in Chapter 2 show that starvation-dependent sensitization in DM1 is mediated by sNPF signaling. Here we show starvation-dependent depression in DM5 is mediated specifically by DTK signaling. Furthermore, hunger modulation of DM5 also influences food finding behavior. We have begun to investigate the molecular mechanism underlying this starvation modulation. Constitutively active insulin receptor abolishes suppression of DM5 in starved flies, and blocking insulin signaling pathway with wortmannin promotes suppression of DM5 in fed flies. We conclude that starvation has opposite effects on different population of ORNs—presynaptic facilitation in DM1 and inhibition in DM5, suggesting a push-pull mechanism to maximize food finding efficacy.

3.2 Introduction

An animal maintains energy homeostasis by changing metabolic hormone and neuromodulator levels, which subsequently modulates the CNS sensitivity to induce appetitive behaviors. In particular, injection of insulin in the murine hypothalamus leads to decreased feeding behavior, suggesting insulin as a satiety signal (Bruning et al., 2000; Woods et al., 1998). Yet, in a natural environment, an animal's food finding behavior precedes feeding behavior, in which the sense of smell plays an indispensable role

(Dethier, 1976). Little is known about whether and how these hormones and neuromodulators can affect an animal's food search behavior.

External food-related stimuli are sensed by the olfactory receptor neurons (ORNs) that express specific receptor genes, and those expressing the same gene converge and synapse onto projection neurons (PNs) in a region called the glomerulus (for review see Su et al., 2009). Activity in a particular single glomerulus leads to specific behaviors—in particular, DM1 is hard-wired for innate attraction behavior to low concentration of food-related odor, apple cider vinegar, and the DM5 glomerulus mediates innate aversion behavior at high concentrations of cider vinegar (Semmelhack and Wang, 2009). Recent studies revealed existence of many neuromodulators in the fly antennal lobe, suggesting dynamic modulation of the glomerular map and therefore an animal's behavioral output (Olsen and Wilson, 2008; Root et al., 2008; Ignell et al., 2009; Dacks et al., 2009). In particular, DTK is expressed in the local interneurons (LNs) and mediates presynaptic inhibition (Ignell et al., 2009).

We have previously shown that that starvation re-tunes the odor map mediated by low global insulin level, and a shift in DM1 activity alone can influence a fly's food search behavior (See Chapter 2). Here, we show that DM5 is suppressed upon starvation due to DTK mediated presynaptic inhibition. DM5 suppression is necessary and sufficient to bias food finding behavior. We also show here that low global insulin level that directly increases DM1 activity has an opposite suppressing effect in DM5. Taken together, these results suggest that starvation engages a push-pull neuropeptide modulation of select olfactory channels to maximize attraction to food odor.

3.3 DM5 activity is starvation dependent and is modulated by tachykinin

Does starvation influence DM5 activity? To test this, we performed two-photon calcium imaging (Wang et al., 2003) to measure PN dendritic calcium responses to a range of cider vinegar concentration from 5% to 80% saturated vapor pressure (SV). Indeed, we found that DM5 activity is suppressed in starved flies, and this suppression is significant at 20% to 80% SV but not at 5% or 10% SV (Figure 3.1A, C, D). This suggests that starvation re-tunes the olfactory map at high food odor concentration by decreasing sensitivity of DM5 at high concentration.

What is the underlying mechanism for this suppression? We hypothesized that DTK signaling could mediate this suppression, since it is a neuromodulator that mediates presynaptic inhibition in ORNs (Ignell et al., 2009). We expressed GCaMP in PNs and RNAi to knockdown DTKR selectively in ORNs using flies bearing the *GH146-LexA*, *LexAOp-GCaMP*, *Or83b-Gal4*, and *UAS-DTKR-RNAi* transgenes. Indeed, genetic knockdown of DTKR in ORNs abolishes starvation-dependent suppression of DM5 response (Figures 3.1C and D). Response of the DM5 glomerulus in starved flies was restored to the same level of fed flies. In contrast, DTKR knockdown in ORNs does not change the normal starvation-enhanced response in DM1 (data not shown). Therefore, we conclude that that DTK signaling is required for starvation-induced suppression of DM5.

