A *DROSOPHILA* MODEL FOR FLAVOR PERCEPTION AND FEEDING DECISION

by

YIWEN ZHANG

(Under the Direction of Ping Shen)

ABSTRACT

The central nervous system (CNS) evaluates internal nutrients levels and external environmental cues to make foraging decisions: to consume enough food for energy needs, yet not too much for the body to handle. Eating disorders or obesity occur when energy balance deviates from body needs, leading to physical and psychosocial morbidity or even fatal consequences.

We have developed a behavioral paradigm in *Drosophila* larvae that provides a quantitative readout for feeding responses. With the help of this system, we revealed that feeding behaviors in Drosophila larvae utilize sophisticated neural modulations, which implied the existence of an elementary form of cognition in this model organism.

Like mammals, fly larvae make feeding decisions according to current body energy level. We have demonstrated that the motivational states are mediated through two octopamine receptors Oamb and Octβ3R downstream of two OA neuron clusters in the subesophageal ganglion. Oamb and Octβ3R exhibit counteracting effects on feeding. While Oamb neurons inhibit overfeeding in the satiated state, Octβ3R neurons promote feeding during food deprivation.

The baseline feeding in satiated larvae can be disrupted by the reward system. Appetizing odor elicits motivation to feed on sweet media. Two dorsal medial neuropeptide F neurons are

responsible for relaying the olfactory stimuli by responding to upstream dopamine signaling through dopamine receptor D1. The behavioral output further relies on a cluster of three NPF receptor neurons in the subesophageal ganglion region, which project to the peripheral enteric systems. Alcohol is also able to alter the feeding response by activating NPF synthesis and release. However, it only intervenes with the pathway for regulating feeding motility, leaving a separate mechanism for modulating food ingestion.

INDEX WORDS: *Drosophila melanogaster*; Feeding behaviors; Flavor perception; Olfactory system; Dopamine; D1 receptor, Neuropeptide F; NPFR1; SOG; Gr43a; Sugar sensation; Motivational states; Octopamine; Oamb; Octβ3R; Ethanol; Alcohol euphoria

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DEDICATION

To my loving family

To you who are reading it

To all the delicious foods
who boosted my dopamine levels
and kept me passionate about life

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I would like to thank all the people who guided me, inspired me, and encouraged me in these six years. Leaving all my family and friends to study abroad was a big decision for me. The part six years turned out to be a journey that helped me to gain better ideas about who I am, to grow from an overprotected girl to an independent human, and to learn how to think.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Feeding-related decision making is a primitive yet exquisite process, involving genetic, endocrine, and neural regulations. In mammals, the individual anatomical brain centers for carrying out separate feeding-related tasks have been identified (1, 2). However, our knowledge remains rudimentary on the cross-modal synergy, such as decoding the vertical information flow through the functionally connected brain centers, as well as understanding the computing and integration of parallel incoming information. The lack of thorough characterization is largely due to the complexity of the mammalian central nervous system on the level of quantity scale (number of neurons) and architecture (each neuron may communicate to thousands of other neurons which forms a mazy network) (3).

Drosophila melanogaster serves as a good model organism since it has a relatively manageable nervous system yet many conserved neurobiological processes, such as neuro-transmitter production and secretion, cellular signaling pathways, and anatomical layouts of certain neural circuits. Previous studies showed that fruit flies exhibited a comprehensive array of feeding behaviors in response to both homeostatic and hedonic signals, suggesting that it can be used as a good model to study neurobiological mechanisms for making feeding decisions.

This chapter will summarize previous major findings on central nervous control of feeding behaviors in mammalian and *Drosophila* models.

1.1 HOMEOSTATIC CONTROL OF FEEDING

1.1.1 *Mammalian CNS feeding center*

Homeostatic control is the regulation of feeding motivation by the internal nutrient levels and it involves CNS control of energy balance. The hypothalamus region of the brain attracted a lot of attention since the classical studies considered it as the satiety and feeding center. Lesioning of the ventromedial hypothalamus (VMH) and the lateral hypothalamus (LH) sub-regions led to overeating and undereating respectively (4-7), indicating the coexistence of two competing mechanisms with opposing effects on feeding in the hypothalamus for the first time. With the new genetic and pharmacological tools available, scientist started to focus on the arcuate nucleus (ARC) since the 1990s, as more and more evidence suggested that it is where the hypothalamic orexigenic and anorexigenic neural circuits originated from (8).

The anorexigenic neurons in the arcuate nucleus release proopiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcripts (CART) to multiple nuclei of the hypothalamus (9, 10). POMC is the precursor for melanocortin peptides including the melanocyte–stimulating hormones (MSH) (11, 12). Synthesized and released MSH inhibits feeding through acting on the downstream melanocortin-4 receptor (MC4R) neuron (13, 14). The CART peptide has been shown to exert an inhibition effect on feeding and weight gaining. The detailed circuit and molecular mechanism for CART is not clear and no receptors for this peptide have been identified (15-17).

The orexigenic neurons in the arcuate nucleus release neuropeptide Y (NPY) and agouti-related protein (AgRP) (18). AgRP works as an antagonist of the melanocortin receptors, and therefore counteracts the anorexigenic effects from POMC peptides (19). NPY belongs to the pancreatic peptide family and is the most abundant neuropeptide widely expressed in the CNS (20, 21). The

hypothalamic NPY exerts an orexigenic effect, as NPY injection to this region led to overeating and obesity in rats (22-24). Hypothalamic NPY cell bodies are restricted in the arcuate nucleus revealed by the mRNA expression (18, 25), while the majority of NPY release takes place in the paraventricular nucleus (PVN), as revealed by immunostaining (21). Food deprivation induces an increased NPY transcription level in the arcuate nucleus, which may in turn activate feeding to restore the energy level (26). NPY has at least 5 receptors in the CNS with distinct expression patterns (27-29), of which well characterized are Y1, Y2, Y4 and Y5 (30). They all belong to the rhodopsin like 7 transmembrane GPCR that couple Gi subunit (31). NPY receptors knockout in rats failed to develop expected feeding phenotypes, probably due to the redundancy of the feeding networks that compensates for the loss during development (32). However, transient manipulation using receptor specific agonists and antagonists in adult rats defined Y1 and Y5 as the receptors involved in regulating feeding (33-36). Y2 and Y4 may act as the autoreceptors at NPF presynaptic terminals, constituting the negative feedback loop to inhibit NPF release (37, 38).

Other than directly talking to the downstream melanocortin and NPY receptor neurons, the lateral information flow between the orexigenic and anorexigenic groups may facilitate the switching between motivational states. NPY/AgRP neurons interact locally with POMC/CART neurons in the arcuate nucleus (39, 40). NPY/AgRP neurons inhibit the POMC/CART neuron by co-release of the *gamma*-aminobutyric acid (GABA) (10). In addition, it has been shown that melanocortin-3 receptors (MC3R) are coexpressed with a subgroup of arcuate NPY/AgRP and POMC/CART neurons (39). MC3R, like postsynaptic MC4R, exerts a physiological anorexigenic function and a neuron activation effect (41). Since applying MC3R agonists failed to alter overall NPY/AgRP activity (40, 42, 43), the expression of MC3R receptor in arcuate

NPY/AgRP neurons may work as an autoreceptor at the presynaptic location to negatively regulate hunger-induced feeding through the antagonistic effect of AgRP. MC3R expression in the arcuate POMC/CART neurons may be involved in forming the reinforcement of the anorexigenic circuit during a satiety state, and receiving the lateral inhibition from NPY/AgRP neurons when hunger strikes.

1.1.2 *Signaling from peripheral*

Which circuit in the hypothalamus feeding center is currently dominating is determined by the levels of the feeding hormones from the peripheral. The blood vessels in the arcuate nucleus have a relatively weak blood-brain-barrier (BBB) thus allowing the direct contact of the ARC neurons to the circulating hormones (44-46), including the 'hunger hormone' ghrelin, and the 'satiety hormones' leptin and insulin. These hormones compete on the CNS feeding center to adjust motivational states (47, 48).

Ghrelin secretion is triggered during food deprivation from the gastrointestinal tracts (49-51). Both peripheral and hypothalamic administration of ghrelin are able to induce a hyperphagia phenotype (52, 53). It directly stimulates the NPY/AgRP neurons through the growth hormone secretagogue receptors (GHSR) to fulfill its orexigenic impact (54-57). Leptin and insulin secretion levels are in proportion to the adipocytes size, and are released by adipocytes and pancreatic beta cells respectively (58, 59). Both NPY/AgRP and POMC/CART neurons express the long-form leptin receptor (LRb) (60, 61) and the insulin receptor (InsR) (62). Leptin and insulin both exert an anorexigic effect by inhibiting the expression of NPY and AgRP, and stimulating POMC synthesis (10, 63-66). Therefore, leptin and insulin were considered critical signals in the adiposity negative feedback (1). Other gut satiation peptides, such as peptide YY (PYY), cholecystokinin (CCK), glucagon-like peptide-1 (GLP-1), and pancreatic polypeptide

(PP), signal to the NTS through the vagus nerve (X) (67-69), and further project to the arcuate nucleus of the hypothalamus (70, 71). The divergent cellular responses of NPY/AgRP and POMC/CART neurons is mediated through different downstream molecular pathways (62, 72-76).

The arcuate nucleus also senses peripheral circulating nutrients, including glucose and fatty acids (1, 77). Previous studies showed that glucose activates POMC neuron firing, which involves an ATP-sensitive potassium channel (78, 79). Fatty acids may inhibit hypothalamic NPF expression through altering the intracellular accumulation of long-chain fatty acyl-CoA (LCFA-CoA) (1, 80). High levels of glucose and fatty acids in the CNS both play an anorexigenic role, paralleling with its physiological implication for abundant nutrients and high energy level.

1.1.3 *Homeostatic control in Drosophila model*

In *Drosophila* larvae, the VUM1 and VUM2 clusters of octopamine (OA, the invertebrate homolog of norepinephrine (NE) (81)) neurons in the SOG region are critical in homeostatic feeding regulation. These two OA clusters exhibit opposite effects on defining motivational states, as VUM1 cluster lesioning generated an increased feeding rate in satiated larvae, while VUM2 cluster lesioning blocked hunger-driven overeating (82). It may parallel the mammalian hypothalamic NE neurons in the paraventricular nucleus (PVN), which receive upstream signaling from the arcuate nucleus neurons (83).

Endocrine hormones involved in homeostatic regulation of feeding are highly conserved in *Drosophila*. The *Drosophila* insulin-like peptides (Dilps) are secreted by the CNS insulin producing cells (IPCs) in proportion to nutrient levels (84, 85). The overexpression of Dilps induced an suppression of feeding in starved larvae (86). The *Drosophila* cytokine Unpaired 2 (Upd2) protein is released by the fat body in response to circulating sugar and fat (87). The

deficiency of body growth and metabolism in Upd2 mutants could be rescued by the expression of human leptin, demonstrating the homology between the two (87). These evidences suggested a considerable similarity between the mammalian and *Drosophila* models.

The physiological role of Upd2 is mediated through a JAK/STAT signaling pathway of GABA neurons, which inhibit the IPCs in the protocerebrum thus elevating Dilps secretion level (87-89). IPCs project axons to the endocrine ring gland as well as the subesophagus region (84). In addition, knockdown of a octopamine receptor Oamb in IPCs led to increased food intake (90). These morphological and functional data raise the possibility about the connection between endocrine modulation and CNS OA feeding control system.

1.2 HEDONIC CONTROL OF FEEDING

1.2.1 *Feeding for reward*

Mammalian Systems

The rewarding value of food can override the homeostatic control system, making animals feed beyond energy needs. Appetizing flavors are perceived through multiple sensory systems, among which the most important are the olfactory and gustatory sensations. Sugar is a natural reward for animals (91, 92) primarily sensed by the taste receptors (93). The gustatory sensation of sweetness is known to promote energy intake and stimulate physiological orexigenic responses including the release of saliva and gastric acid (94-96). Olfactory perception for appetizing food odors also plays an important part in stimulating appetite and is critical in initiation of feeding (97-99). Other than these universal mechanisms, humans also learnt to associate complex cues to food reward, such as visual and auditory cues, or the language (100, 101). The high rewarding value of food potentiates the development of addictive behaviors by working on the mesolimbic

reward pathway like other drugs of abuse (102-104). Addiction-like behaviors toward food were also observed in rodent models, which showed a high motivation toward highly palatable food and exhibited withdrawal symptoms when the reward is discontinued (105-107).

Drosophila Systems

The invertebrate *Drosophila melanogaster* showed a descent level of behavioral complexity toward non-homeostatic stimulations. The responses generated include food intake increase triggered by moderate sugar and salt in satiated larvae (108, 109), developing anticipation for nutrients when exposed to food related cues (110), showing preference toward rewarding and avoidance toward aversive gustatory stimuli (111, 112), and acquiring olfactory and visual memories when associated with innate food rewards (113, 114). Using a behavior paradigm, our lab has previously identified a hedonic feeding-like phenotype in *Drosophila* larvae (115). When stimulated with appetitive odor cues, satiated larvae increased the rate of mouth hook contraction on sugar medium, which correlates an increase in food intake amount. Both gustatory and olfactory stimuli are required for this food reward aroused feeding, suggesting a multi-modal processing for flavor perception.

1.2.2 *Gustatory system for flavor perception*

Mammalian Systems

In humans, gustatory information is sensed by the gustatory receptor neurons (GRNs) in the taste buds, and are innervated by pseudounipolar neurons which project to the nucleus of the solitary tract in the brain stem through the facial nerve (VII), the glossopharyngeal nerve (IX), and the vagus nerve (X) (116). The gustatory information is then relayed to the gustatory cortex in the brain insular lobes after synapsing in the thalamus (Fig.1.1) (117).

Sweet, bitter and amino acid tastes are detected by the G-protein-coupled receptors (GPCRs) in mammals, while sour and salty tastes are mediated through ionotropic receptors (IRs) (118). Coding of different tastes on the gustatory receptors level conforms to the valence labeled line model (116), meaning that multiple chemicals can activate the same GRNs and thus are grouped into the same taste quality. Spatial representation for different taste qualities remains in higher brain centers. Studies in mice demonstrated a topographic segregation for different tastes in the gustatory cortex (119).

Drosophila Systems

The gustatory organs in adult flies are distributed on different body parts including proboscis, legs, and internal taste organs near the pharynx. In female flies, gustatory receptors (GRs) are also expressed in the ovipositor and they function as tools that choose a nutrient rich environment to lay eggs on (120). *Drosophila* larvae has three external gustatory organs: dorsal organ (DO), terminal organ (TO), and ventral organ (VO), accompanied by three internal pharyngeal gustatory organs: Dorsal Pharyngeal sense organs (DPS), ventral pharyngeal sense organs (VPS), and posterior pharyngeal sense organs (PPS). The three internal gustatory organs persist into adult pharyngeal organs (121). *Drosophila* GR neurons send axons directly to the subesophageal ganglion (SOG) region which is functionally similar to mammalian brainstem (Fig.1.1) (114, 122). Information about *Drosophila* gustatory higher brain centers was less documented. Putative gustatory interneurons either directly project to pharyngeal muscles for regulating the feeding motor program, or to higher brain centers for more complex functions (120, 123-125). Recently, the antennal mechanosensory and motor center (AMMC) in adult flies was suggested as the secondary CNS center for relaying sweet taste (126). The mushroom body,

the brain center for olfactory memory formation (127, 128), is innervated by gustatory interneurons near AMMC (124) and might be an integration center for odor and taste signals. Gustatory receptors in *Drosophila* are ionotropic (IRs) (129, 130). Compared with mammals, *Drosophila* showed a higher chemical convergency level on GRs. Appetizing tastes toward sugar, amino acids, and low salt concentrations activate the same type of GRNs, while aversive tastes like bitterness and sour flavor are perceived by the same GR group (131-133). In *Drosophila*, most of the GRs mediate aversive tastes to avoid toxins. Moreover, aversive gustatory response is more dominant than appetitive response given that some bitter tasting chemicals can inhibit sugar sensing GRNs (134). The spatial information for gustatory signals in the first CNS taste center, the SOG, is organized on two levels. The first level of SOG regionalization is based on different gustatory organs. GR neurons from the same gustatory organ have a relatively conserved projection pattern in the SOG (120, 135-137). Secondly, within projections from the same organ, axons relaying attractive taste inputs and aversive taste inputs are further separated into different regions (120, 135, 138).

1.2.3 *Olfactory system for flavor perception*

Mammalian Systems

In mammals, odors are received by the olfactory receptor neurons (ORNs) in the nasal olfactory epithelium. The ORNs send axons to the glomerulus in the olfactory bulb through the olfactory nerve to interact mitral neurons, which carry information to olfactory cortex regions (Fig.1.2) (139, 140).

Mammalian ORs are G-protein coupled receptors (GPCRs) (141). The binding of odorants to ORs activates adenylyl cyclase and a downstream cascade of activations, leading to the opening of ion channels and membrane depolarization (142). Each odorant activates a repertoire of ORs

and the combination of activated ORs determines the quality of odorants. This feature of odor perception is described as a 'distributive model' (116, 143), and it enables the perception for a large scale of odor molecules in different doses with a limited number of OR types. Spatial information from different types of ORNs remains separated in the olfactory bulb (144) and the olfactory cortex (145). Detailed analysis in the higher CNS centers using mammalian models is difficult due to the huge amount of cell numbers.

Drosophila Systems

Drosophila detects odorants by the ORNs in the antenna and the maxillary palp in adults (146, 147), and in the dorsal organ in larvae (148). ORNs project to the antenna lobe located in the deutocerebrum to form synapses with projection neurons, which are parallel to mammalian mitral neurons. Projection neurons further relay the information to the lateral horn region (LH) and the mushroom body (MB) in the brain lobes (Fig.1.2) (122).

Drosophila ORs also belong to the seven-transmembrane domain protein family but share no homology to the mammalian GPCRs (122). Instead, *Drosophila* ORs are ligand gated ionotropic channels which can be opened directly when binding to the odorants (129, 149, 150). The identification for different odor molecules is less efficient compared to mammals, as single odorant activates only one or a few corresponding ORs (147), which is close to the 'labelled line model' in *C.elegans* olfactory system (151). Axons from different types of ORNs remain spatially separated in the antenna lobe in *Drosophila*. Each glomerulus in the antenna lobe only receives olfactory inputs from one type of upstream ORN and odor classification is thus kept within the pattern of glomeruli activities (147, 152). Projection neurons also form stereotype arborizations in the mushroom body and the lateral horn regions (153, 154). By receiving olfactory information from multiple glomeruli and sending axons to multiple higher brain

regions (155, 156), these secondary olfactory neurons initiate the convergence and divergence in *Drosophila* odor perception. How the higher brain centers further decode the olfactory information and translate it into behavioral outputs remains unclear.

1.2.4 *CNS circuit for hedonic feeding*

Mammalian Systems

The CNS control of hedonic response for food involves two components: one pathway regulates appetite, or anticipation and motivation for food reward; another pathway assesses palatability, or carries out sensation and calculation for food reward (157, 158). Dopamine and opioids circuits in the midbrain and limbic regions have been identified as key regulators. Previous studies on mice suggested that these two pathways are independent of each other, yet are usually coordinated in exhibiting the hyperphagia responses toward highly rewarding foods (159, 160). The mesolimbic dopamine pathway, the shared target for many drugs of abuse (161, 162), is considered crucial for the 'wanting' of food (163, 164). It may link the sensory system with the endocrine regulation by forming connections with the insular and olfactory cortex regions as well as the hypothalamus (163, 165-168). The dopaminergic cell bodies originate from the ventral tegmental area (VTA) and project to the nucleus accumbens (NAc) (169). An increase of dopamine release in the NAc was observed when animals tasted sugar solution (170-173). Electrophysiological activity of dopaminergic neurons was also detected during food-driven bar pressing activity in monkeys (174). Perfusion of dopamine into the NAc led to increased sugar consumption, probably through increasing the food approaching speed and locomotor activity (174-176). Animals also generate a self-administration of dopamine receptor agonists into the NAc region, and this addiction-like behavior is mediated through D1 and D2 receptors (177-179).

However, pharmacological lesioning of the mesolimbic dopamine circuit failed to block the affective reaction expression in mice if the sugar reward is directly delivered into the mouth (158), indicating a separate pathway for 'liking' of food.