We next asked if the DM5 modulation holds true for another odor. Ethyl butyrate is a pure chemical odorant that activates a closely related set of glomeruli that are activated by cider vinegar (Hallem and Carlson, 2006). We therefore examined DM5 activity with ethyl butyrate as an odor source. Indeed, we observed a similar starvation-dependent suppression of DM5 response as seen with cider vinegar, and this suppression

required DTK signaling (Figure 3.2). Thus, we conclude that this starvation-induced modulation may be a general property of DTK signaling in DM5 independent of odor identity.

3.4 Tachykinin signaling is both necessary and sufficient for food finding behavior

What is the behavioral consequence of DTK-mediated suppression of DM5? We hypothesized that suppression of DM5, an aversive glomerulus (Semmelhack and Wang, 2009), should improve flies' food finding efficacy. In order to investigate this, we used the single fly assay described in Chapter 2 to measure food search behavior. We knocked down DTKR expression in ORNs by expressing RNAi in starved flies bearing the *Or83b-Gal4* and *UAS-DTKR-RNAi* transgenes, and measured the latency required for flies to reach the target. We found that knocking down DTKR in ORNs results in a significantly longer latency in food finding; about 45% of control flies find the odor source within 10 minutes while only 25% of DTKR knockdown flies do so (Figure 3.3A, B). Thus, DTK signaling in ORNs is important for food search behavior in starved flies.

Can modulation in DM5 alone influence food finding behavior? To test this, we expressed DTKR-RNAi in Or85a neurons, which project to DM5 glomerulus (Couto et al., 2005; Fishilevich and Vosshall, 2005). Indeed, DTKR knockdown in DM5 alone is sufficient to mimic the behavioral impairment observed in receptor knockdown in many ORNs (Figure 3.3C, D). This suggests that DTKR expression in DM5 is necessary for food search behavior in the starved state.

Next, we asked whether overexpression of DTKR in the DM5 sensory neurons can bias food search behavior. We ectopically expressed DTKR in DM5 in fed flies

bearing *Or85a-Gal4* and *UAS-DTKR-OE* transgenes. Over-expression of DTKR in DM5 alone is sufficient to significantly increase a fly's food search behavior; 16% of fed control flies find the food odor source within 10 minutes while 27% of fed flies with ectopic DTKR expression do so (Figure 3.3E, F). However, DTKR overexpression in fed flies does not trigger food search behavior to the same level seen in starved flies, and this is likely to be due to low DM1 activity in fed flies (data not shown). Together, our behavioral data suggest that DTKR expression and the subsequent DM5 suppression is necessary and sufficient to bias food search behavior.

3.5 DM5 modulation is concentration dependent

In a natural environment, flies are exposed to a wide range of different odorant concentrations during their pursuit to find food. High threshold for DM5 activation suggests that DM5 modulation may be concentration dependent. To investigate the function of DTK signaling at various concentrations, we ectopically expressed DTKR in Or85a neurons and measured their food finding latency in response to 1%, 5%, 25% and 75% cider vinegar. Fed flies were used in order to prevent DM1 facilitation from affecting food finding behavior. Ectopic expression of DTKR in DM5 did not enhance food search when 1% cider vinegar was placed as an odor source (Figure 3.4). This suggests that DM5 modulation serves no role in food search when the odor concentration is low. However, food search is significantly enhanced in overexpression flies when the concentration goes from 5% to 25% (Figure 3.4). Interestingly control and DTKR overexpression flies both exhibited reduced attraction to the 75% cider vinegar (Figure 3.4), which may indicate that this concentration is sufficient to activate DM5 even with a

high amount of presynaptic inhibition. Thus, we conclude that the food finding behavior mediated by DM5 modulation is concentration dependent.

3.6 Tachykinin is released from LNs to suppress DM5

Where is the source of DTK for the hunger modulation of DM5 response? Previous studies have shown that *GH298-Gal4* line labels a subpopulation of LNs in *Drosophila* antennal lobe that is immunoreactive to DTK and GABA (Wilson and Laurent, 2005; Ignell et al., 2009). We therefore hypothesized that DTK is released from these LNs to suppress DM5. We expressed DTK-RNAi in these LNs and measured PN response of DM5 in flies bearing *GH146-LexA, LexAOp-GCaMP, GH298-Gal4,* and *UAS-DTK-RNAi* transgenes (Figure 3.5A). Indeed, peptide knockdown abolished DM5 suppression in starved flies (Figure 3.5B), suggesting the GH298 LNs provide the direct source of DTK required for starvation-induced modulation at DM5.

3.7 Insulin signaling modulates DM5 sensitivity

What is the mechanism by which starvation induces DTK signaling to suppress DM5? Insulin is a satiety signal in fruit flies and insulin receptor is expressed in many ORNs (See Chapter 2: Figure 2.8). It is possible that the same global metabolic cue that suppresses sNPF signaling could also decrease DTK signaling. To investigate the role of insulin in DTK signaling, we examined PN activity of DM5 in flies with blocked insulin signaling. Flies were fed overnight with sugar and wortmannin, a drug that selectively blocks PI3K, an enzyme involved in insulin signaling. We observed that the peak $\Delta F/F$ in both DM1 and DM5 glomeruli in wortmannin-fed flies mimicked that of starved flies,

confirming the drug's effectiveness to mimic the low insulin level found during starvation (Figure 3.6A, B). Strikingly, expression of DTKR-RNAi in ORNs eliminated the effect of wortmannin selectively in DM5 (Figure 3.6A, B), suggesting that DM5 suppression in wortmannin-fed flies required DTK signaling. Conversely, expression of constitutively active insulin receptor (InR-CA) in the ORNs of starved flies prevented the starvation-dependent suppression of DM5 (Figure 3.6C). Thus, we conclude that insulin modulates DTK signaling and subsequently DM5 sensitivity.

What is the molecular mechanism by which DTK signaling increases upon starvation? Because ectopic expression of receptors in DM5 rescues starved food finding behavior in fed flies (Figure 3.3F), we hypothesized that DTKR expression increases in the ORNs upon starvation. We measured the level of DTKR mRNA using quantitative RT-PCR in surgically isolated antennae of fed and starved flies. Indeed, we found that the receptor mRNA is increased by approximately four-fold in starved flies (Figure 3.6D). Thus, we conclude that DTK signaling is amplified upon starvation by increasing the receptor expression in the ORNs.

3.8 Discussion

Together with Chapter 2, we present here a physiological and behavioral characterization of two opposite modulatory mechanisms in the fly olfactory system. Upon starvation, presynaptic facilitation by sNPF increases acivity of the DM1 glomerulus, whereas presynaptic inhibition by DTK suppresses DM5 activity. Surprisingly, both modulation mechanisms are engaged by the same metabolic cue, insulin. This study reveals that a global cue such as insulin can in re-tune the odor map in

a push-pull fashion to maximize a natural behavior such as food finding. Moreover, this study encourages investigation of other odor-driven natural behaviors that may be affected by an animal's internal physiological state, such as mating status.

3.9 Methods

Two-photon calcium imaging. GCaMP imaging was performed as previously described (Root et al., 2008; Wang et al., 2003). In odor experiments, a constant airflow of 1 l/min was applied to the antennae via a pipe of 12 mm diameter. Odor onset was controlled by mixing a defined percentage of carrier air with air redirected through odor bottles as previously described (Root et al., 2008; Semmelhack and Wang, 2009). Starved flies were starved overnight with water for 17-24 hr.

Behavior assay. Female flies that were 2-5 days old and presumed non-virgin were used for all experiments. Single flies were introduced into chambers that were 60 mm in diameter and 6 mm in height. The chamber was illuminated by 660 nm LEDs. Flies were tracked at 2 Hz with custom software written in Labview (V.8.5, National Instruments), and analysis was performed with Igor Pro (V.6, Wavemetrics, Inc) using a custom macro. Latency is defined as the elapsed time before an individual fly spends more than 5 seconds within a 5 mm distance from the odor source, which minimizes false positives due to random entry into the odor zone. Apple cider vinegar was diluted 1:100 in 1% low melting temperature agarose for 1%, 1:25 for 5%, 1:4 for 25%, and 1:1.33 for 75%. 5 μl were placed in the center of the chamber.

Pharmacology. Wortmannin (LC Laboratories, Woburn, MA) was dissolved in DMSO at stock concentrations of 10 mM. Flies were fed overnight in vials containing 4% sucrose solution alone, or that plus 25nM wortmannin.