It has been suggested that the opioid circuit in the ventral striatum is critical for assessing the rewarding value of foods (180-182). The specific role of opioid peptides in generating the 'liking' effects was focused on two types of phenomenon: 1. The manipulation of the limbic opioids circuit activity interfered with the sensing of palatability. Opioids receptors agonists and antagonists were used and the measurement for food palatability was achieved by stereotyped motion and expression in mice or by questionnaire rating in human (183-187). 2. Disruption of the limbic opioid circuit reshaped the preference toward highly rewarding food (183, 188). The μ-receptors in the nucleus accumbens was identified as the essential downstream target for the opioid peptides in sensing the natural food rewards (189-193). Therefore, NAc, expressing both dopaminergic and opioid receptors, acts as a key region to regulate hedonic feeding.

Drosophila Systems

The *Drosophila* CNS response to food reward may also be divided into two circuits representing 'wanting' and 'liking' respectively.

OA neurons are essential in assessing food reward, since they are required during the acquisition stage in olfactory learning when sugar unconditioned stimulus pairs with odor conditioned stimuli (194). Moreover, activation of OA neurons is sufficient to replace natural sugar stimulus to generate conditioned odor preference (194). The OA neurons mediating sugar reward act through Oamb receptors on a cluster of downstream DA neurons during reinforcement. However, general DA signaling is not considered 'rewarding' as it pairs with aversive olfactory learning induced by electric shock stimulus instead (195, 196).

The previous study in our lab identified a cluster of DA neurons required in appetizing odor-induced feeding increase on glucose. Appetizing odor activates the DA neurons but failed to generate a hyperphagia phenotype without the presence of gustatory stimulus, suggesting a role of DA in potentiating the motivation toward food (115).

DA mediated reward feeding requires the D1 receptor and the neuropeptide F (NPF, the invertebrate homolog of NPY) system (115). Invertebrate NPF was first discovered in Monieza expansa and was assigned the name because it replaces the conserved C-terminal Y (tyrosine) in NPY with F (phenylalanine) (197). In *Drosophila*, NPF was identified in 1999 (198) and its receptor NPFR1 was characterized in 2002 (199). Blocking of the NPF/NPFR1 system failed to alter hunger-induced feeding (82), yet disturbed the food quality-related feeding responses. Larvae with NPF deficiency showed a lower resistance to aversive cues, including rough and bitter tasting food and a deleteriously cold environment (200-203). It suggested that the NPF system may play a key role in mediating the motivation to feed and sensing food palatability.

In the following chapters, I will discuss three feeding phenomena and the corresponding mechanisms in *Drosophila* larvae, including a food reward-induced overeating, a hunger-induced feeding activation, and a drug-induced feeding hyper-motility.

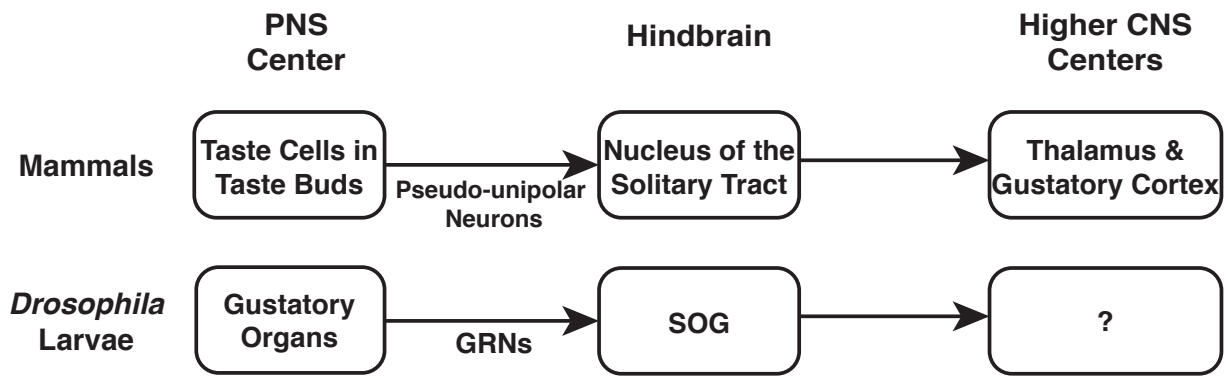


Figure 1.1 *Comparison of the gustatory systems between mammals and Drosophila larvae.*

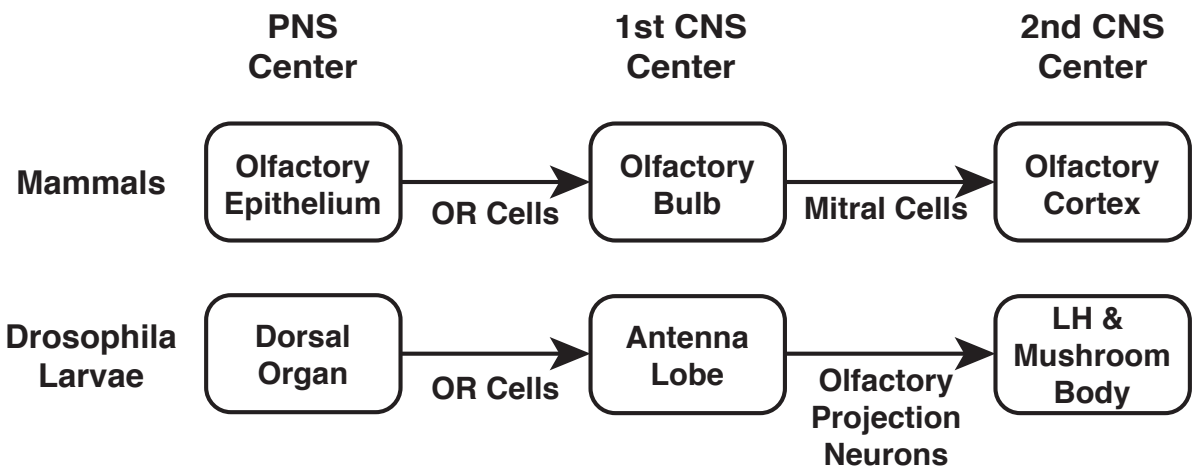


Figure 1.2 *Comparison of the olfactory systems between mammals and Drosophila larvae.*

CHAPTER 2

PERCEPTUAL RECOGNITION OF APPETITIVE ODOR STIMULI IN A *DROSOPHILA* MODEL

2.1 INTRODUCTION

The rewarding value of food contributed by multiple appetitive sensory cues allowed animals to distinguish the proper energy source among the massive environmental messages. However, in the modern world, especially in developed countries where high-calorie foods are abundant, the primitive craving for food can drive overconsumption and cause obesity, increasing the risks for type II diabetes and cardiovascular diseases (204). Appetizing odors, as predictive cues for food, directly regulate food intake (98, 205). Though the molecular and circuit mechanisms for odor detection have been well studied, how the olfactory information is processed in the higher brain centers and assigned rewarding values remains poorly understood.

Drosophila olfactory sensation shares a considerable level of similarity to the mammalian models including the design of the primary peripheral olfaction center (143, 148, 206), the circuit from the peripheral to the CNS projection neurons (122, 147), and the spatial segmentation in the secondary CNS olfactory center (145, 155, 156). The simplicity of fruit fly's nervous system and the powerful genetic tools provide us opportunities to gain valuable insights for the more sophisticated perceptual processes of sensory information.

We have previously identified an appetizing feeding phenomenon in *Drosophila* larvae that mirrored mammalian hedonic feeding behaviors (115). When exposed to a banana like scent,

pentyl acetate (PA), prior to accessing glucose food, larvae displayed an aroused appetitive state by increasing feeding rate and food intake amount. A cluster of four dopaminergic (DA) neurons were found to be essential for this odor-induced feeding arousal, acting as the third order olfactory neurons relaying the incoming olfactory information from the second order projection neurons. In addition, we also showed the involvement of the neuropeptide F (NPF, a fly homolog for mammalian neuropeptide Y or NPY) system in regulating the hedonic feeding toward sensory stimuli. This suggested the conserved neurobiological roles of the neurotransmitters in feeding regulation. As in mammals, the mesolimbic dopaminergic reward pathway is important in food-cues induced feeding (163, 207, 208), and hypothalamus NPY is one of the key orexigenic neurotransmitters in maintaining energy balance (29, 209).

In this work, we investigated the role of NPF neurons in reward feeding on the single cell resolution, and further looked into the downstream NPF receptor neurons response to NPF signaling. We have dissected a complete neuronal circuit from the peripheral sensation of environmental signals to CNS processing of the information, and to executing behavioral reactions. Our findings suggest that studies using the fly larva platform can pave the way for new discoveries of complex neural processes in the mammalian models.

2.2 RESULTS

2.2.1 *Roles of two dorsomedial NPF neurons in odor-aroused feeding*

Our lab has previously showed that the *Drosophila* NPF system is required for odor-induced appetitive feeding response (115). With the anatomical analysis, we found the dendrites of four brain lobe NPF neurons are enriched in the lateral horn region, where the 3rd order olfactory dopaminergic neurons (the DL2 DA cluster) axons project (Fig.2.1A). The presumptive synaptic

connection in the lateral horn region between dopaminergic neurons and NPF neurons were revealed by the GFP Reconstitution Across Synaptic Partners (GRASP) technique (210, 211) (Fig.2.1B). To confirm the functional connection, we knocked down the Dop1R1 (the dopamine receptor functionally involved in odor-aroused feeding) activity in NPF neurons and observed the loss of odor function (Fig.2.1C), suggesting that NPF neurons are downstream of DL2 DA neurons in forming the neural circuit for processing olfactory perception to regulate feeding.

To determine which subset(s) of NPF neurons is required to induce the appetitive arousal, we performed single NPF neuron laser lesioning. Lesioning of the two dorsomedial NPF (dmNPF) neurons abolished odor-stimulated feeding, while the feeding increase persisted in dorsolateral NPF (dlNPF) lesioned larvae. Furthermore, genetic activation of the whole NPF system or with lesioned dlNPF neurons led to increased feeding in the absence of odor stimuli, while larvae with lesioned dmNPF neurons failed to exhibit such a response (Fig.2.1E left). This information demonstrated that the two dmNPF neurons are required for odor-induced feeding response, and are also sufficient to induce appetitive arousal. Knocking down of Dop1R1 activity in NPF neurons failed to block the appetitive arousal induced by NPF activation (Fig.2.1E right), suggesting that the activation of NPF neurons can bypass the upstream excitation from the dopaminergic neurons.

2.2.2 *Cellular responses of two dorsomedial NPF neurons to appetizing odor*

To monitor the cellular response of dmNPF neurons to appetizing odor stimuli, we applied optical electrophysiology by expressing a fluorescent voltage sensor protein Arclight in *NPF-Gal4* neurons (212, 213) (Fig.2.2A). The larval dorsal organ (DO) was exposed to a pump carrying air or PA flow, and Arclight fluorescent intensity change in dmNPF soma was captured by a fast speed camera at the rate of 100 Hz for 20 seconds at different time points.

Depolarization of the membrane potential was coupled with a decrease of fluorescence, while hyperpolarization was visualized by an increase of fluorescent signal. Baseline of the dmNPF soma remained silent when air flow was delivered to the dorsal organ region. After PA was delivered, the dmNPF neuron failed to generate an immediate response, but gradually developed continuous depolarization events after prolonged odor stimuli. The excitatory activity of dmNPF persisted during the 10 min of odor stimulation. After removing the odor source, the dmNPF neuron did not stop firing immediately, but gradually quieted down and returned to baseline level after 5 min (Fig.2.2B). Comparison of the fluctuation and power of the low frequency range confirmed that dmNPF activity after 5 min PA treatment is significantly increased (Fig.2.2C, D).

2.2.3 *sog0NPFR1 as the downstream effector*

Knocking down of the NPF activity in NPF neurons abolished the odor-aroused feeding, suggesting the NPF signaling from dmNPF is required to form the behavioral response (Fig.2.3A). The axon of dmNPF wired through the entire CNS. To understand the impact of regional NPF release, we performed axon lesioning on dmNPF neurons at two different sites: at the beginning of the axon, and right below the subesophagus ganglion (SOG) (Fig.2.3B, C). Cutting off the upstream signaling at the region before the SOG blocked the odor-aroused feeding, however, loss of connection to the ventral nerve cord (VNC) region did not alter the feeding response (Fig.2.3D). Thus, NPF release along the axon in the brain lobes may play a key role in joining the olfactory input and feeding output.

We found that four sog0NPFR1 neurons, a cluster of NPF receptor (NPFR1) neurons adjacent to brain lobe dmNPF axon, are required for odor-stimulated feeding, which likely serves as the downstream target of dmNPF in relaying the olfactory reward signals (Fig.2.3E, F). NPFR1 RNA *in situ* failed label cell bodies (Fig.2.S1). To verify the functional relationship between the

dmNPF neurons and sog0NPFR1 neurons, we performed double lesioning surgeries (Fig.2.3G). In surgery 1, lesioning of one side dmNPF with the corresponding sog0NPFR1 (contralateral side) failed to block the feeding increase, suggesting that one complete circuit of dmNPF and sog0NPFR1 is adequate for odor-aroused appetite. In surgery 2, lesioning of the dmNPF with the ipsilateral sog0NPFR1 mimicked the loss of double side dmNPF or sog0NPFR1, suggesting a unilateral functional connection between the contralateral dmNPF and sog0NPFR1 neurons (Fig.2.3H).

2.2.4 *Morphological mapping of individual sog0NPFR1 neurons*

The sog0NPFR1 cluster includes four cells, with the #4 lateral neuron relatively distal to the #123 neurons (Fig.2.S2A). Their axons leave the CNS through the antenna nerve (AN) and reach multiple locations in the peripheral (Fig.2.S2B-D). Mosaic analyses using the FLP-Out Gal80 technique (210) revealed that dendrites from the sog0NPFR1 neurons are restricted in the local area. Axons from the #123 neurons travel posteriorly after reaching the frontal nerve junction and innervate the esophagus, ending at the ring gland or the foregut region. #4 sog0NPFR1 projects anteriorly and reaches the mouth hook region (Fig.2.4A-D). Immunostaining revealed that #123, but not #4, sog0NPFR1 neurons are serotonergic and likely belong to the enteric nervous system (214) (Fig.2.4E). These findings suggest the sog0NPFR1 may be divided into sub-clusters that contain and release different neurotransmitters and regulate different behavior outputs.

2.2.5 *Functional mapping of individual sog0NPFR1 neurons*

To further understand the functional role of the sog0NPFR1 cluster, we performed laser lesioning on different sog0NPFR1 neurons. We found that the #123 sub-cluster is required for executing the feeding increase, while lesioning of #4 sog0NPFR1 alone failed to abolish the PA-

elicited feeding increase (Fig.2.5A). We found a *GMR29H01-Gal4* line (215)that drives a similar GFP expression pattern in the sog0 region but loses the projection to the mouth hook (Fig.2.5B). With the GFP double expression using *GMR29H01-Gal4* and *NPFR1-Gal4*, we confirmed that the GMR29H01-Gal4 labels three neurons in sog0 region overlapping #123 sog0NPFR1 neurons (Fig.2.5C). Functional knockdown of NPFR1 activity in *GMR29H01-Gal4* neurons abolished the odor-induced feeding increase. This information supports the necessity of #123 sog0NPFR1 neurons, and further argues that the NPF receptor activity is essential in conducting the upstream olfactory effect.

2.3 DISCUSSION

We have identified a CNS circuit for processing the appetizing food odor information and executing feeding behavior adjustments. It comprises a cluster of dopaminergic neurons, a pair of Dop1R1 neurons expressing NPF, and a cluster of NPFR1 enteric neurons expressing serotonin. This provides a *Drosophila* model for studying sensory perception, and may improve our understanding of the complex perceptual interpretation process between the sensory inputs and behavior outputs in mammals.

2.3.1 *The dose-dependent response to single odorant on dmNPF cellular level*

As mammals, fruit flies exhibit a dose-dependent response toward odorants probably due to activation of different olfactory receptor repertoires (216, 217). Behaviorally, fruit fly larvae generated an inverted U-shaped feeding response toward the simple odorant PA (Fig.2.S3A), suggesting a different mechanism under high non-appetizing odor stimulation. To gain insights about cellular response of the NPF circuit under high dosage of odorant, we monitored the membrane potential activity change in dmNPF neurons using Arclight. Except a few large

depolarization peaks immediately following a high dose of PA supply, dmNPF failed to generate repeated excitatory responses during the 10 min of odor application (Fig.2.S3B-D), demonstrating an inverted U-shape cellular response of dmNPF toward odor concentration gradient.

Our study suggested that an inhibitory mechanism, upstream of the dmNPF, was involved when larvae were exposed to a high dosage of PA. The inhibitory effect may reside in the same circuit, on the activity level of the receptor neurons, dopaminergic neurons, or the dopamine receptors. It may also work through a parallel circuit, by counteracting the excitatory signals or blocking the circuit connectivity. On the other hand, how dmNPF activity correlated with the feeding behavior implied that dmNPF is a center for odor-induced reward feeding, as its activity likely defines the status for hedonic feeding.

2.3.2 *Functional roles of the sog0NPFR1 neurons*

Anatomical analysis showed that the axons of the #123 sog0NPFR1 sub-cluster become part of the recurrent nerve (RN) and directly innervate the esophagus. This sub-cluster belongs to the serotonergic enteric nervous system, which, when activated, is sufficient to induce an increased esophagus movement (214). Functional analysis revealed the necessity of the #123 sog0NPFR1 sub-cluster in transducing the positive input from the olfactory circuit to a reinforced feeding outcome. However, the *Drosophila* larvae remained at a normal baseline feeding rate when the signal from this sub-cluster was ablated. We propose that a separate pathway exists for maintaining the baseline feeding activity. In the presence of the incoming appetizing odor, #123 sog0NPFR1 neurons activated the foregut ingestive motility, thus altering the coordinated feeding behaviors.

2.3.3 *The gustatory circuit involved in odor-aroused feeding*

To test the circuit mechanism for odor-aroused appetite, the mouth hook contraction rate was quantified when larvae were fed on the agar media containing 10% glucose. Odor failed to activate the feeding response on the non-sweet food media, suggesting the involvement of a gustatory component (115). Gr43a is currently the only known sugar receptor in Drosophila larvae, and is required for the chemotaxis response toward glucose (111). The expression of this receptor was restricted in two gustatory neurons in the peripheral pharyngeal region, with the axon directly projecting to the SOG region (Fig.2.6A). Inhibition of the Gr43a neurons with the inward-rectify potassium channel Kir2.1 (218) or laser lesioning abolished the odor-induced feeding increase on glucose food, indicating the gustatory sensation of glucose through Gr43a neurons are required for exhibiting the reward feeding phenotype (Fig.2.6B, C).

We found that *NPFR1>GFP.nls larvae* showed a similar expression pattern to Gr43a neurons in the pharynx. The double GFP.nls expression driven by the *Gr43a-Gal4* and *NPFR1-Gal4* indicated that the pharyngeal *NPFR1-Gal4* neurons co-localized with the Gr43a neurons (Fig.2.6D). Functional knockdown of NPFR1 in *Gr43a-Gal4* neurons abolished the odor-aroused feeding (Fig.2.6E), suggesting that the sugar receptor neurons express the NPF receptor, and the odor-induced glucose overconsumption is mediated through the NPF receptor activity in Gr43a neurons.

How glucose works on the Gr43a neurons is not clear yet, since the Gr43a neuron failed to show response toward 10% glucose stimuli in the soma region by calcium imaging (Fig.2.S4A, B). However, these two cells can be directly activated by fructose stimuli, revealed by calcium imaging and optical electrophysiology (Fig.2.S4).

We have noticed that anatomically, the axon terminal of the Gr43a neurons is adjacent to dmNPF axons and sog0NPFR1 neurons. Thus, the SOG region, where the olfactory circuit, the gustatory

circuit, and the feeding executive circuit converged, provides a possible integration center for cross-modal sensory information. The voltage fluorescent sensor Mneon (219) showed that the co-presence of NPF and glucose stimulate Gr43a axon terminal firing (Fig.2.7). It suggested that appetizing olfactory stimuli may potentiate gustatory neurons through the NPF signaling in the SOG region. Combining with previously known information, there is likely a functional connection between the dmNPF neurons and the NPFR1 receptors on Gr43a axon terminal. Interactions between different sensory inputs are also observed in human, such as olfactory or gustatory stimuli can enhance the sensation of each other (220, 221). Drosophila larvae may serve a useful model for further decoding the neural mechanisms for perception of flavor, and other complex cognitive processes.