Transgenic flies. The following fly stocks were used: *Or83b-Gal4* (Kreher et al., 2005); *Or83b-LexAVP16* (Lai and Lee, 2006); *Or42b-Gal4* and *Or85a-Gal4* (Fishilevich and Vosshall, 2005); *GH146-Gal4* (Stocker et al., 1997); *UAS-GCaMP (*Wang et al., 2003*)*; *LexAop-GCaMP* (Root et al., 2008)*; UAS-DTKi, UAS-DTKRi, GH298-Gal4, UAS-DTKR-GFP* (Ignell et al., 2009); *UAS-sNPFR1* (Lee et al., 2008); *UAS-InR-CA* (obtained from the Bloomington stock center #8263).

Quantitative RT-PCR. RNA was isolated from antennae of 50 female flies for each sample. The RNeasy kit (Qiagen) was used to isolate RNA and the reverse transcription was performed using the Retroscript kit (Ambion) with random decamers. This cDNA was subjected to PCR analysis using SYBR green detection on an iCycler thermocycler (Biorad). All values are normalized against *rp49* as a control gene.

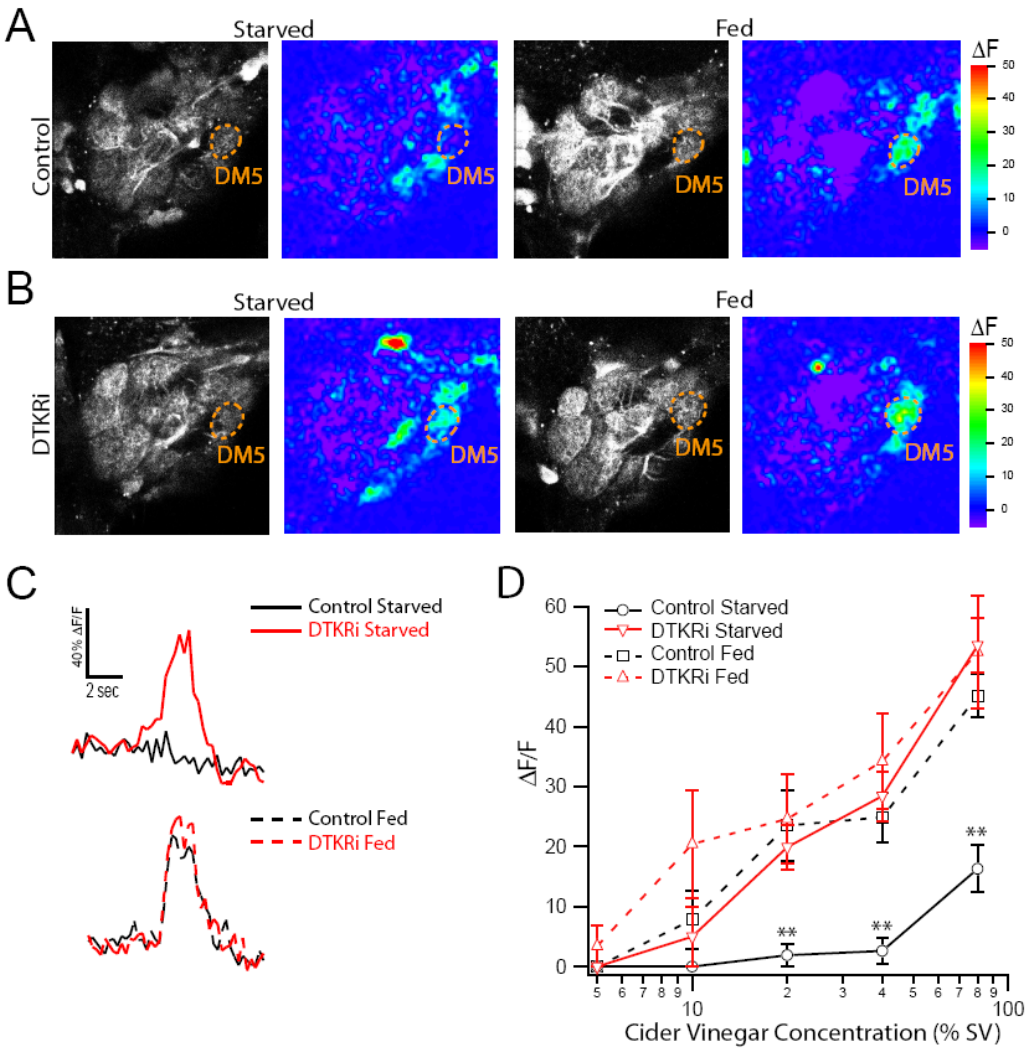

Figure 3.1. DM5 activity is starvation dependent and is modulated by tachykinin.