2.4 MATERIAL AND METHODS

2.4.1 *Fly Strains, Media, and Larval Growth*

The fly rearing and the egg collections were performed as previously described with slight modifications (115, 222). After a 2.5 h synchronized egg collection, eggs were kept in a 12-hour light/dark cycle in an incubator at 25 °C. Larvae were transferred to a fresh apple juice plate with yeast paste at the age of 48–52 h (<80 larvae per plate). 74-76 h old larvae were used for feeding assays. The fly lines used included w^{1118}, UAS-*DenMark* (223), UAS-*syt.eGFP* (224), *TH-Gal4* (225), UAS-*GFP.nls*, UAS-*Arclight* (213), UAS-NPF^{dsRNA}, *hsFLP*, tubP>Gal80>, *Gr43a-Gal4*, *GMR29H01-Gal4* (226), UAS-*CaMPARI* (227), UAS-*kir2.1* (218), and were obtained from Bloomington Drosophila Stock Center at Indiana University. UAS-*Dop1R1* (V107058) was obtained from the Vienna Drosophila RNAi Center. *NPF-Gal4*, *NPFR1-Gal4*, and UAS-

NPFR1^{dsRNA} (228) were described previously. UAS-*CD4::spGFP*$^{1-10}$, LexAop-*CD4::spGFP*^{11} (211) were kindly provided by K. Scott.

2.4.2 *Behavioral experiments*

The rate of larval food intake was quantified by following a previously published protocol with slight modifications (201, 229). 10% glucose food was prepared by dissolving 5g D-glucose (Fisher Chemical) in 45 ml ddH_2O, and then mixing with 6 g agar powder (US Biological). The glucose food was prepared on the same day of the feeding assay, and soaked in room temperature for 4 h before assays to reach an even texture.

The feeding assay was performed in a 35-mm Petri dish containing 0.5 g of food paste. 10 to 20 early third-instar larvae were transferred to the center of the assay plate to settle for 1 min, and then each plate was videotaped for 2 min. The number of MHCs per 30 s was scored and analyzed. All assays were analyzed under blind conditions. At least three separate trials were conducted for each test.

Olfactory stimulation was induced by exposing the 3rd instar larvae to 2 min pre-incubated 7.5 µl pentyl acetate (Sigma) for 5 min. dTrpA1 activation was achieved by pre-feed larvae on 31 ℃ yeast paste for 20 min prior to feeding assays.

2.4.3 *Immunohistochemistry*

Brains from w^{1118} larvae 74-76 h after egg laying were dissected out and immediately fixed in 4% paraformaldehyde for 30min at room temperature. NPF immunostaining was performed as previously described (115, 230). Antibodies used include chicken anti-GFP (1:1,000, Invitrogen), rabbit anti-NPF (1:2,000) (230), and rabbit anti-5HT (1:500, Sigma) (231). Rabbit anti-NPF was incubated with C8 overnight before use. Alexa Fluor-568 goat anti-rabbit (1:2,000;

Invitrogen), and Alexa Fluor-488 goat anti-chicken (1:2000, Invitrogen) were used as the secondary antibodies. Images were collected using a Zeiss LSM 710 confocal microscope.

2.4.4 *Targeted Laser Lesion*

The laser lesion was performed using a previously published protocol with slight modification (115, 232). Early second-instar larvae (52 h after egg laying) were rinsed and transferred to 150 µl double-distilled H2O on a microscope slide, and placed into the anesthetization chamber (90-mm Petri) with 1ml ether. After 2.5 minutes, slides were removed and larvae were covered with a coverslip. NPF/NPFR1 neurons were shown under 40x magnification by a nucleus GFP reporter (UAS-*GFP.nls*). The laser beam was focused on individual nuclei or axon and was applied as a burst of 20 shots at a rate of 3 Hz. Neurons lesioned with laser showed the loss of GFP signal. The group of larvae that went through all the treatments except laser ablation served as the mock group. After the laser treatment, the larvae recovered on fresh apple juice plates with yeast paste for 24 h before the assay.

2.4.5 *Mosaic analysis*

Imaging of individual sog0NPFR1 neurons was achieved by using the FLP-out Gal80 technique (115, 210). *hsFLP; NPFR1-Gal4; tubP>Gal80>/*UAS-*mCD8-GFP* larvae were raised in 25 ℃ to induce random GFP expression. 3rd instar larval CNS tissues were dissected and GFP signal was amplified with immunohistochemistry staining. CNS tissues were then screened for single sog0NPFR1 GFP expression. Images were collected using a Zeiss LSM 710 confocal microscope.

2.4.6 *Calcium imaging*

Monitoring of Gr43a neuron calcium level was achieved by the photo convertible calcium integrator CaMPARI (227). 3rd instar larvae were fed on water, 10% glucose, or 5% fructose for

5 min, and irradiated with the 405 nm LED light array (200 mW/cm2, Loctite) for 5s to activate the irreversible green-to-red color conversion. Individual larva was dissected followed by immediate imaging using a Zeiss LSM 510 confocal microscope. Quantification of green and red signal intensity was achieved by ImageJ. Cell body of Gr43a neurons were manually selected and green-to-red total intensity ratio was calculated.

2.4.7 *Optical electrophysiology and data processing*

NPF>UAS-*Arclight* larvae were used for Arclight imaging of dmNPF neurons after odor excitation. CNS preparation was the same as the calcium imaging previously described (115). The dissected CNS was incubated in Drosophila PBS. Appetizing odor dosage was prepared by incubating 150ul PA for 1 hour in a sealed 20L foam box, which is equal to 11ul in the 1.5L odor chamber. Non-appetizing high odor dosage was prepared by incubating 800ul PA in 20L box. Odor was delivered by pumping to larval head region at a rate of 0.36L/min. 150ul PA was fully vaporized after 1-hour incubation, generating a starting concentration of 6.6mg/L. The odor concentration change after 10-minute odor pumping would be less than 18% in theory. 800ul PA had reached saturated vapor concentration since it had PA liquid residue in the box after 1-hour incubation and also after 10-minute odor delivery.

Gr43a>UAS-*Mneon* larvae were used for monitoring Gr43a soma and axon terminal. For the negative control group, larvae were fed on water for 5 min and tested in HL6 (232) with no sugar. The positive control group was fed on 5% fructose and tested in HL6 containing 240mM fructose. For applying NPF alone, larvae were fed on water and tested in HL6 containing 1 μm NPF. For applying NPF and glucose treatments simultaneously, larvae were fed on 10% glucose containing 1 μm NPF, Mneon intensity was recorded in HL6 containing 240mM glucose and 1 μm NPF.

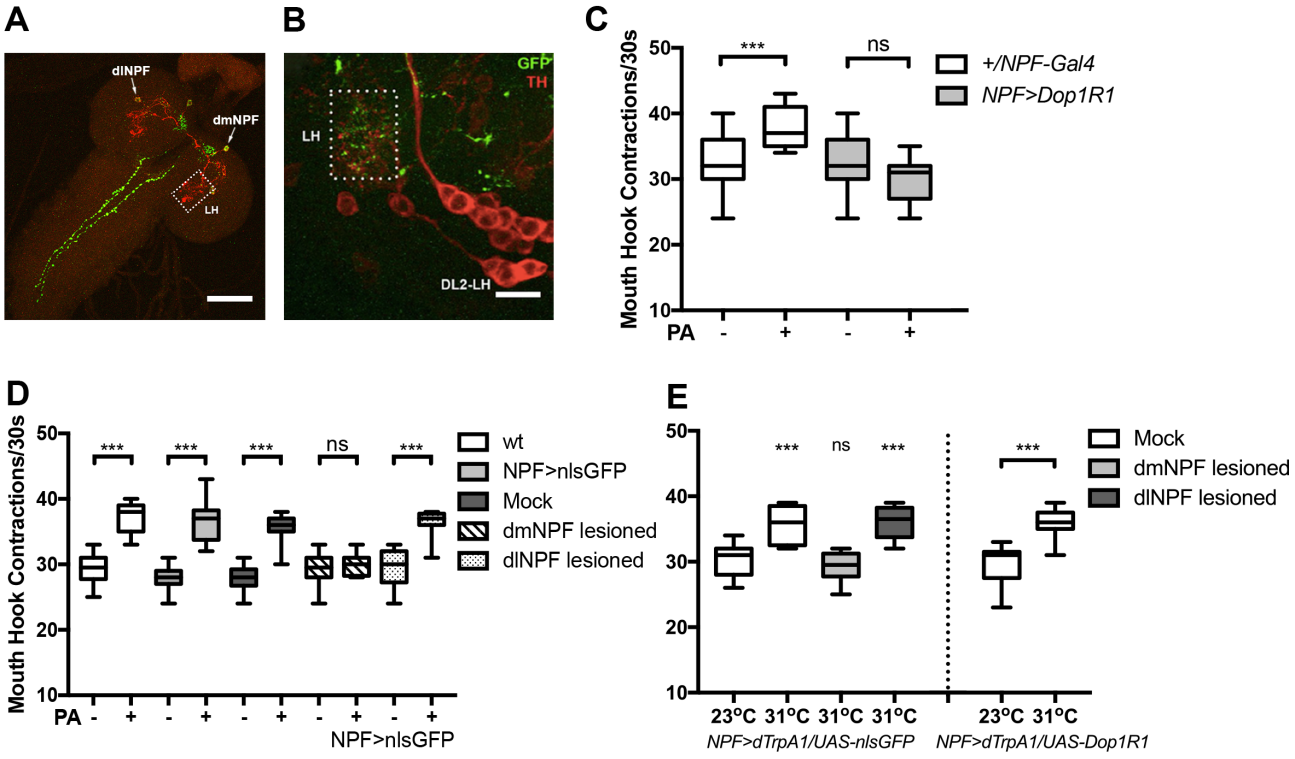
Larval CNS was imaged under 40X water immersion lens using a light microscope (Zeiss Axio Examiner). The NeuroCCD-SM camera and Turbo-SM software (RedShirtImaging) were used to record and output Arclight traces. Images were recorded at a rate of 100 Hz for NPF soma and at 10ms exposure time. At each time point, 2000 frames were continuously collected. For Gr43a neurons, 1000 frames were collected at 250 Hz. The Background was subtracted from all the frames before the average intensity of the regions of interest was documented. 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 by:

$$I_{t,corrected}=I_t+(A-Ae^{(-at)}).$$

$I_{t,corrected}$ was then divided by the average value of $I_{t,corrected}$ of the corresponding time series to normalize each curve. Standard deviation and fast fourier transform were both computed with normalized intensity (212, 213).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Mock
dmNPF lesioned
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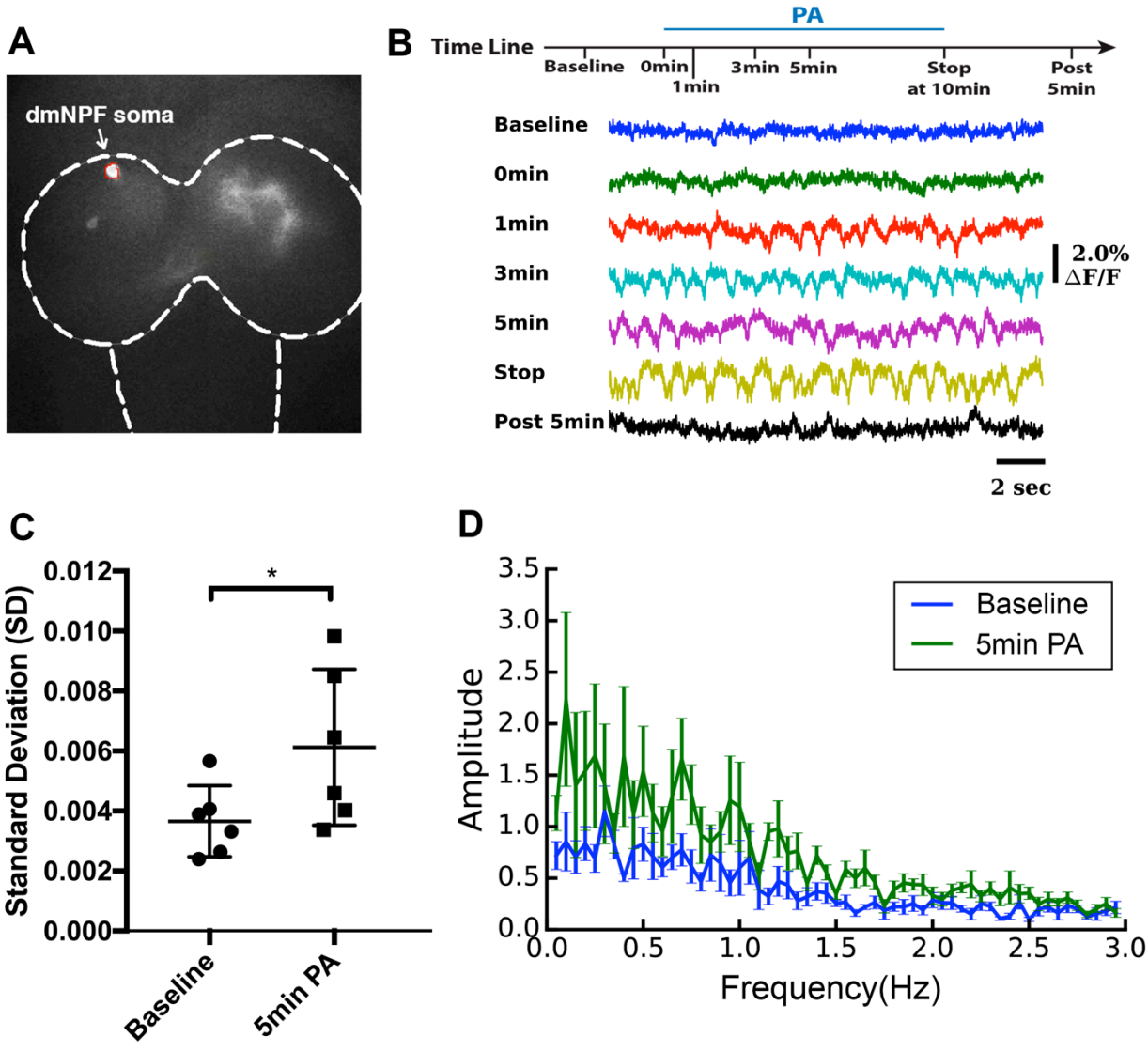
23°C 31°C 31°C 31°C
NPF>dTrpA1/UAS-nlsGFP
23°C 31°C
NPF>dTrpA1/UAS-Dop1R1

Figure 2.1 *Two dmNPF neurons relaying appetizing olfactory signals (the anatomical and functional evidence).* (A) Four brain lobe NPF neurons and their dendritic and axonal regions showed by expressing DenMark and syt.eGFP driven by NPF-Gal4. The dendrites from the dmNPF neurons were found in the lateral horn region (marked by the dotted box). Scale bar=50μm. (B) The presumptive synaptic connection between the DL2 dopaminergic neurons and the dmNPF neurons in the lateral horn region was revealed by the split GFP technique. Genotype: *TH-Gal4*; *NPF-LexA*/UAS-*CD4::spGFP1-10*; LexAop-*CD4::spGFP11*. The lateral horn region is marked by the dotted box (image provided by Yuhan Pu). Scale bar=20μm. (C) Functional knockdown of Dop1R1 activity in NPF neurons abolished odor-aroused feeding response (n≥11, data provided by Yuhan Pu). One-way ANOVA was performed followed by Sidak's multiple comparison test. (D) Lesioning of the dmNPF neurons abolished odor-aroused feeding response. Appetitive feeding persisted in larvae with lesioned dlNPF neurons (n≥14). One-way ANOVA was performed followed by Sidak's multiple comparison test. (E) Genetic activation of NPF neurons by exposing 3rd NPF>dTrpA1 larvae in 31 ℃ for 20 min led to an increase in feeding rate. The excessive feeding was abolished when dmNPF neurons were lesioned, but was not affected by dlNPF lesioning (n≥9). One-way ANOVA was performed followed by Tukey's multiple comparison test. Functional knockdown of Dop1R1 activity failed to block the impulsive feeding driven by NPF activation (n≥17). One-way ANOVA was performed followed by Sidak's multiple comparison test. ***P<0.001.


A
dmNPF soma
B
Time Line
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Baseline
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1min
3min
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Stop
at 10min
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5min
Baseline
0min
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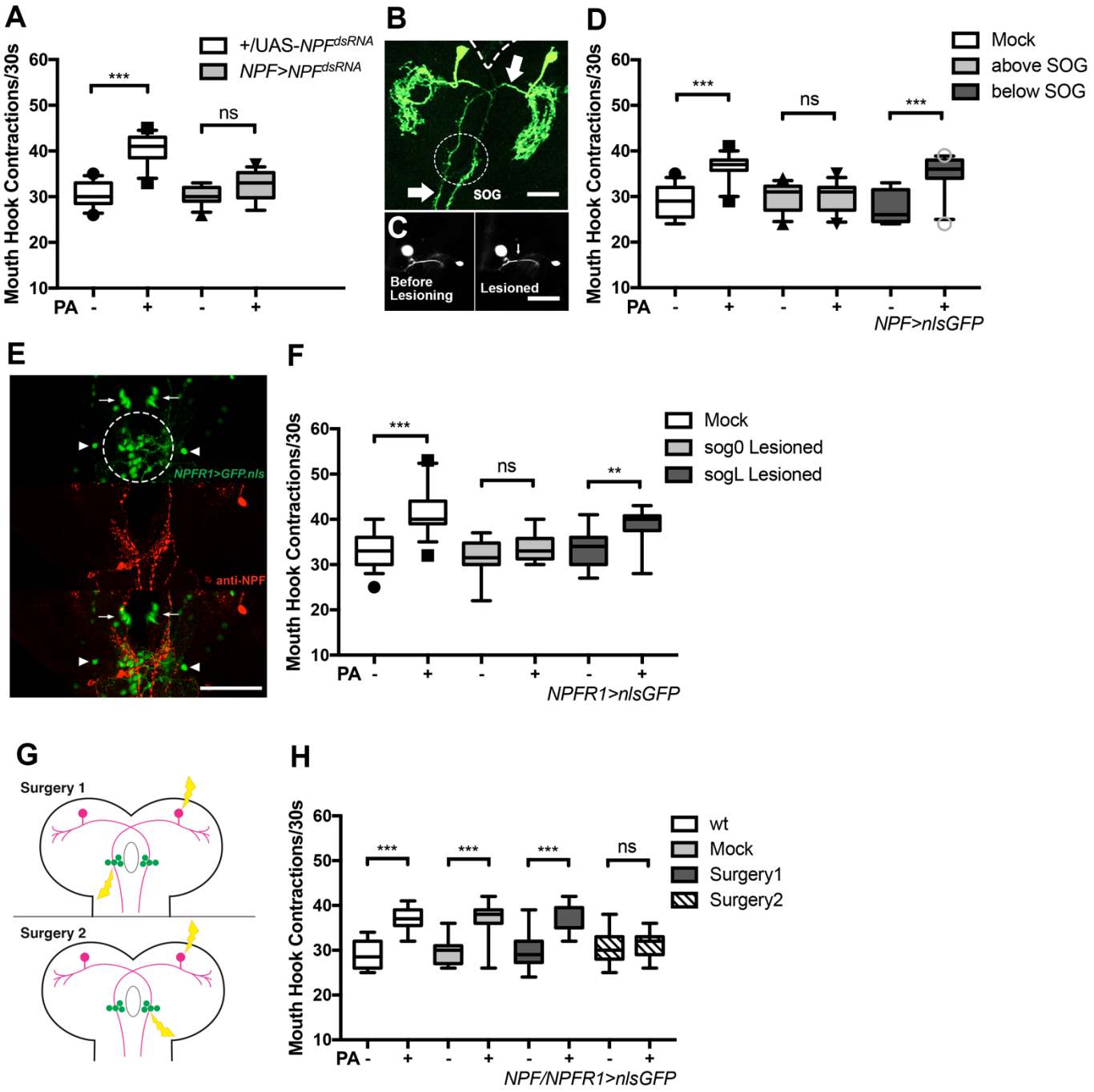
3min
5min
Stop
Post 5min
2.0%
ΔF/F
2 sec
C
Standard Deviation (SD)
0.012
0.010
0.008
0.006
0.004
0.002
0.000
*
Baseline
5min PA
D
Amplitude
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0.0
0.5
1.0
1.5
2.0
2.5
3.0
Frequency(Hz)
Baseline
5min PA

Figure 2.2 *Appetizing odor stimuli activates dmNPF depolarization.* (A) The dmNPF neuron revealed by UAS-*Arclight* expression directed by *NPF-Gal4*. The CNS is outlined by the white dotted line. Other CNS NPF neurons were out of focal plane. (B) 20s of Arclight intensity represented membrane potential change was recorded in the dmNPF neuron starting at different time points during appetitive PA treatments. Normalized membrane potential activities of the representative tissue are shown. (C) Standard deviation (SD) of the membrane potential activity was calculated for the normalized baseline and 5min appetizing PA treated time point (n=6). Mean of the standard deviation after 5min appetitive odor treatment was significantly higher than the baseline activity. Statistical analysis was performed using Paired Student's-t test. *P<0.05. (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. Amplitude after 5 min appetitive dose treatment within the low frequency range (<3 Hz) showed a significant increase compared with baseline. Two-way ANOVA was performed. For appetitive dose treatment, P<10e-12.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+/UAS-NPF^dsRNA
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NPF>nlsGFP
E
NPFR1>GFP.nls
anti-NPF
F
Mock
sog0 Lesioned
sogL Lesioned
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PA
NPFR1>nlsGFP
G
Surgery 1
Surgery 2
H
wt
Mock
Surgery1
Surgery2
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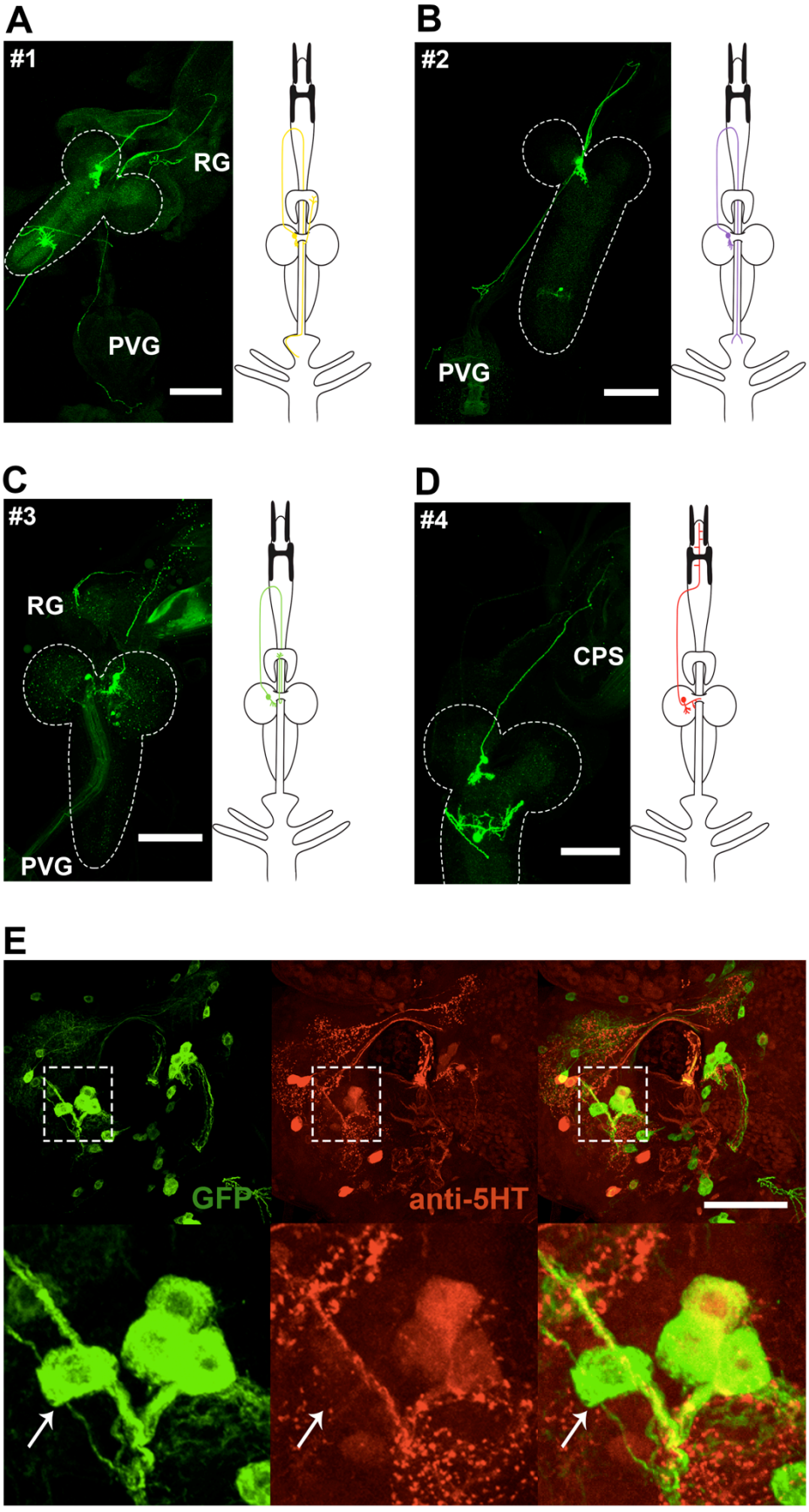
***
***
ns
PA
NPF/NPFR1>nlsGFP

Figure 2.3 *A sub-cluster of SOG NPFR1 neurons as dmNPF downstream target in mediating odor induced feeding.* (A) Functional knockdown of NPF activity in NPF neurons abolished odor-aroused feeding (n≥12). (B) dmNPF axon projects contralaterally and passes through the The SOG region (marked by the white dotted circle). Axon lesion was targeted at two sites pointed by the white arrows. Scale bar=25μm. (C) An example of an axon before and after the laser lesioning. Scale bar=25μm. (D) Lesioning of the dmNPF axon above the SOG region abolished the appetitive response to odor, while lesioning below the SOG region failed to block the odor-induced feeding increase (n≥9). (E) NPF receptor neurons above the SOG region were revealed by expression of UAS-*GFP.nls* directed by *NPF-Gal4*. NPF neurons are labelled by immunohistochemistry with the NPF antibody previously described (230). sog0NPFR1 clusters are indicated by white arrows. SOG lateral NPFR1 neurons are indicated by the white triangles. SOG region is marked by the white dotted circle. Scale bar=50μm. (F) Targeted lesioning of the sog0NPFR1 clusters abolished the odor stimulated feeding response (n≥12). (G) Schematic illustration of the dmNPF/sog0NPFR1 double lesioning. Targeted sites are labelled by the yellow arrows. In surgery 1, contralateral dmNPF and sog0NPFR1 were simultaneously lesioned. In surgery 2, ipsilateral dmNPF and sog0NPFR1 were lesioned. (H) Ipsilateral lesioning of dmNPF and sog0NPFR1 abolished the appetitive response to PA treatment (n≥12). Statistical analysis was performed using One-way ANOVA followed by Sidak's multiple comparisons test. **P<0.01, ***P<0.001.


A
#1
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PVG
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PVG
C
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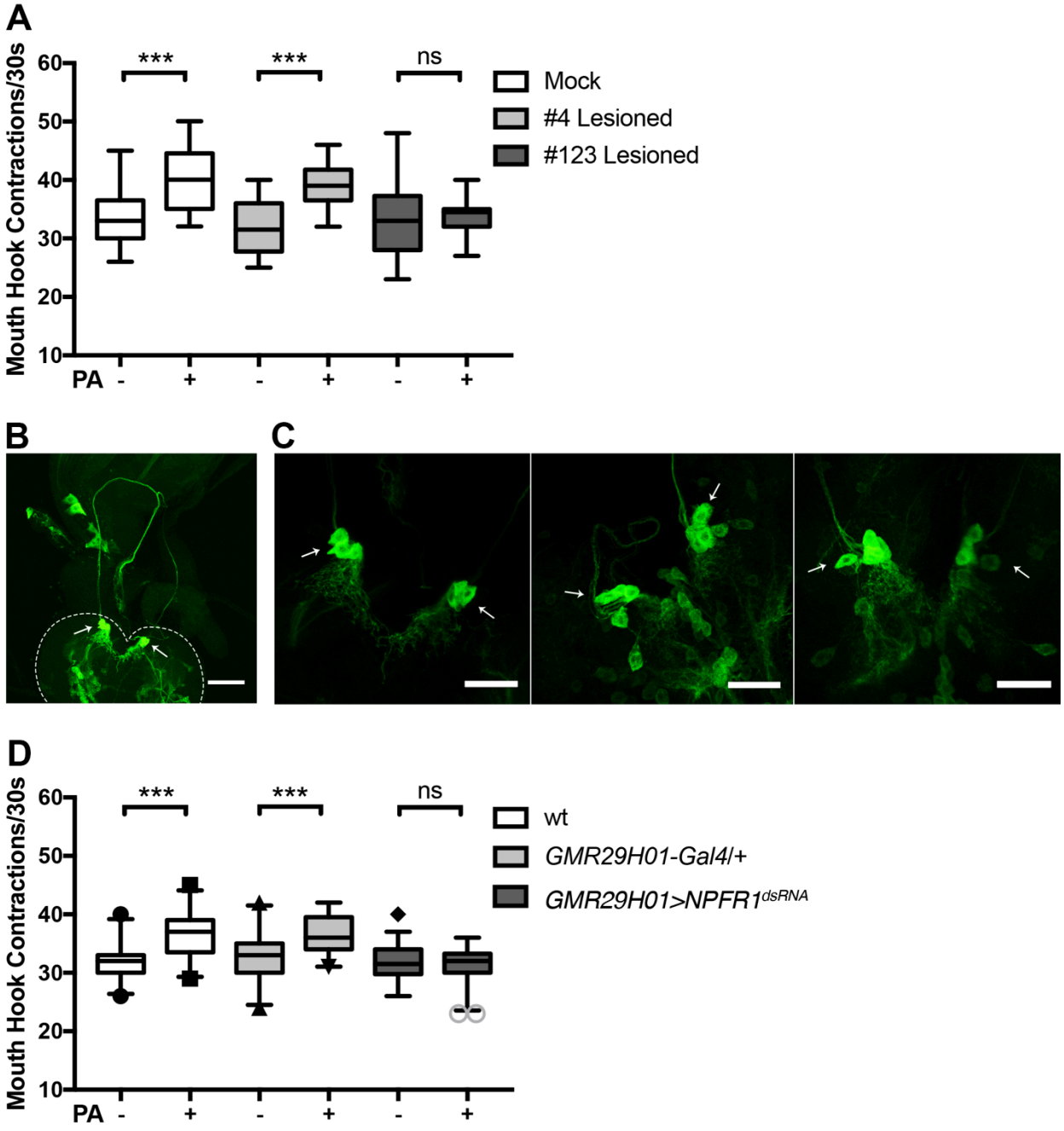
PVG
D
#4
CPS
E
GFP
anti-5HT

Figure 2.4 *Morphological mapping of single sog0NPFR1 neurons.* (A-D) Mosaic analysis of sog0NPFR1 individual neurons using hsFLP;;NPFR1>mCD8-GFP/tub>Gal80>. #1, #2, #3 sog0NPFR1 neurons project axon to front nerve junction and head posteriorly to the ring gland or the proventriculus. #4 sog0NPFR1 neuron projects anteriorly and reaches the mouth hook region. CNS are outlined by white dotted circles. Scale bar=100μm. PVG: proventriculus ganglion; RG: ring gland; CPS: cephalopharyngeal skeleton. (E) sog0NPFR1 co-localization with SOG serotonergic neurons. Green: NPFR1-Gal4 driven UAS-mCD8GFP. Red: anti-5HT revealed serotonergic neurons. Sog0NPFR1 cluster is marked by the white dotted box. #123 sog0NPFR1 neurons are 5HT positive neurons. #4 sog0NPFR1 (pointed by the white arrow) does not show 5HT immunostaining signals. Scale bar=50μm.


A
Mouth Hook Contractions/30s
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PA - + - + - +
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#4 Lesioned
#123 Lesioned
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Mouth Hook Contractions/30s
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PA - + - + - +
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GMR29H01-Gal4/+
GMR29H01>NPFR1dsRNA

Figure 2.5 *Functional mapping of sog0NPFR1 sub-clusters.* (A) Lesioning of the #123 sub-cluster abolished PA induced appetitive feeding response. Single #4 lesioning failed to block the appetizing olfactory effect (n≥12). (B) SOG expression pattern of *GMR29H01>mCD8-GFP* larvae. Nerves of the SOG cluster project to the frontal nerve junction and travel posteriorly. CNS is outlined by the white dotted circle. Scale bar=50μm. (C) Co-localization of #123 sog0NPFR1 neurons and GMR29H01-Gal4 neurons. GMR29H01-Gal4 drives GFP expression in a tight cluster of three neurons. Double expression of mCD8-GFP driven by *GMR29H01-Gal4* and *NPFR1-Gal4* mimicked *NPFR1-Gal4* single expression. Sog0 neurons are indicated by the white arrows. Scale bar=25μm. (D) Functional knockdown of NPFR1 activity in GMR29H01-Gal4 neurons abolished odor-aroused feeding (n≥20, data provided by Yuhan Pu). Statistical analysis was performed using One-way ANOVA followed by Sidak's multiple comparisons test. ***P<0.001.

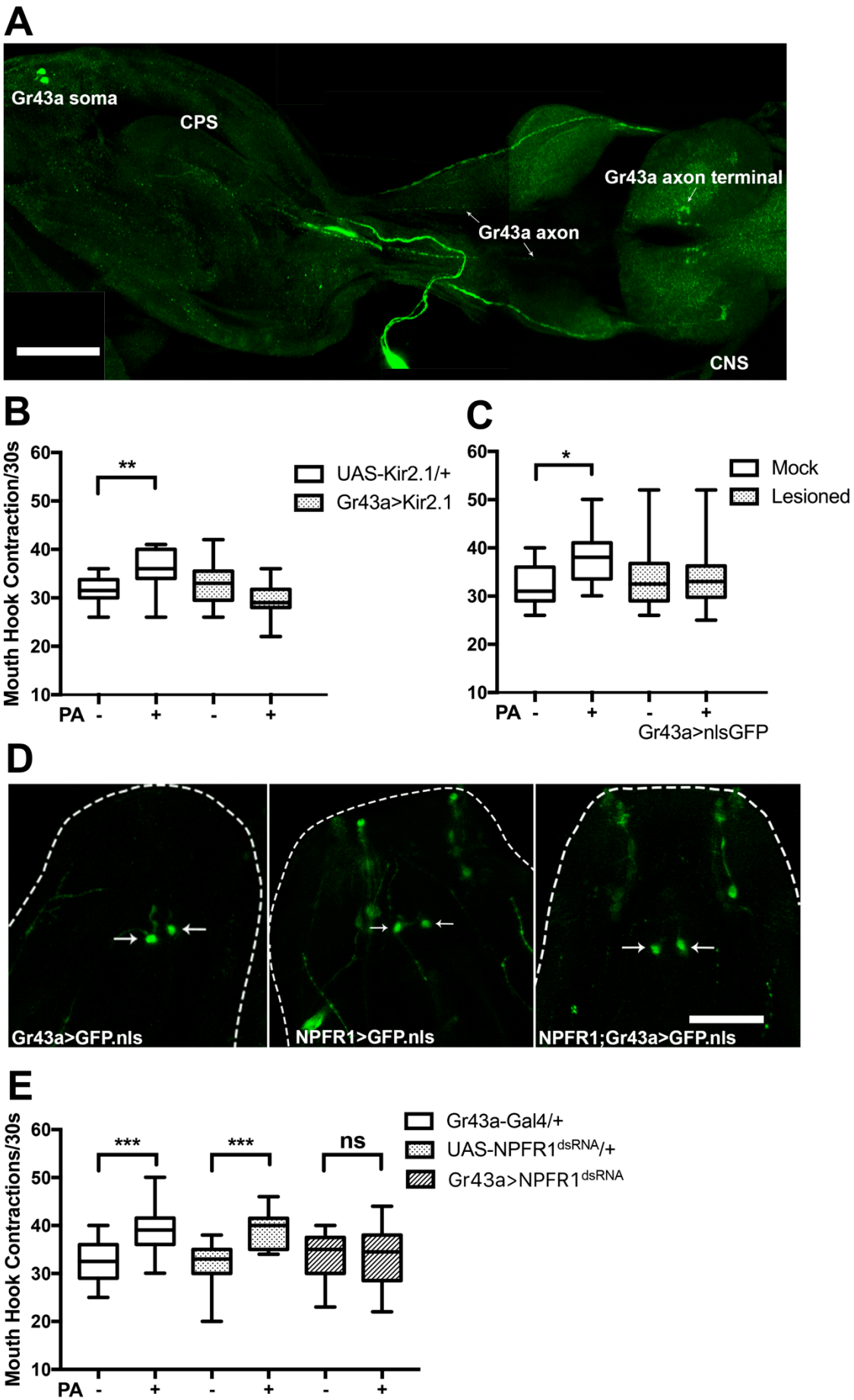

A
Gr43a soma
CPS
Gr43a axon terminal
Gr43a axon
CNS
B
Mouth Hook Contraction/30s
**
UAS-Kir2.1/+
Gr43a>Kir2.1
PA - + - +
C
*
Mock
Lesioned
PA - + - +
Gr43a>nlsGFP
D
Gr43a>GFP.nls
NPFR1>GFP.nls
NPFR1;Gr43a>GFP.nls
E
Mouth Hook Contractions/30s
***
***
ns
Gr43a-Gal4/+
UAS-NPFR1dsRNA/+
Gr43a>NPFR1dsRNA
PA - + - + - +

Figure 2.6 *Gr43a sugar receptor neurons are involved in odor-aroused glucose feeding.* (A) Expression of GFP-mCD8 in *Gr43a-Gal4* larvae. Two Gr43a neurons in the posterior pharyngeal sense organs (PPS) project axon to CNS SOG region. CPS: cephalopharyngeal skeleton. Scale bar=100μm. (B) Inhibition of Gr43a neuronal activity by Kir2.1 blocked odor-aroused appetite (n≥13). (C) Laser lesioning of two PPS neurons abolished odor-aroused appetite (n≥14). (D) Double GFP.nls expression driven by *Gr43a-Gal4* and *NPFR1-Gal4.* Both single Gal4 expression and double Gal4 expression showed two neurons in the PPS organ. PPS neurons are labeled by the white arrow. Scale bar=100μm. (E) Knocking down of NPFR1 activity in Gr43a neurons abolished odor-induced feeding increase (n≥20). Statistical analysis was performed using One-way ANOVA followed by Sidak's multiple comparisons test. *P<0.05, **P<0.01, ***P<0.001.

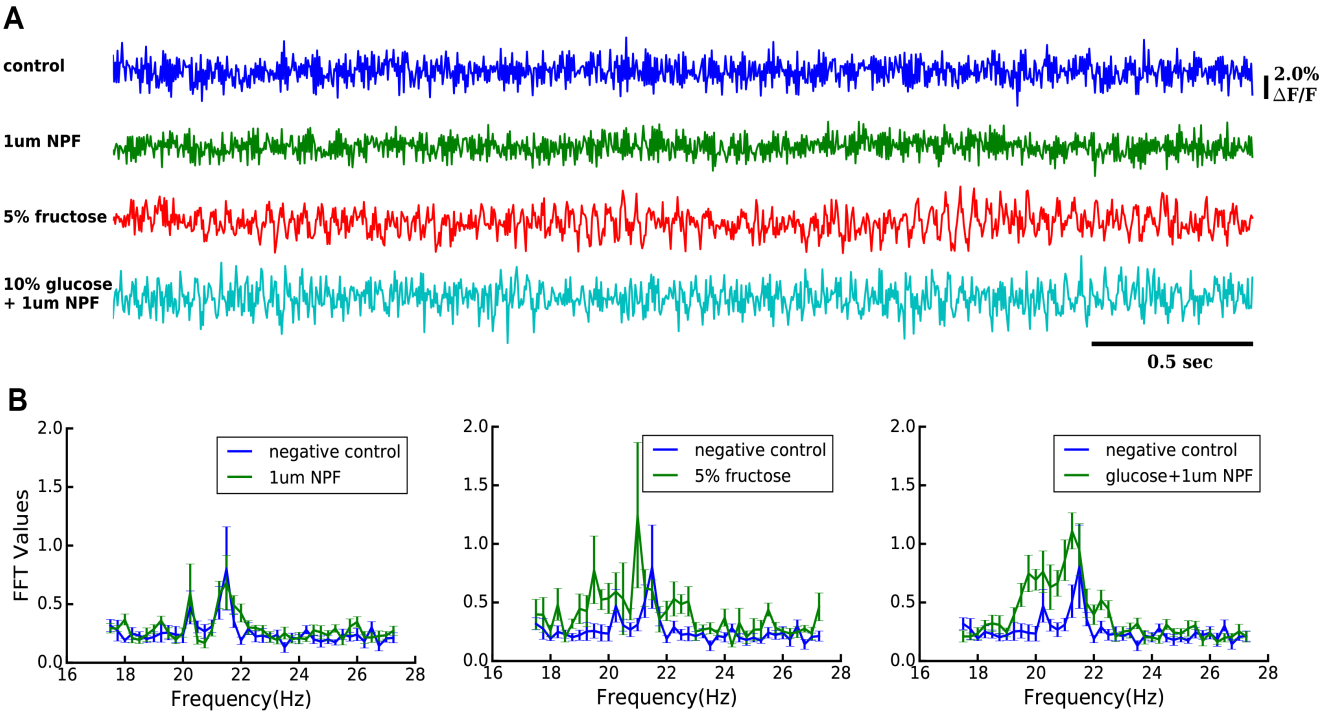

A
control
1um NPF
5% fructose
10% glucose
+ 1um NPF
2.0%
ΔF/F
0.5 sec
B
FFT Values
negative control
1um NPF
Frequency(Hz)
negative control
5% fructose
Frequency(Hz)
negative control
glucose+1um NPF
Frequency(Hz)

Fig 2.7 *Co-presence of NPF and glucose activates Gr43a axon terminal firing.* (A) Mneon intensity in Gr43a axon terminal was recorded under different treatment conditions. Directly adding NPF to axon terminal failed to stimulate axon firing. Pre-feeding of 5% fructose and 10% glucose with NPF both led to axon firing. (D) Power spectrums were calculated and compared between the negative control group and the treated groups. NPF application alone failed to generate a change in axon firing activity. 5% fructose and 10% glucose plus NPF pre-consumption showed increased amplitude around 21 Hz ($n \geq 4$). Two-way ANOVA was performed, $P<0.05$.