A and B, Two-photon imaging of PN calcium activity in response to cider vinegar stimulation on DM5 plane of the antennal lobe in starved and fed flies. Control = *GH146LexA, LexAOp-GCaMP/+; Or83b-Gal4/+*, DTKRi = *GH146LexA, LexAOp-GCaMP/UAS-DTKR-RNAi; Or83b-Gal4/UAS-DTKR-RNAi*. Gray-scale images show antennal lobe structure while pseudocolored images reveal odor-evoked activity at 80% SV. **C**, Representative traces of fluorescence change over time for DM5 excited by cider vinegar at 80% SV. **D**, Peak ΔF/F across a range of cider vinegar concentrations for DM5 glomerulus. **D**, n=5-10 for each condition; error bars show SEM. **P<0.01; t-test.

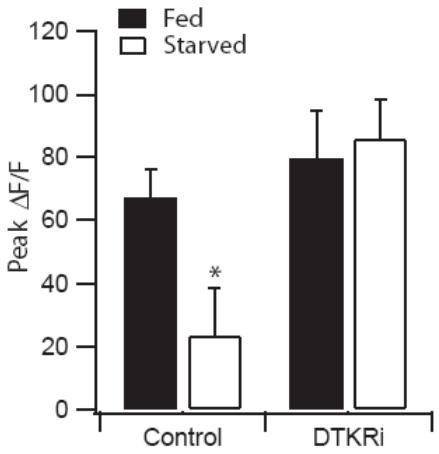

Figure 3.2. DM5 response to ethyl butyrate is starvation dependent.
PN dendritic response to 0.4% SV 1:100 ethyl butyrate in the DM5 glomerulus. Control = *GH146LexA, LexAOp-GCaMP/+; Or83b-Gal4/+*, DTKRi = *GH146LexA, LexAOp-GCaMP/UAS-DTKR-RNAi*. DM5 is suppressed only in starved control flies. n=5 for each condition; error bars show SEM. *P<0.05; t-test.