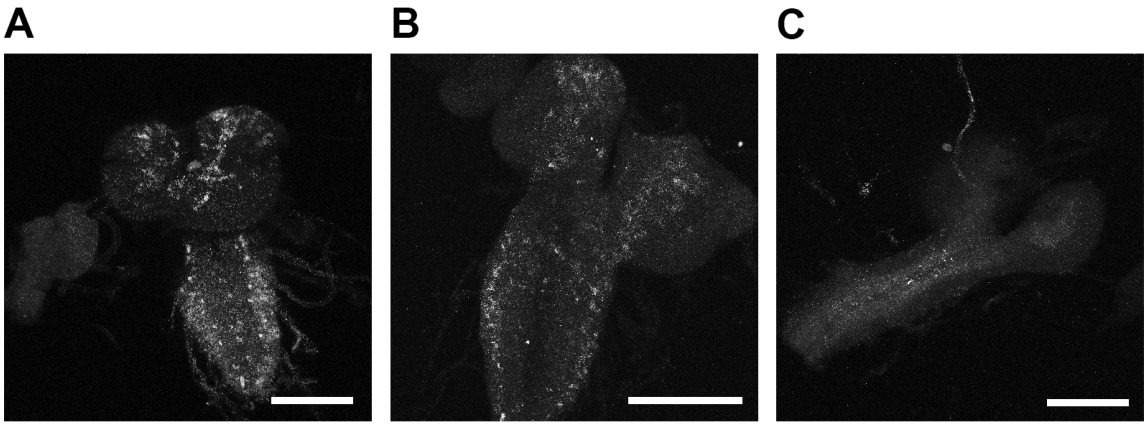

A
B
C

Figure 2.S1 *Visualization of NPFR1 mRNA in the CNS.* (A) NPFR1 RNA in wild type larval CNS. (B) NPFR1 RNA in heterozygous NPFR1 mutant larval CNS. Genotype: KO#50A/+. (C) NPFR1 RNA in homozygous NPFR1 mutant larval CNS. Genotype: KO#50A. Scale bar=100μm.

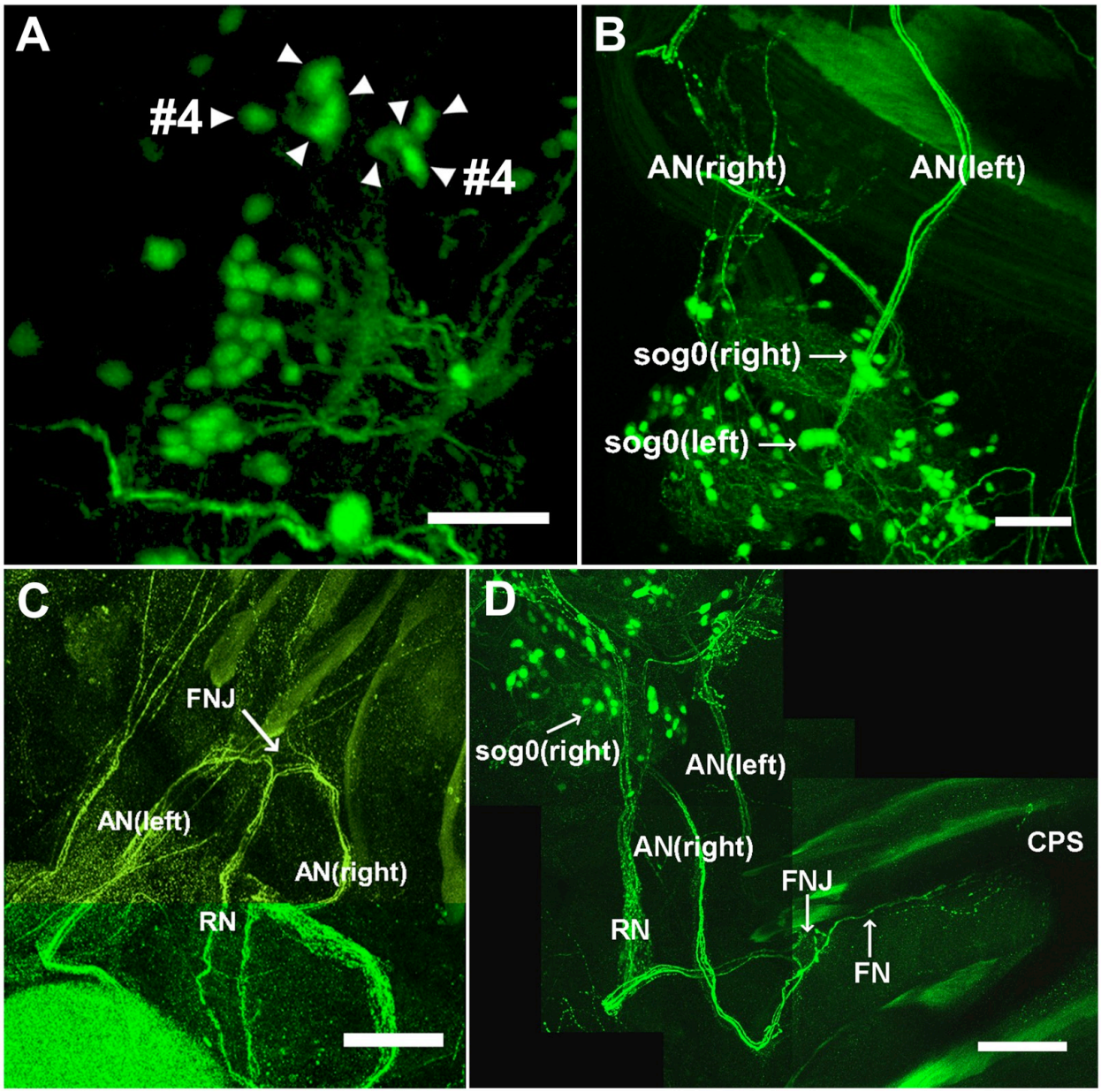

A
#4
#4
B
AN(right)
AN(left)
sog0(right)
sog0(left)
C
FNJ
AN(left)
AN(right)
RN
D
sog0(right)
AN(left)
AN(right)
CPS
FNJ
RN
FN

Figure 2.S2 *Morphology of the sog0NPFR1 cluste.* (A) sog0NPFR1 cell bodies were revealed in *NPFR1>GFP.nls*. Four cell bodies on each side are indicated by the white triangles. Scale bar=50μm. (B, C, D) Axons from sog0NPFR1 cluster exit the CNS through the antenna nerve (AN). After reaching the frontal nerve junction, some axons project posteriorly through the recurrent nerve (RN). One branch further projects anteriorly into the mouth hook region. Genotype: *NPFR1>mCD8-GFP*. (B) Scale bar=50μm. (C) Scale bar=25μm. (D) Scale bar=100μm.

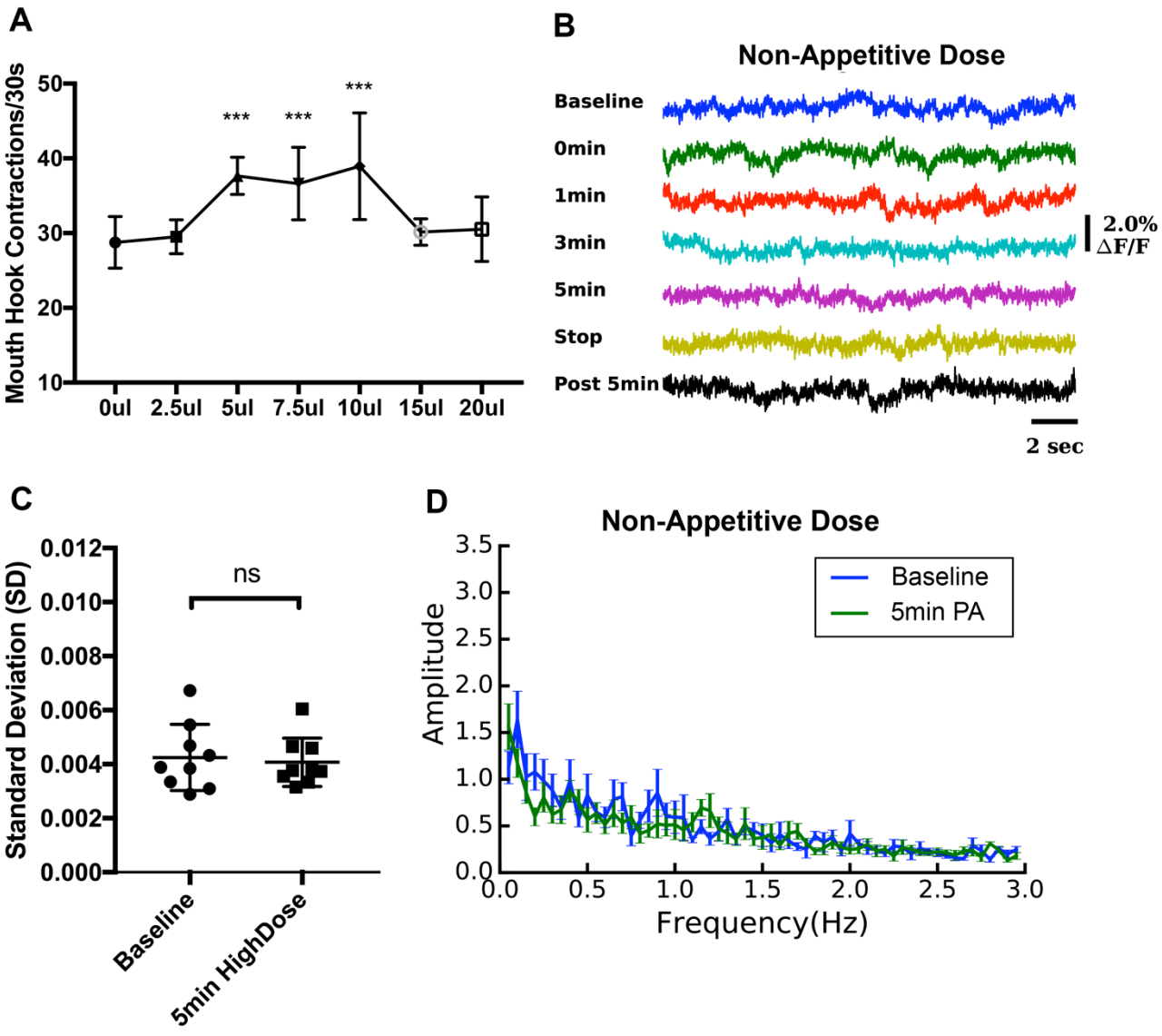

A
Mouth Hook Contractions/30s
50
40
30
20
10
***
***
***
0ul
2.5ul
5ul
7.5ul
10ul
15ul
20ul
B
Non-Appetitive Dose
Baseline
0min
1min
3min
5min
Stop
Post 5min
2.0%
ΔF/F
2 sec
C
Standard Deviation (SD)
0.012
0.010
0.008
0.006
0.004
0.002
0.000
ns
Baseline
5min HighDose
D
Non-Appetitive Dose
Amplitude
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Baseline
5min PA
0.0
0.5
1.0
1.5
2.0
2.5
3.0
Frequency(Hz)

Fig 2.S3 *Behavioral and cellular response to high dosage non-appetizing odor stimuli.* (A) Dose-dependent feeding response toward PA stimuli (n≥19, data provided by Melissa Palombo). Statistical analysis was performed using One-way ANOVA followed by Dunnett's multiple comparisons test. ***P<0.001. (B) dmNPF Membrane potential activity recorded at different time points during high dosage non-appetizing PA treatment. (C) Standard deviation (SD) of the membrane potential activity was calculated for the normalized baseline and 5min non-appetizing PA treated time point (n=9). Mean of the standard deviation after 5min non-appetitive odor treatment was not significantly different than the baseline activity. Statistical analysis was performed using Paired Student's-t test. (D) Amplitude after 5 min non-appetitive dose treatment within the low frequency range (<3 Hz) showed no significant change compared with baseline. Two-way ANOVA was performed.

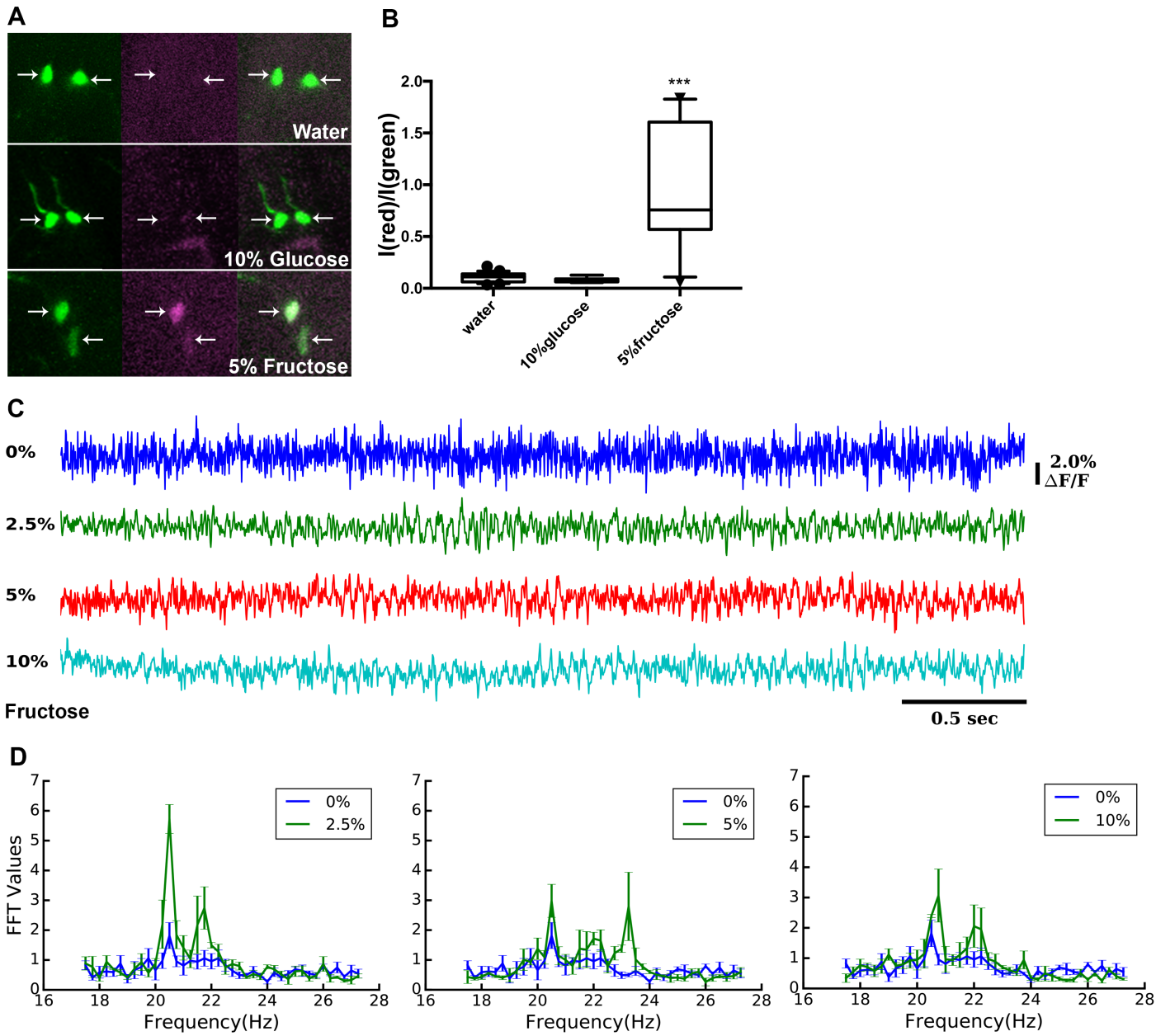

A
Water
10% Glucose
5% Fructose
B
I(red)/I(green)
2.0
1.5
1.0
0.5
0.0
***
water
10%glucose
5%fructose
C
0%
2.5%
5%
10%
Fructose
2.0%
ΔF/F
0.5 sec
D
FFT Values
0%
2.5%
5%
10%
Frequency(Hz)

Fig 2.S4 *Gr43a neurons are selectively responsive to fructose stimuli.* (A) Gr43a neuron calcium level change indicated by the Calcium Modulated Photoactivatable Ratiometric Integrator (CaMPARI). Gr43a neurons were labelled by the white arrows. Larvae fed on 5% fructose showed a positive cellular response, while larvae fed on 10% glucose and water showed negative response. (B) Quantification of red to green ratio in Gr43a neurons ($n \geq 13$). Fructose treated group is significantly higher than the water control group. Statistical analysis was performed using One-way ANOVA followed by Sidak's multiple comparisons test. ***$P<0.001$. (C) Mneon intensity in Gr43a neuron was recorded under different dosage of fructose feeding conditions. 2.5%, 5%, 10% fructose consumption activated Gr43a soma firing. (D) Power spectrums were calculated and compared between the negative control group and fructose treated groups. 2.5%, 5%, 10% fructose treated groups showed increased amplitude around 21 Hz ($n=4$). Two-way ANOVA was performed, $P<0.05$.

CHAPTER 3

GENETIC INFLUENCES ON VULNERABILITY TO SUGAR OVERCONSUMPTION IN A *DROSOPHILA* MODEL

3.1 INTRODUCTION

Sugar is a vital energy source that is highly rewarding. A carbohydrate-rich meal triggers a rapid insulin release that restores blood or hemolymph sugar to the baseline level in both mammals and invertebrates (233-235). However, the regulatory capacity of the insulin system is limited. Long term sugar overconsumption, frequently caused by eating disorders such as binge eating in humans, will likely leads to diabetic disorders (236). At present, our understanding of genetic and neural mechanisms underlying sugar eating disorders remains limited, partly because of the complexity of the nervous system of traditional animal models.

Drosophila larvae are surrounded by readily accessible sugar-rich food most of their lives. These animals appear to regulate their sugar intake and metabolism through two conserved signaling systems. First, our previous study has shown that targeted lesioning of a small subset of norepinephrine-like octopamine (OA) neurons from the larval hindbrain-like subesophageal ganglia (SOG) led to increased feeding of glucose-containing liquid food under well-nourished conditions (237). In addition, an insulin-mediated regulatory mechanism has been identified that is essential for suppressing the surge of blood sugar level (238). These findings have prompted us to propose that *Drosophila* larvae may offer a useful model to investigate genetic influence on the vulnerability to sugar overconsumption.