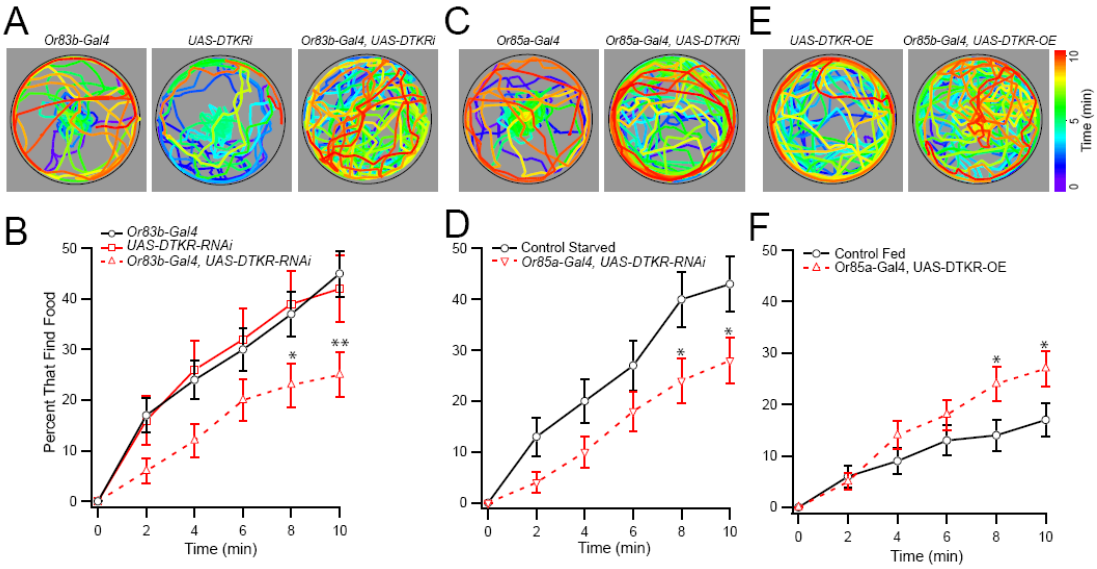

Figure 3.3. DM5 activity is necessary and sufficient for food search.
A, A behavioral assay was used to measure the latency of odor-guided food finding. Flies were introduced into a dark arena containing a food odor, cider vinegar, in the center. The coordinates of single flies for representative control flies (left two) and those expressing *DTKR-RNAi* (right) in the ORNs. **B**, The latency of food search is quantified as the cumulative percentage of flies that find the odor source as a function of time. **C,** The coordinates of representative control flies (left) and those expressing *DTKR-RNAi* in DM5 (right). **D**, The latency of food finding as a function of time. **E,** The coordinates of representative control flies (left) and those ectopically expressing *DTKR-OE* in DM5 (right). **F**, The latency of food finding as a function of time. n=66-165 flies for each condition. Error bars show SEM. *P<0.05, **P<0.01; z-test for proportions comparing the top curves to the bottom curve in **B, D, F**.

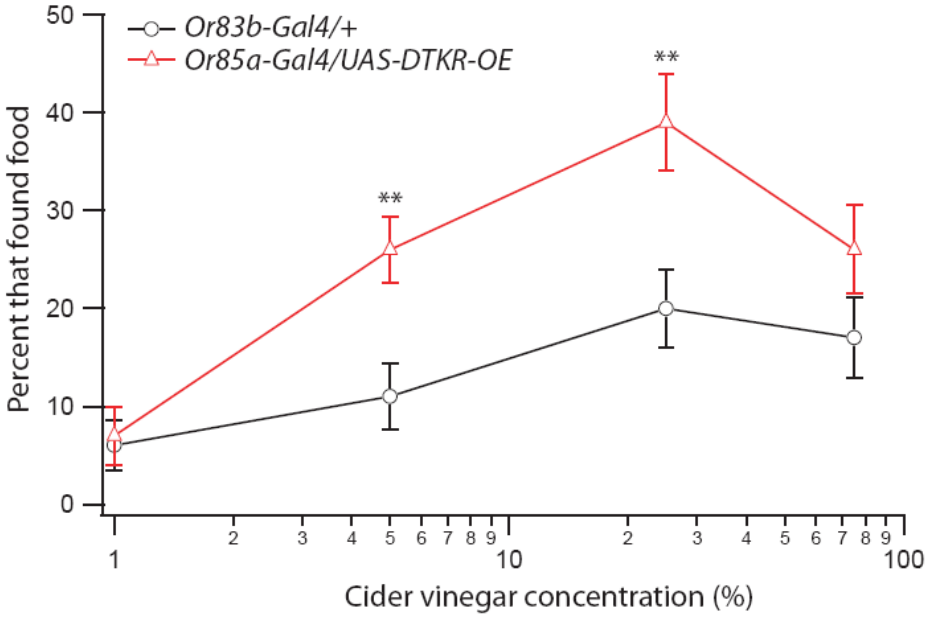

Figure 3.4. Tachykinin modulation of DM5 is concentration dependent.
The latency of food search is quantified as the cumulative percentage of flies that find the odor source as a function of cider vinegar concentration. Inducing DM5 modulation in fed flies does not have an effect at 1% cider vinegar, but significantly increases food search performance at 5% and 25% cider vinegar. n=72-165 for each. Error bars show SEM. **P<0.01; z-test for proportions comparing the top curve to the bottom curve.