In this work, we show that the *Oamb* gene, which encodes an α-adrenergic-like receptor for OA, defines a major genetic pathway for preventing sugar overconsumption in well-nourished fly larvae. We also provide evidence that controlled intake of sugar food by larvae in adaptation to energy needs requires coordinated regulation by two distinct OA receptors, each defining a separate neural circuit. Based on these findings, we propose that the noradrenergic-like system defines an ancient regulatory mechanism for prevention of sugar overload.

3.2 RESULTS

3.2.1 *Conditional Knockdown of and OA Receptor Activity Led to Sugar Overconsumption*

The fly genome encodes an α-adrenergic-like receptor Oamb (or Oa1) and three β-adrenergic-like receptors, Octβ1R (or Oa2), Octβ2R, and Octβ3R (239, 240). Given that lesioning of OA neurons in the SOG led to sugar food overconsumption in fed larvae, we decided to probe the potential regulatory roles of OA receptors in controlled sugar intake by conditionally knocking down the activity of each of the four receptors. This was achieved by expressing the double-stranded RNA of each receptor using a mifepristone-inducible pan-neural *GS-elav-GAL4* in fed larvae. We found that functional knockdown of Oamb, but not other subtypes, led to a significant increase in larval feeding response to 10% glucose liquid food (Fig.1), suggesting that the normal neural activity of Oamb is acutely required to prevent sugar overconsumption in fed larvae.

3.2.2 *Genetic Analysis of Sugar Consumption Regulation by Oamb*

We postulate that genetic factors including those related to the Oamb pathway may have major influences on sugar consumption, and genetically tractable fly larva could be useful for investigating underlying genetic mechanisms. To test this hypothesis, we first examined how genetic variations in the Oamb receptor activity might affect larval feeding response to sugar

food. We found that in the presence of the glucose medium, both of the *Oamb* mutant lines tested showed significantly increased feeding responses under fed conditions, phenocopying the *GS-elav-GAL4/*UAS-*Oamb*^{dsRNA} fed larvae (Fig.2). In addition, *elav-GAL4/*UAS-*Oamb*^{dsRNA} fed larvae, which constitutively express the *Oamb* dsRNA in the nervous system, also showed a similar increase in the feeding rate. Together, these findings suggest that a genetic change that results in a reduction in the Oamb pathway activity can have a major effect on the level of sugar consumption.

3.2.3 *Selective Regulation of Sugar/Carbohydrate Consumption by Oamb*

These findings raised the question of whether Oamb-deficient fed larvae display excessive feeding activity in the presence of other types of palatable food. To examine this, we also tested the feeding responses of *elav-GAL4/*UAS-*Oamb*^{dsRNA} fed larvae to liquid media containing 0.5% tryptone or 3% oleic acid. We found that Oamb-deficient larvae showed a normal baseline level of feeding response to the protein- or fatty acid-rich media (Fig.2). Therefore, these results suggest that the Oamb receptor defines a feeding circuit that selectively prevents overconsumption of food enriched in carbohydrate but not protein or fat under well-nourished conditions.

3.2.4 *Functional Mapping of the Neural Oamb Activity*

As a first step towards characterization of the underlying circuit mechanism, we first functionally knocked down *Oamb* activity in genetically defined subsets of neurons previously implicated in the control of feeding behavior under fed conditions (115, 238, 241). However, expression of *Oamb* dsRNA in neurons that produce serotonin, dopamine and insulin-like peptides failed to yield any significant increases in the glucose food response of fed larvae (Fig.3A). Subsequently, we constructed a new *GAL4* driver (*1.6-Oamb-GAL4*) using a 1.6-kb promoter fragment from the

Oamb gene. We found that *1.6-Oamb-GAL4/*UAS-*Oamb*^{dsRNA} fed larvae showed a significant increase in the feeding response, similar to that of *elav-GAL4/*UAS-*Oamb*^{dsRNA} fed larvae (Fig.3A). Using a nuclear GFP reporter, we found that this line predominantly labeled a limited number of neurons in the brain lobes as well as the subesophageal and ventral ganglia (Fig.3B).

3.2.5 *Functional Mapping of the Neural Octβ3R Activity*

Our previous work showed that conditional knockdown of Octβ3R, a β-adrenergic-like OA receptor, in the larval nervous system attenuated hunger-driven feeding response to sugar food (82). To evaluate the functional relationship between the Oamb and Octβ3R circuits, we constructed a *1.8-Octβ3R-GAL4* driver using a 1.8-kb *Octβ3R* promoter fragment. We found that *1.8-Octβ3R-GAL4/*UAS-*Octβ3R*^{dsRNA} larvae failed to show hyperphagic response to sugar food in food-deprived conditions (Fig.4A). Furthermore, this *1.8-Octβ3R-GAL4* directed the GFP reporter expression in two central neurons in the tritocerebrum of larvae that do not overlap with *1.6-Oamb-GAL4* neurons (Fig.4B). Together, our findings suggest that two separate OA subprograms, mediated by distinct subsets of central neurons, underlie the opposite regulatory effects of OA on sugar consumption under different motivation states (satiation and hunger).

3.3 DISCUSSION

We have shown that two of the four OA receptors encoded by the *Drosophila* genome mediate the dual role of the OA system in modulation of feeding of readily available sugar food under different motivational states. An α-adrenergic-like receptor Oamb is acutely required for prevention of sugar overconsumption in fed larvae, while a β-adrenergic-like receptor Octβ3R promotes hyperphagic response to the sugar food. Our findings suggest that the

adrenergic-like system of invertebrate animals is a crucial regulator that links the motivational state to the adaptive consumption of sugar, a vital energy source.

3.3.1 *The impact of genetic deficiencies in the Oamb gene on sugar consumption*

Sugar food preference is known to vary among individuals, and our understanding of how genetic factors contribute to such variations remain limited (242-244). We have shown that functional deficiency of the *Oamb* gene caused significant increases in the sugar food consumption in fed larvae. These results raise the possibility that mutations in an array of genes involved in the OA/Oamb pathway may also have similar effects on sugar food consumption. Therefore, our findings suggest that the fly larva may be a useful platform for investigating the contributions of genetic factors to variations in sugar consumption among individual animals. It would also be interesting to test whether genetic variations that affect the function of norepinephrine system may underlie the genetic predisposition to crave for sugar-rich food in mammals.

3.3.2 *The functional relationship between Oamb and Octβ3R sub-circuits*

Our previous study provided evidence for a potential interaction between the OA/Oamb- and OA/Octβ3R-mediated sub-circuits in modulation of sugar consumption by fly larvae (237). It has shown that two separate subsets of OA neurons (named VUM1 and VUM2, respectively) in the hindbrain-like region are required for the control of sugar food ingestion. Targeted lesioning of VUM1 resulted in sugar overconsumption in fed larvae, while targeted lesioning of VUM2 blocked Octβ3R-dependent, hyperphagic response. Further, targeted lesioning of VUM2 also attenuated Octβ3R-dependent, hyperphagic response to sugar food. However, how VUM1 and VUM2 neurons functionally interact with each other remains unclear. In this work, our evidence supports the notion that VUM1 neurons are acutely active in fed larvae but silenced under

prolonged food deprivation (Fig.5). In fed larvae, VUM1 may indirectly suppress a VUM2-dependent sub-circuit through its signaling to Oamb neurons. It is possible that the VUM1/Oamb neuronal pathway may exert the inhibitory effect on the VUM2/Octβ3R neuronal pathway at the level of the Octβ3R neurons or their downstream targets. Further experiments will be needed to determine how the OA/Oamb and OA/Octβ3R sub-circuits interact to co-regulate sugar consumption under different motivational states.

3.3.3 *Control mechanisms for carbohydrates intake in flies and mammals*

Carbohydrates are vital energy sources to animals across evolution. Despite considerable evolutionary divergence, the control mechanisms for carbohydrate intake in insects and mammals may share similar molecular and neural mechanisms. For example, OA neurons from the hindbrain-like SOG region are known to be associated with sugar sensation in insects. Treatment of OA promotes honey bee's feeding response toward sucrose (245), and is able to increase the reward value of food resources (246). It has also been reported that OA is necessary and can even replace sugar stimuli in forming appetitive olfactory memories in Drosophila (247, 248). Similarly, a group of norepinephrine (the vertebrate counterpart of OA) neurons in the brainstem of rats are responsive to glucose level (249-251) required for regulating carbohydrates-specific food ingestion (252).

It is proposed that precise control of feeding is achieved through different affinities between agonists and different receptors, and the relative activity level of α1 and α2 receptor neurons determines the feeding consequences (253). In rats, antagonistic effects of altering food intake are mediated through different downstream receptor neurons located in the paraventricular nucleus of hypothalamus (253, 254). NE signaling promotes feeding through α1 receptors (255, 256), while its activation of α2 receptors inhibits food intake (257, 258). In *Drosophila* larvae,

we have also identified two separate OA circuits exerting opposite effects in regulating feeding. Similar to mammalian models, two different downstream receptors are found exhibiting antagonistic effects. Both *1.6-Oamb-GAL4* and 1.8-*Octβ3R-GAL4* neurons are present in a larval brain region anterior to the OA neurons. It would be interesting to determine whether this region represents a functional equivalence of the mammalian hypothalamus. Furthermore, satiation status in rats affects an animal's feeding decisions by altering both NE release adrenoceptor levels (23, 259). We postulate that the OA system is also subject to modulation by endocrine hormones and nutrients levels, and it may define a key control site in the central nervous system where multi-sensory integration and feeding regulation takes place.

3.4 MATERIAL AND METHODS

3.4.1 *Fly Strains, Media, and Larval Growth*

The fly rearing and the egg collections were performed as previously described (222). After a 2.5-h synchronized egg collection, eggs were kept in a 12 hour light/dark cycle in an incubator at 25 °C. Larvae were transferred to a fresh apple juice plate with yeast paste at the age of 48–52 h (<80 larvae per plate). The fly lines used included $Oamb^{286}$ (260, 261), $Oamb^{MB00297}$ (262, 263), UAS-*GFP.nls*, UAS-*mCD8-GFP*, *GS-elav-Gal4*, UAS-$Octβ2R^{dsRNA}$, UAS-$Octβ3R^{dsRNA}$, *TH-Gal4*, *VGlut-Gal4*, *TRH-Gal4*, *dIlp2-Gal4*, were obtained from Bloomington Drosophila Stock Center at Indiana University. UAS-$Oamb^{dsRNA}$ (#2861) (264), UAS-$oa2^{dsRNA}$ (#47896) (264) were obtained from the Vienna Drosophila RNAi Center.

3.4.2 *Transgenic Constructs*

A 1.8 kb genomic DNA fragment containing the 5' regulatory region of *Octβ3R* was cloned by PCR with two the primers, 5'-AGGTGACACACACCACATCG-3' and 5'-

CTGAGTCTCGGCCAAGTCC-3'. The Octβ3R-Gal4 construct was made by subcloning the PCR product into the pCaSpeR-Gal4 vector at the EcoR I site.

To construct the Oamb-Gal4 driver line, a 1.6 kb DNA fragment containing the 5' regulatory sequence for the Oamb gene was amplified by 5'-atacatactagaattctctgaaagctgcgggata-3' and 5'-gggcgagctcgaattccggcaagaaccgttagttc-3' and cloned into the pCaSpeR-Gal4 vector at the EcoR I site. The purified construct was injected to w1118 background (BestGene Inc).

3.2.5 *Behavioral Assay*

The rate of larval food intake was quantified by following a previously published protocol with slight modifications (201, 229). 10% (W/W) glucose food was prepared by mixing 45 ml ddH_2O, 5g D-glucose (Fisher Chemical), and 6 g agar powder (US Biological). 3% (V/V) fatty acid food was prepared by mixing 45 ml ddH_2O, 1.4 ml oleic acid (Sigma-Aldrich), and 6 g agar powder. 0.5% (W/W) tryptone food was prepared by mixing 45 ml ddH_2O, 0.23 g tryptone (Sigma-Aldrich), and 6 g agar powder. The feeding assay was performed in a 35-mm Petri dish containing 0.5 g of food paste.

For assays, 10 to 20 early third-instar larvae were transferred to the center of the assay plate, and then each plate was videotaped for 2 min. The number of MHCs per 30 s was scored and analyzed. All assays were analyzed under blind conditions. At least three separate trials were conducted for each test. Statistical analyses were performed using one-way ANOVA followed by Tukey's multiple comparisons test.

3.2.5 *Immunohistochemistry*

Brains from larvae 76 h after egg lay were dissected out and the immunostaining were performed as previously described (115) by using chicken anti-GFP (1:1,000; Invitrogen), Alexa 488-goat

anti-chicken (1:2,000; Invitrogen). Images were collected using a Zeiss LSM510 META confocal microscope.

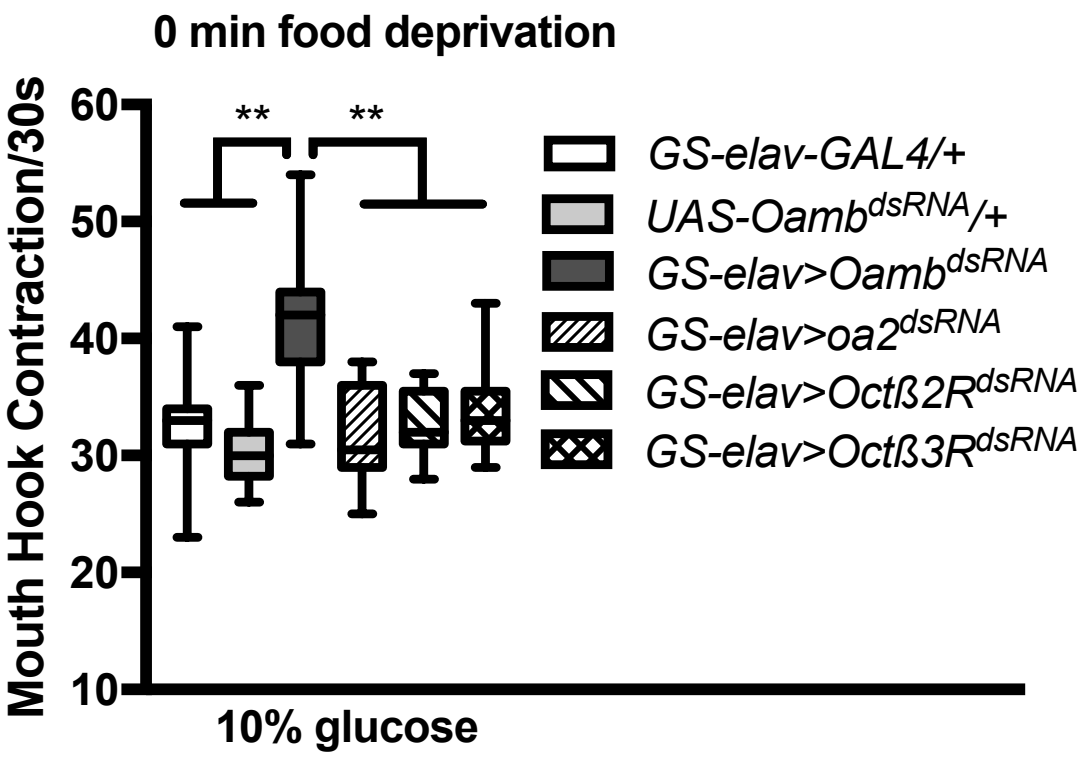

0 min food deprivation
Mouth Hook Contraction/30s
60
50
40
30
20
10
**
**
10% glucose
GS-elav-GAL4/+
UAS-Oamb dsRNA /+
GS-elav>Oamb dsRNA
GS-elav>oa2 dsRNA
GS-elav>Octß2R dsRNA
GS-elav>Octß3R dsRNA

Figure 3.1 *Conditional knockdown of Oamb activity in the nervous system leads to increased feeding of sugar food in well-nourished larvae.* Glucose feeding rate of fed larvae was increased after conditional knockdown of receptor Oamb in the nervous system. For this and other figures, feeding activities were scored under blind conditions, and data were analyzed using One-way ANOVA followed by Tukey's multiple comparisons test. **$P<0.0001$; $n\geq12$.

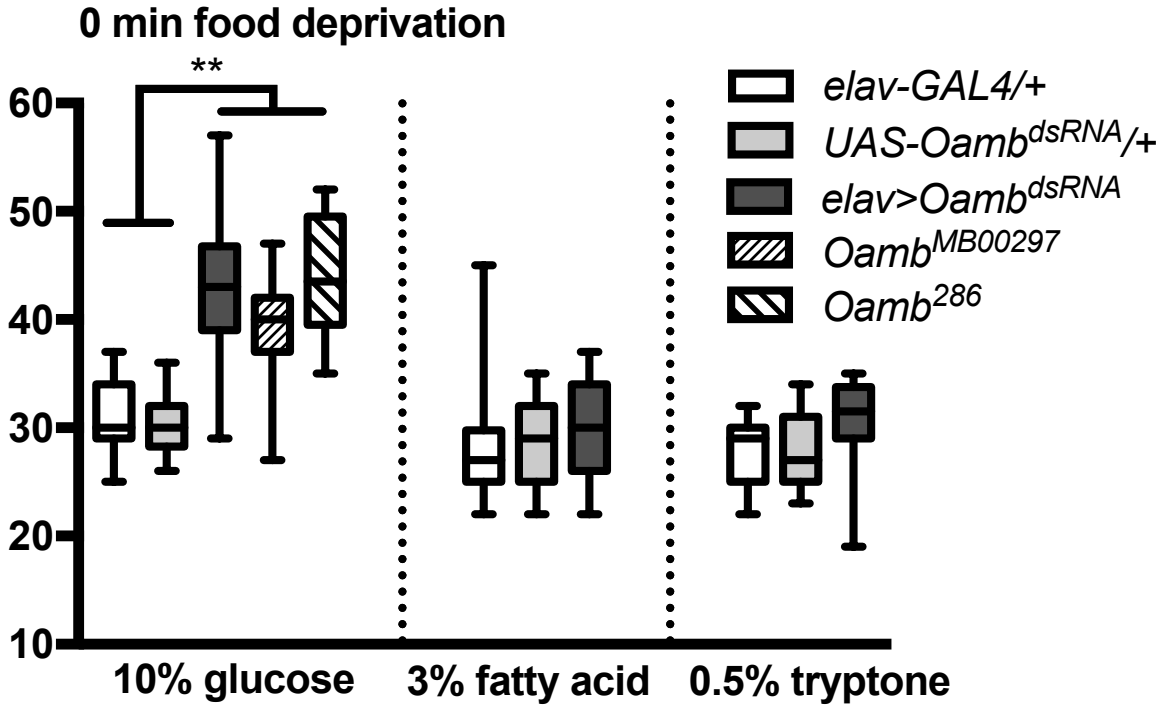

0 min food deprivation
**
60
50
40
30
20
10
10% glucose
3% fatty acid
0.5% tryptone
elav-GAL4/+
UAS-Oamb^dsRNA/+
elav>Oamb^dsRNA
Oamb^MB00297
Oamb^286

Figure 3.2 *Genetic analysis of Oamb activity in well-nourished larvae.* Oamb286 and OambMB00297 (a null and an insertion allele, respectively) showed increased feeding rate in the glucose medium. Pan-neural expression of the double stranded RNA (dsRNA) of Oamb elevated glucose feeding but failed to alter feeding rate on oleic acid and tryptone. **$P<0.0001$; $n\geq12$.

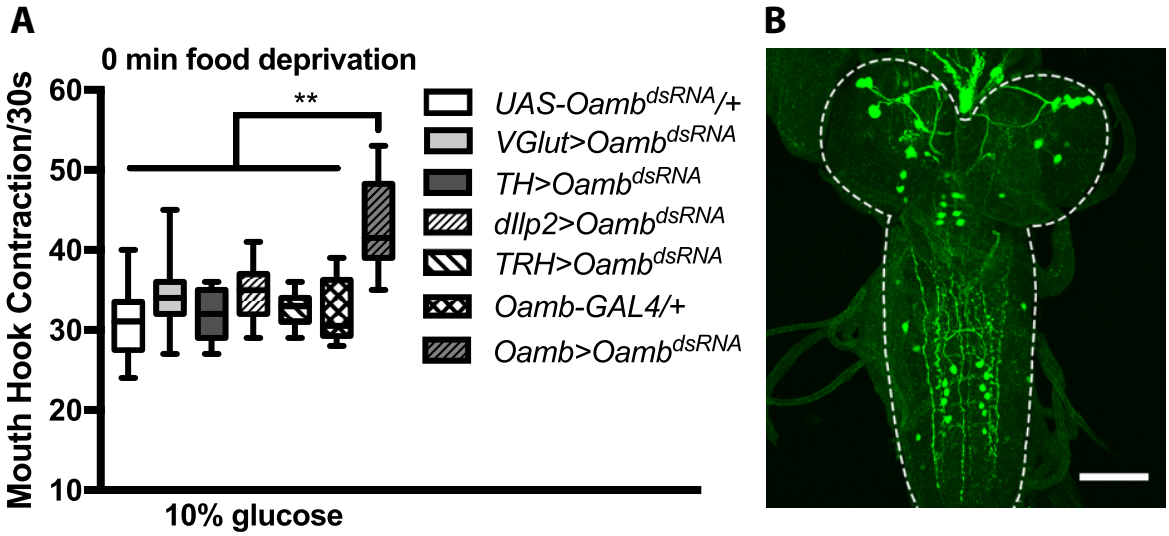

A
0 min food deprivation
Mouth Hook Contraction/30s
60
50
40
30
20
10
**
10% glucose
UAS-Oamb^dsRNA/+
VGlut>Oamb^dsRNA
TH>Oamb^dsRNA
dIlp2>Oamb^dsRNA
TRH>Oamb^dsRNA
Oamb-GAL4/+
Oamb>Oamb^dsRNA
B

Figure 3.3 *Functional knockdown of Oamb receptor activity in various subsets of neurons using different Gal4 drivers.* (A) Oamb-Gal4 driven Oamb knockdown mimicked pan-neural Oamb knockdown. (B) Immunofluorescence of GFP expressed in 1.6-Oamb-Gal4 neurons. The CNS tissue is outlined by white dotted line. Scale bar=50um, **P<0.0001

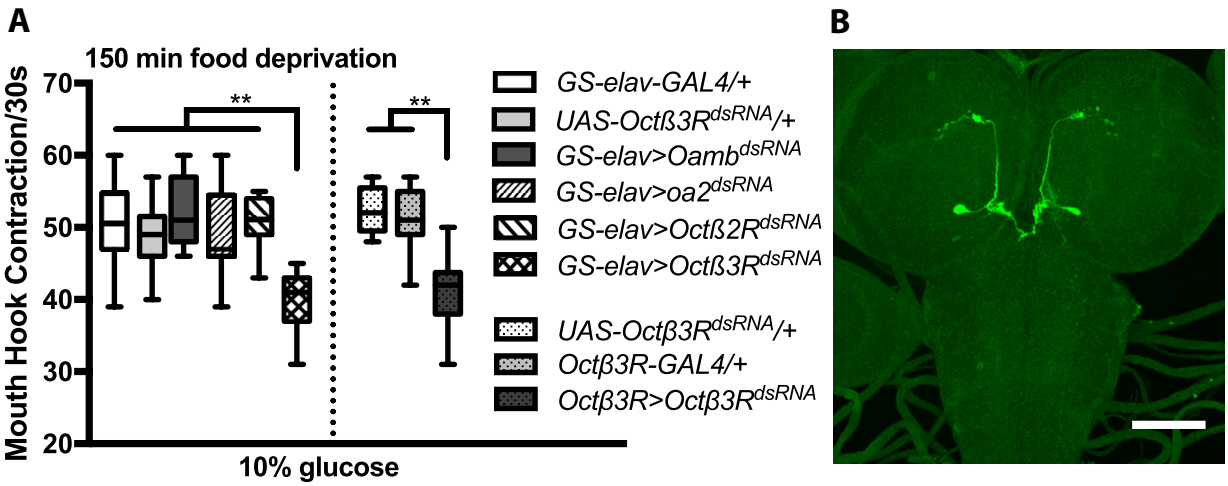
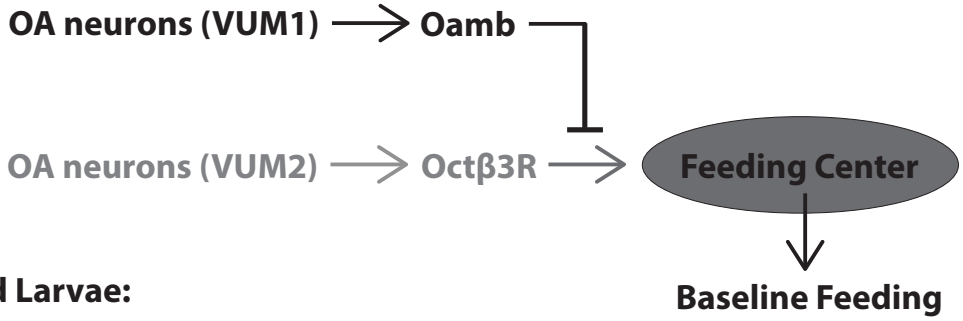

A
150 min food deprivation
Mouth Hook Contraction/30s
70
60
50
40
30
20
**
**
10% glucose
GS-elav-GAL4/+
UAS-Octß3R dsRNA /+
GS-elav>Oamb dsRNA
GS-elav>oa2 dsRNA
GS-elav>Octß2R dsRNA
GS-elav>Octß3R dsRNA
UAS-Octβ3R dsRNA /+
Octβ3R-GAL4/+
Octβ3R>Octβ3R dsRNA
B

Figure 3.4 *Conditional knockdown of Octβ3R receptor activity suppressed hunger-driven increases in sugar consumption.* (A) The rate of glucose feeding in fasted larvae was suppressed after conditional pan-neuronal knockdown of receptor Octβ3R (n≥12). Functional knockdown of Octβ3R in 1.8-*Octβ3R-GAL4* neurons also attenuated hunger-drive feeding in fasted larvae. (B) Immunofluorescence of GFP in 1.8-*Octβ3R-gal4* neurons. Scale bar=50um, **P<0.0001; (n≥9).

Satiated Larvae:



Starved Larvae:

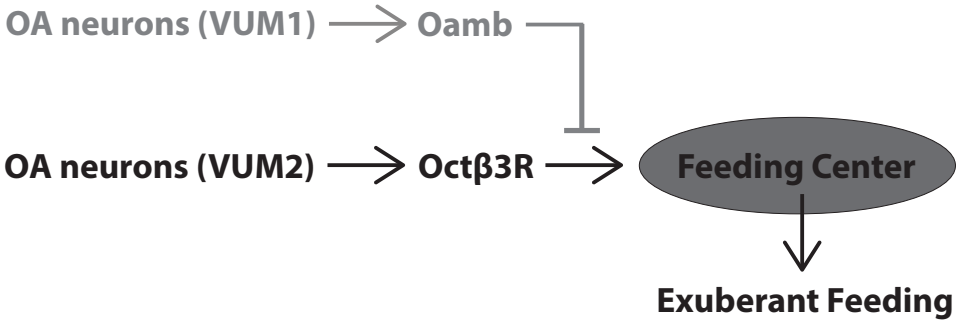


Figure 3.5 *A schematic presentation of a working model for the roles of the Oamb and Octβ3R sub-circuits under different motivational states.* Active neural circuits are in black, and inactive circuits are in light grey.

CHAPTER 4

A *DROSOPHILA* MODEL FOR STUDING STIMULATING EFFECT OF ALCOHOL

4.1 INTRODUCTION

Excessive consumption of alcohol is a world-wide problem, as it creates large burdens on the economy (265) and remains a major threat to health. According to a report from the Centers for Disease Control and Preventions (CDC), in 2001, over consumption of alcohol contributed to 75,766 deaths and 2.3 million years of potential life lost in the United States (266). However, the rewards the brain gains from alcohol keeps generating an increasing amount of needs despite all the well-known negative consequences.

The rewarding effect of alcohol is associated with the mesolimbic dopaminergic pathway in mammals, which serves as the common substrate for many drugs of abuse including nicotine and opiates (161). In humans and rodents, alcohol displays biphasic roles on behavior and physiology dependent on the dosage ingested (267). Before it reaches the sedating dosage, the euphoria and stimulating stage generated by moderate alcohol consumption is considered correlative to the positive reinforcement process in developing addiction (268).

The *Drosophila* model is gaining popularity for alcohol research due to the similar behavioral profile and highly conserved neurobiological mechanisms (269). Adult flies exhibit biphasic responses toward acute ethanol exposure similarly to that in mammals. Ethanol vapor initially induces hyperactivity in fly adults, followed by sedation if the treatment continues (270, 271). Repeated ethanol exposure induces addition-like behaviors in flies, including a developing

preference toward ethanol containing media, increasing tolerance (272, 273), and withdrawal-like symptoms (274, 275). On top of these, it is likely that some conserved neuropeptides and receptors, including dopamine (276), serotonin (277), γ-aminobutyric acid (GABA) (278), neuropeptide Y/neuropeptide F (NPF) (228), and epidermal growth factor receptor (EGFR) (279), play a conserved role in regulating alcohol regulated behaviors in mammals and in *Drosophila*. Here, we have identified a behavior paradigm for studying the stimulating effect of alcohol in *Drosophila* larvae. With the relatively manageable complexity of the larval central nervous system (CNS), we found that two neuropeptide F (NPF) neurons in the brain lobes are essential in mediating ethanol induced feeding motility increase. This may serve as a seed for further understanding the mechanisms of alcohol reward in humans and developing innovative pharmacological interventions for drug addiction.

4.2 RESULTS

4.2.1 Ethanol Pre-consumption Induced Feeding Rate Increase

Moderate alcohol consumption is considered excitatory and leads to increase in locomotor activity in rodents (267, 268). We have previously found that using ethanol as a solvent to deliver drugs to *Drosophila* larvae generated an increased baseline of feeding rate. To test whether ethanol can also exert a stimulating effect in *Drosophila* larvae, we fed larvae 10% ethanol for various amounts of time before quantifying the mouth hook contraction (MHC) rate on 10% liquid glucose food (Fig.4.1A). 3rd instar larvae showed a significant increase of MHC rate after exposed to 10% ethanol for 2 min, 20 min, 30 min, 2 h, 5 h, and 22 h (Fig.4.1B). When hemolymph alcohol level was tested, the effective pretreatment time no longer than 2 h correlates with an increased hemolymph ethanol concentration at 0.03%-0.04%, a level very

close to the euphoria range in human blood alcohol test (280). If ethanol pre-feeding was prolonged, the stimulating feeding behavior persists, however, hemolymph alcohol concentration dropped to baseline. We considered 20 min to 2 h of 10% ethanol feeding as the acute effective dosage, and 5 h, 22 h as the chronic effective dosage as larvae may start to adapt to the ethanol media and develop tolerance.

4.2.2 *Ethanol Effect is Not Mediated Through Olfaction, Gustation, or Hunger*

Larval baseline feeding activity can be enhanced by rewarding sensory cues such as appetizing odor and sugar (115), as well as food deprivation (82). To investigate whether ethanol induced hyper-activity is contributed by the odor of ethanol, we repeated the behavioral experiments with an odorant receptor coreceptor (orco) mutant or83b^{1}. We found the loss-of-function mutant exhibited a similar response curve compared to wild type larvae (Fig.4.2A). When we reduced glucose concentration during the assay stage to 0.5% and 0%, it also failed to abolish the ethanol induced feeding rate increase. This indicates ethanol's effect is independent of gustatory stimuli (Fig.4.2B). In addition, larval feeding rate after 30 min starvation showed a slight increase but not as high as the 30 min ethanol pretreatment group (Fig.4.2C), which failed to explain the feeding rate increase purely with motivational states. Thus, ethanol induced stimulating effect cannot be attributed to any of the previously known factors. Instead, it should be specifically due to the pharmacological effect of the molecule itself.

4.2.3 *CNS NPF Neurons in Ethanol Induced Hyper-motility*

To identify which neurons are involved in ethanol stimulated feeding motility, we expressed a temperature sensitive *shibire* allele (281) in major neurotransmitter Gal4 lines. We found that inhibition of NPF neurons under restrictive temperature abolished ethanol's effect on feeding (Fig.4.3A). (Inhibition of glutamatergic neurons also blocked ethanol induced feeding rate

increase. CNS glutamatergic neurons are not further characterized for two reasons: 1. glutamatergic is one of the most common neuron transmitters in the CNS and generates a busy pattern; 2. a large portion of glutamatergic neurons belong to the motor neuron group (282, 283), which may affect feeding motility in a different pathway.)

To understand whether CNS NPF activity is altered by ethanol, we tested NPF neurons response on different aspects. On the transcription level, we failed to see a change of NPF mRNA level in the CNS. Three transcription variants have been reported for *NPF* gene. We designed intron-spanning primers targeting each variant (Fig.4.S2A), and observed relatively higher transcription levels of variant a and c while variant b is relatively rare in the CNS (Fig.4.S2B). None of the variants showed a change in transcription level after acute or chronic ethanol feeding.

Immunohistochemistry staining showed an increase of NPF expression level in the CNS after 30 min ethanol feeding (Fig.4.3C, D). To investigate whether the increased NPF peptide level is due to activated synthesis or inhibited release, we first visualized and quantified NPF release level by expressing atrial natriuretic peptide fused GFP (ANF-GFP) in NPF neurons (284-286). We verified the reliability of ANF-GFP as a neuropeptide release level indicator by co-expressing a heat-activated cation channel dTrpA1 (287, 288). 10 min, 20 min and 30 min activation of NPF neurons correlated with a significant decrease of GFP level (Fig.4.S3). When comparing ANF-GFP level in untreated and treated larvae, we learnt that 30 min ethanol feeding resulted in a decrease of GFP, indicating an activation of NPF release (Fig.4.3E, F). Based on an increased release level and an increased remaining peptide level, we concluded that both NPF synthesis and release are activated during ethanol feeding.

4.2.4 *Functional Analysis of Single NPF Neuron*

There are two pairs of NPF neurons in the brain of 3rd instar larvae, dorsal medial NPF (dmNPF) and dorsal lateral NPF (dlNPF) (Fig.4.S2C). To determine which subset of NPF neurons are responsible for ethanol induced hyper-motility, we applied laser ablation on targeted NPF pairs. We found that lesion of the dmNPF pair mimicked the inhibition of the whole NPF system, suggesting that dmNPF neurons are required for ethanol induced hyper-motility. While dmNPF dendrites anatomically overlaps dlNPF axons, the rest of dmNPF can be divided into five separate regions (Fig.4.S4). To test local influence of ethanol on dmNPF neurons, we quantified NPF peptide and NPF release level in different regions of dmNPF. We have noticed an overall decrease of ANF-GFP and increase of NPF expression (Fig.4.4B, C), however, the SOG axon region exhibited the largest change in ANF-GFP release (56% GFP intensity decrease) and NPF level increase (6.4-fold increase). These findings suggested that ethanol serves as a stimulant for dmNPF on the levels of neuronal and molecular activity. However, we cannot conclude whether the uneven local response is due to a regional activation effect of ethanol or a re-distribution of neurotransmitters.

Whether transcription level in an individual NPF neuron is altered may be achieved by RNA *in situ* hybridization. We have successfully visualized NPF mRNA in individual neurons with the RNAscope technique (ACDbio) (Fig.4.S2C). Further studies are required to quantify single cell NPF transcription level between 0 min and 30 min ethanol feeding groups.

4.3 DISCUSSION

We have discovered that short term feeding on 10% ethanol leads to an increase in feeding motility, and this stimulating effect is mediated through activating two dorsal medial NPF neurons in the brain lobes. Like *Drosophila* adults, *Drosophila* larvae can also be used as a

model to study the stimulating effect of ethanol. The relative simplicity of larval nervous system enabled us to apply precise control and study the circuit mechanisms on single neuronal level.

4.3.1 *A Conserved Role of NPF/NPY in Flies and Mammals*

In mammals, neuropeptide Y (NPY, the vertebrate homolog of NPF), is an abundant neuropeptide distributed widely in the CNS (20, 21). It is known as an anxiolytic peptide (38, 289, 290) and is considered closely related to alcohol-dependence. NPY expression was significantly higher in rat strain selected for high alcohol preference compared to non-preferring rats (291). In addition, NPY mutant mice showed higher alcohol consumption and resistance, while mice overexpressing NPY showed an opposite trend compared to wild type (292). NPY neurons essential for suppressing alcohol binge drinking were later targeted to the amygdala (25, 293, 294), a brain structure involved in emotional reactions. As injection of NPY to the amygdala region brought ethanol intake amount to baseline level in alcohol preferring rats (295), it is likely that the initial NPY expression level in the amygdala determines the alcohol dependence in an individual animal.

Our previous studies showed that the fly NPF system displayed a conserved anxiolytic-like role, as larvae with NPF deficiency showed a lower resistance to aversive cues including bitter food and deleteriously cold environment (203, 228, 238). NPF is also involved in regulating neurobiological response to ethanol. Adult flies deficient in the NPF system showed decreased ethanol sensitivity (228, 270, 277). Interestingly, sexual deprivation in male flies reduced NPF expression, which in turn triggers enhanced alcohol preference (296). One possible explanation is that feeding on ethanol exerts anxiolytic effect by activating NPF synthesis and release, therefore, lowered stress induced by mating deprivation.

In mammals, ethanol may stimulate feeding behaviors since alcohol preferring rats showed an increased NPY level in the hypothalamus (271), where NPY plays an orexigenic role. Our findings suggested the fruit fly NPF system can also be conserved in regulating the stimulating effect of ethanol. Further investigation on detailed mechanisms of alcohol induced euphoria-like states in *Drosophila* larvae may provide novel insights for understanding the positive reinforcement process and addictive effect of alcohol.

4.3.2 *Ethanol Induced Feeding Rate Increase is Independent of Food Ingestion Increase*

Whether alcohol plays an orexigenic or anorexigenic role has long been controversial in mammalian studies (297-302). Previously, our lab has shown that feeding rate increase driven by hunger and appetizing odor correlates well with food intake amount increase (82, 115). To test whether ethanol pre-consumption also leads to an increase in food intake, we measured the amount of food ingested by feeding larvae erioglaucine disodium salt (a blue food dye) with the liquid glucose media. After 30 min 10% ethanol pretreatment, the food amount larvae ingested during the test stage was significantly decreased (Fig.4.S5). When we fed larvae with a lower concentration of ethanol of 2% and 5% instead, neither an increase or decrease in food intake was observed. Whether ethanol exerts an appetizing effect under certain conditions needs to be carefully investigated with different pretreatment time, concentrations, and various food media. What we can conclude from the known information is that ethanol may disrupt another pathway in regulating food ingestion.

Previous work on the *Drosophila* larvae feeding motor system has shown that food approaching and food ingestion activities are controlled by different muscle groups innervated by different nerves (303-305). The scooping motion is achieved by mouth hood elevator (MHE) and mouth hood depressor (MHD), the mouth hood extension and retraction is controlled by the labial

retractor (LR), both of which are innervated by the maxillary nerve (MN). The sucking of food into the esophagus is achieved by a group of cibarial dilator muscles (CDM), which are innervated by the antennal nerve (AN). The antennal nerve also innervates the enteric system which controls the esophagus and gut movement, thus essential for food ingestion (214). Under regular conditions, MN and AN, food approaching and food ingestion, should be coordinated in order to fulfill complete feeding cycles (303). The inconsistent change toward ethanol pretreatment showed these two aspects of feeding behaviors can be isolated and independently regulated. Ethanol might stimulate MN while inhibiting AN activity. To verify this hypothesis, electrophysiology or optical electrophysiology monitoring single nerve responses toward ethanol needs to be done.

4.4 MATERIAL AND METHODS

4.4.1 *Fly Strains, Media, and Larval Growth*

The fly rearing and the egg collections were performed as previously described with slight modifications (115, 222). After a 2.5 h synchronized egg collection, eggs were kept in a 12-hour light/dark cycle in an incubator at 25 °C. Larvae were transferred to a fresh apple juice plate with yeast paste at the age of 48–52 h (<80 larvae per plate). 74-76 h old larvae were used for feeding assays. The fly lines used included w^{1118}, $Or83b^{1}$ (306), UAS-$shibire^{ts1}$ (281), *NPF-Gal4* (228), *Th-Gal4* (225), *Tdc2-Gal4* (307), *VGlut-Gal4* (308), *Trh-Gal4* (309), UAS-*GFP.nls*, and were obstained from Bloomington Drosophila Stock Center at Indiana University. *NPF-Gal4* (228), UAS-ANF-GFP (284) were described previously.

4.4.2 *Quantification of Feeding Rate*

The rate of larval food intake was quantified by following a previously published protocol with slight modifications (201, 229). 10% glucose food was prepared by dissolving 5g D-glucose (Fisher Chemical) in 45 ml ddH_2O, and then mixing with 6 g agar powder (US Biological). 0% glucose food was prepared by mixing 48 ml ddH_2O and 6 g agar powder, 0.5% glucose food was prepared by mixing 48 ml ddH_2O, 0.25g D-glucose, and 6 g agar powder. Each food was prepared on the same day, and soaked in room temperature for 4 h before assays to reach an even texture.