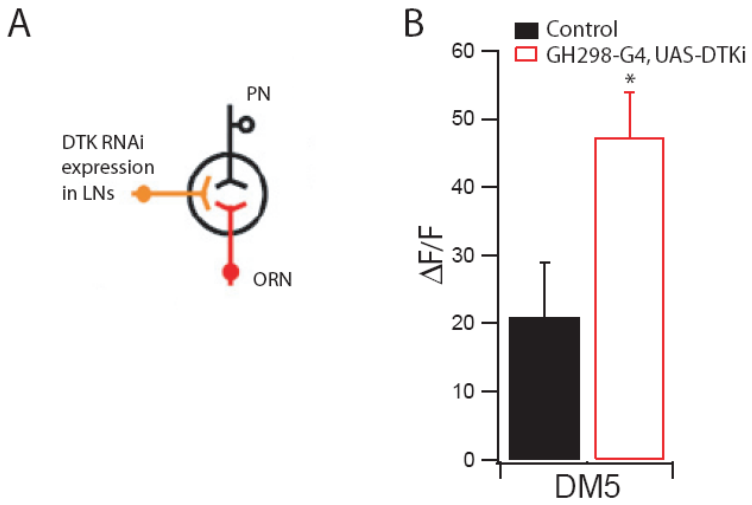

Figure 3.5. DTK is released from GH298 neurons.
A, schematic diagram of LNs expressing DTK-RNAi transgene. **B,** PN dendritic response to 80% SV cider vinegar in the DM5 glomerulus for the starved flies. Control = *GH146LexA, LexAOp-GCaMP/+; Or83b-Gal4/+*, GH298-G4, UAS-DTKi = *GH146LexA, LexAOp-GCaMP/UAS-DTK-RNAi; GH298-Gal4/UAS-DTK-RNAi*. n=5 for each condition; error bars show SEM. *P<0.05; t-test.

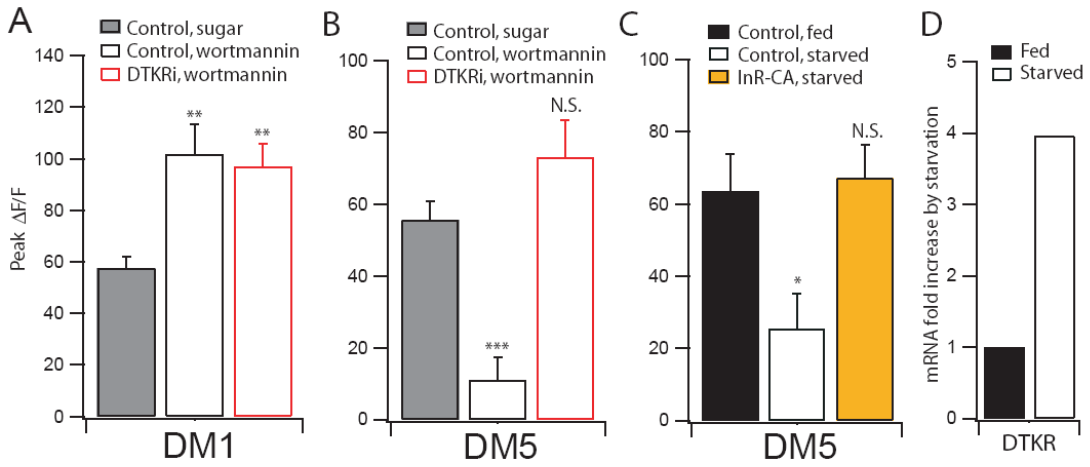

Figure 3.6. Insulin suppresses tachykinin signaling in DM5.

A, PN dendritic response to 0.2% SV cider vinegar in the DM1 glomerulus for control flies fed sucrose overnight and those fed sucrose plus 25nM wortmannin. Knockdown of DTKR in ORNs had no effect in DM1 activity in wortmanin-fed flies. n=5 each. **B,** PN dendritic response to 80% SV cider vinegar in the DM5 glomerulus for the same groups from **A**. Knockdown of DTKR in ORNs recovered a sugar-fed response of DM5 activity in wortmanin-fed flies. n=5 each. **C,** PN dendritic response to 80% SV cider vinegar in the DM5 glomerulus for control flies and those expressing InR-CA in ORNs. n=5 each. **D,** Quantitative RT-PCR analysis of starvation-induced changes in DTKR mRNA expression in the antennae. Results are the average of four replicates, each of which was measured in triplicate and normalized to a control gene (rp49). **A, B, C,** n=5 for each condition; error bars show SEM. *P<0.05, **P<0.01, ***P<0.001; t-test.

Chapter 3 References

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