The feeding assay was performed in a 35-mm Petri dish containing 0.5 g of food paste. 10 to 20 early third-instar larvae were transferred to the center of the assay plate to settle for 1 min, and then each plate was videotaped for 2 min. The number of MHCs per 30 s was scored and analyzed. All assays were analyzed under blind conditions. At least three separate trials were conducted for each test.

4.4.3 *Ethanol Pretreatment*

To prepare the 10% (W/W) ethanol food, 2 g of dry yeast powder was added to 3.2 ml boiling water and mixed immediately to prepare the yeast paste. 0.8 ml 99.5% ethanol (Pharmco-Aaper) was added after the yeast paste cooled down to room temperature. To prepare 0% ethanol, 2% ethanol, 5% ethanol containing yeast paste, the ethanol amount added was 0ml, 0.16 ml, 0.4ml. ddH_2O amount used was changed to 4ml, 3.84ml, 3.6ml, accordingly. 40 µl red food dye was added to each food paste to visualize the food ingested into the gut of *Drosophila* larvae. Larvae were added onto the yeast paste at different time points before the behavioral assays. For 5 h ethanol pretreatment, fresh ethanol food was provided every two hours, and for 22 h long-term ethanol pretreatment, fresh ethanol food was provided at 22 h, 8 h, 6 h, 4 h, 2 h before the

behavioral assays to minimize ethanol concentration change caused by consumption and evaporation.

4.4.4 *Quantitative Real Time RT-PCR*

20 brains from w^{1118} larvae 74-76 h after egg laying were dissected out into a 1.5ml tube. The procedures of RNA extraction, first-strand cDNA synthesis, and qRT-PCR were described previously (270). The relative RNA quantification was normalized against *rsp17*.

Different forward primers were designed for three variants of NPF mRNA. Variant a: 5'-cagttgaa ccagaactatgtgcc-3'; Variant b: 5'-agaacgaattcagaactatgtgcc-3'; Variant c: 5'-aactcccagttgaaccagc c-3'. A shared reverse primer was designed: 5'-ttgacatcgttctttcgcgg-3'. Real time RT-PCR and data analysis were carried out with 7500 Real-Time PCR System from Applied Biosystems.

4.4.5 *NPF Immunohistochemistry and Quantification*

Brains from w^{1118} larvae 74-76 h after egg laying were dissected out and immediately fixed in 4% paraformaldehyde for 30min at room temperature. The immunostaining was performed as previously described (115, 230). Rabbit anti-NPF was incubated with C8 overnight before use, and Alexa568 goat anti-rabbit (1:2,000; Invitrogen) was used as secondary antibody. Images were collected using a Zeiss LSM 710 confocal microscope.

Tissues to be quantified were processed and stained in parallel. Quantification of IHC intensity was achieved by ImageJ. A threshold of 30 was set to exclude the background noises (255 as the maximum intensity). Pixels with intensity above 30 were considered positively stained areas. Average intensity and total positive area were recorded for whole CNS tissues and local areas. For local NPF quantification, dmNPF neuron was manually divided into five regions according to morphology (Fig.4.S4). SOG region in different tissues was selected by a circle of the same

size, anterior VNC and posterior VNC were divided by the midpoint of the axon posterior to the SOG region.

4.4.6 *ANF-GFP Visualized NPF Release*

Brains from *NPF>ANF-GFP* larvae 74-76 h after egg laying were dissected out and immediately fixed in 4% paraformaldehyde for 30min at room temperature. Fixed tissues were then rinsed with 1xPBT six times and for 20 min each time. Images were collected using a Zeiss LSM 710 confocal microscope.

Tissues to be quantified were processed in parallel. Method for GFP intensity quantification was the same as NPF IHC quantification described in 4.4.5.

NPF>ANF-GFP/dTrpA1 larvae were used for verifying ANF-GFP as a neuropeptide release indicator. Larvae were kept under 31 ℃ for various amounts of time and sacrificed immediately for GFP quantification. Local release level quantification was achieved by the similar method described in 4.4.5.

4.4.7 *Targeted Laser Lesion*

The laser lesion was performed using a previously published protocol with slight modification (115, 232). Early second-instar larvae (52 h after egg laying) were rinsed and transferred to 150 µl double-distilled H2O on a microscope slide, and placed into the anesthetization chamber (90-mm Petri) with 1ml ether. After 2.5 minutes, slides were removed and larvae were covered with a coverslip. NPF neurons were shown under 40x magnification by a nucleus GFP reporter (UAS-*GFP.nls*). The laser beam was focused on individual nuclei and was applied as a burst of 20 shots at a rate of 3 Hz. Neurons lesioned with laser showed the loss of GFP signal. The group of larvae went through all the treatments except laser ablation served as the mock group. After the

laser treatment, the larvae recovered on fresh apple juice plates with yeast paste for 24 h before the assay.

4.4.8 *RNA in situ Hybridization*

Brains from w^{1118} larvae 74-76 h after egg laying were dissected out and immediately fixed in 4% paraformaldehyde for 30min at room temperature. After rinsing with 1xPBT for 3 times, 5 min each time, the tissues went through 25%, 50%, 75% and 100% methanol dehydration for 5 min each. The tissues were then incubated in fresh 100% methanol at 20 ℃ for overnight. NPF mRNA *In situ* hybridization was achieved by applying the RNAscope technique from ACDBio (Fluorescent Multiplex Kit). After methanol was removed and air-dried from the tissues, pretreatment 3 was added and tissues were incubated at room temperature for 20 min. Tissues were then rinsed with 1xPBT 3 times for 5 min each, followed by hybridization and signal amplification steps. Images were collected using a Zeiss LSM 710 confocal microscope. Probe toward bacterial gene DapB was used as the negative control probe. NPF probe was designed against the following sequence, which covers two common exons (exon 3 and exon 4, Fig.4.S2A) for all three NPF mRNA variants, and is intron spanning to increase signal to noise ratio:

5’-aactatgtgccaaacaatgcgttgcatcctggttgcctgtgtggcccttgccctcctagccgccggctgccgagtggaggcgtccaact
ccagacctccgcgaaagaacgatgtcaacactatggctgatgcctacaagttcctgcaggatctggacacctactacggcgacagagccc
gcgttcggttcggaaagcgcggatcgctgatggatatcctgaggaatcacgagatggacaacataaatctaggaaaaaatgccaacaatg
gaggagaatttgctcgcggttttaatgaggaggagatattctaaatccattttagacgaccatggcaacgtcactaactcatgatgatagttatt
agcatacgcatttttatttaaattgtttttcggggcaatagtttaacgtgctgggaaagaacaagtagttgcagctacagaaataagtatttactct
agtcttgatgtcggttgaataaatgaattaccccaat-3’.

NPFR1 probe was designed against the following sequence, which are shared by all four NPFR1 mRNA variants:

5’-acgcaacgcgccccggcaaacagcaaatcaggcggatatatcggcgggactagggtatataagcaggagatgcgcaccaaaagcc
aacagatggtcgctgactgtgcacgcgtgtggttatcggagatcagtaaacagcccaactaaacaccgaaacttactgtaataaaaaaaaac
gggaaataagcgaaataatcaaaatgcggccgcatacttatttataattttgaggcggccgagcaccggggccccaaactctttggatctgc
acggaatccagaattccgagagagcaaaaacacaaagcgaagtcccgtgagtgcattccaagttgaaaactaagtgagcaactgctgcttt
ggcagccggaaaaacagagattcactcgtgtcactcgcagaaggaaaaacaagaaccgacggccaggaaaacaatacggtaccacgca
ctatagtaaatatatagcatacatatccccagggcgaaggagattgccaggacgatgataatcagcatgaatcagacggagcccgcccagc
tggcagatggggagcatctgagtggatacgccagcagcagcaacagcgtgcgctatctggacgaccggcatccgctggactaccttgac
ctgggcacggtgcacgccctcaacaccactgccatcaacacctcggatctgaatgagactgggagcaggccgctggacccggtgcttatc
gataggttcctgagcaacagggcggtggacagcccctggtaccacatgctcatcagcatgtacggcgtgctaatcgtcttcggcgccctag
gcaacaccctggttgttatagccgtcatccggaagcccatcatgcgcactgctcgcaatctgttcatcctcaacctggccatatcggacctact
tttatgcctagtcaccatgccgctgaccttgatggagatcctgtccaagtactggccctacggctcctgctccatcctgtgcaaaacgattgcc
atgctgcaggcactttgtattttcgtgtcgacaatatccataacggccattgccttcgacagatatcaggtgatcgtgtaccccacgcgggaca
gcctgcagttcgtgggcgcggtgacgatcctggcggggatctgggcactggcactgctgctggcctcgccgctgttcgtctacaaggagct
gatcaacacagacacgccggcactcctgcagcagatcggcctgcaggacacgatcccgtactgcattgaggactggccaagtcgcaacg
ggcgcttctactactcgatcttctcgctgtgcgtacaatacctggtgcccatcctgatcgtctcggtggcatacttcgggatctacaacaagctg
aagagccgcatcaccgtggtggctgtgcaggcgtcctccgctcagcggaaggtggagcgggggcggcggatgaagcgcaccaactgc
ctactgatcagcatcgccatcatctttggcgtttcttggctgccgctgaacttttcaacctgtacgcggacatggagcgctcgccggtcactca
gagcatgctagtccgctacgccatctgccacatgatcggcatgagctccgcctgctccaacccgttgctctacggctggctcaacgacaact
tcc-3’.

4.4.9 *Hemolymph Ethanol Quantification*

20 w^{1118} larvae 74-76 h after egg laying for each treatment group were washed and placed in a 1.5 ml tube with 100 µl ddH_2O. Tissue went through flash freeze in -80°C and was homogenized with a pellet for 30 seconds on ice. After Repeating the homogenizing process three times, tissue was centrifuged at 13000 rpm for 10 minutes at 4 ℃. 20 µl of the middle transparent liquid phase was used for measuring the ethanol concentration. The lower phase and the upper phase were discarded.

Quantification of ethanol concentration is done by using the ethanol assay kit from Abcam (ab65343). All the measurements were done within 30 min after sacrificing the larvae to avoid oxidation caused color change. Light absorbance was averaged out of three measurements for each group. Each treatment condition was repeated three times under parallel conditions.

4.4.10 *Food Ingestion Assay*

The food ingestion assay was carried out by feeding early 3rd instar larvae 10% glucose liquid media containing 1% erioglaucine disodium salt (Sigma-Aldrich) for 2 min. 20 larvae were rinsed and collected for each group, and were place in a 1.5 ml tube with 100 µl ddH_2O. Larvae were flash frozen in -80°C and homogenized with a pellet for 30 seconds on ice. After Repeating the homogenizing process three times, tissue was centrifuged at 13000 rpm for 10 minutes at 4 ℃. 20 µl of the middle transparent liquid phase was used for measuring the ethanol concentration. The lower phase and the upper phase were discarded. Food dye amount was analyzed spectrophotometrically for absorbance at 625 nm. Larvae fed in undyed food were used for measuring the background absorbance level.

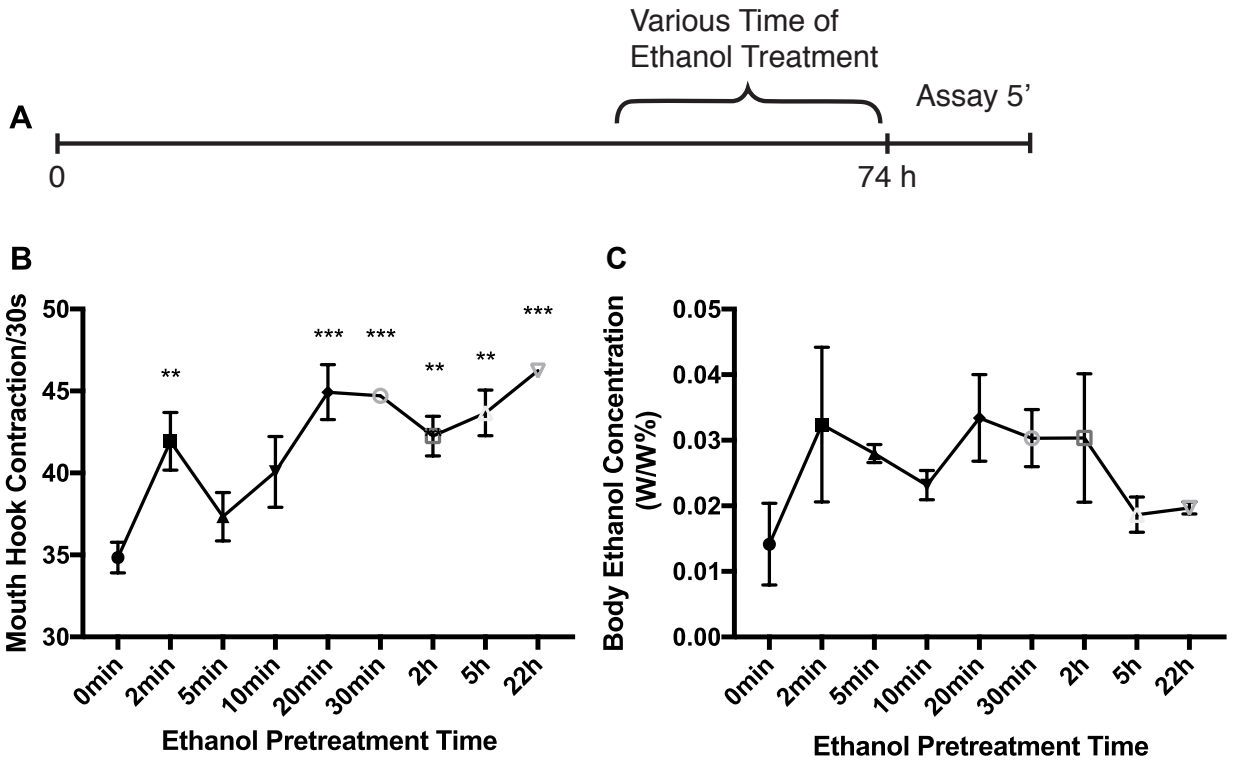

A
Various Time of
Ethanol Treatment
Assay 5'
0
74 h
B
Mouth Hook Contraction/30s
50
45
40
35
30
**
***
***
**
**
***
0min
2min
5min
10min
20min
30min
2h
5h
22h
Ethanol Pretreatment Time
C
Body Ethanol Concentration (W/W%)
0.05
0.04
0.03
0.02
0.01
0.00
0min
2min
5min
10min
20min
30min
2h
5h
22h
Ethanol Pretreatment Time

Figure 4.1 *Ethanol ingestion results in mouth hook hyper-motility.* (A) Schematic drawing of experimental procedures for behavioral assay. (B) Early third instar *Drosophila* larvae were placed on 10% ethanol food for different amounts of time before feeding rate was measured on 10% liquid glucose media. Mouth hook contraction rate of individual larvae was counted within 30 seconds ($n \geq 12$). One-way ANOVA was used followed by Dunnett's multiple comparison test. The groups ingested 10% ethanol for 2 min, 20 min, 30 min, 2 h, 5 h, and 22 h showed a significantly higher feeding rate on liquid glucose media compared to 0 min ethanol pretreatment group. $^{**}P<0.01$, $^{***}P<0.001$. (C) Larval body ethanol concentration was measured after feeding on 10% ethanol for different amounts of time.

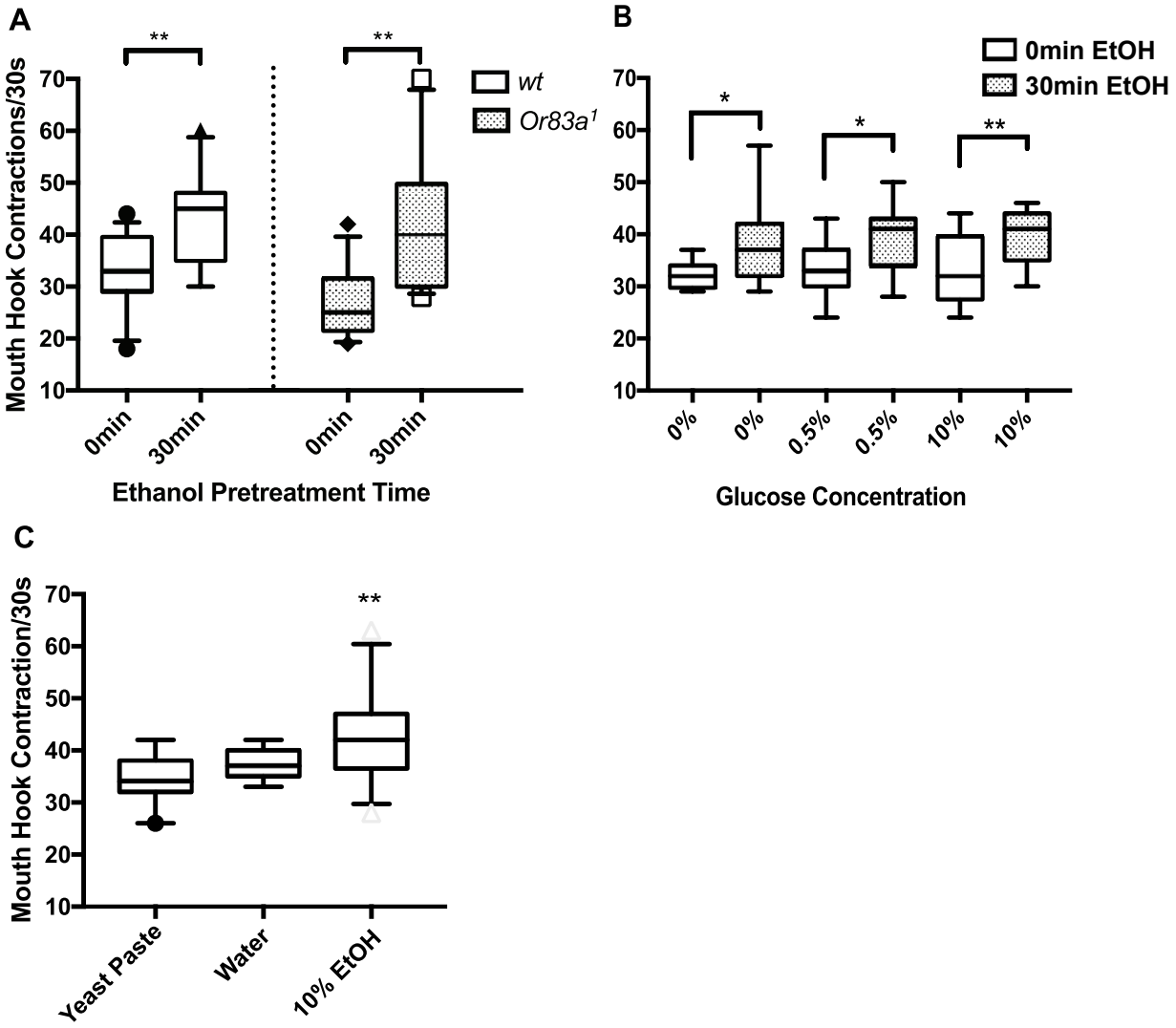

A
Mouth Hook Contractions/30s
70
60
50
40
30
20
10
**
**
wt
Or83a1
0min
30min
0min
30min
Ethanol Pretreatment Time
B
0min EtOH
30min EtOH
*
*
**
0%
0%
0.5%
0.5%
10%
10%
Glucose Concentration
C
Mouth Hook Contraction/30s
70
60
50
40
30
20
10
**
Yeast Paste
Water
10% EtOH

Figure 4.2 *Sensory and Homeostatic factors are not involved in ethanol induced feeding rate increase.* (A) Olfactory mutant larvae showed a similar response to 30 min ethanol treatment compared to wild type larvae (n≥12). Statistical analysis was performed using One-way ANOVA followed by Sidak's multiple comparison test. (B) Feeding rate increase induced by 30 min ethanol ingestion persisted when tested on non-sweet liquid media and 0.5% liquid glucose media (n≥11). Student's-t test was performed within each glucose concentration group. (C) 30-minute starvation failed to induce a significant feeding rate increase (n≥19). Statistical analysis was performed using One-way ANOVA followed by Tukey's multiple comparison test. *P<0.05, **P<0.01, ***P<0.001.

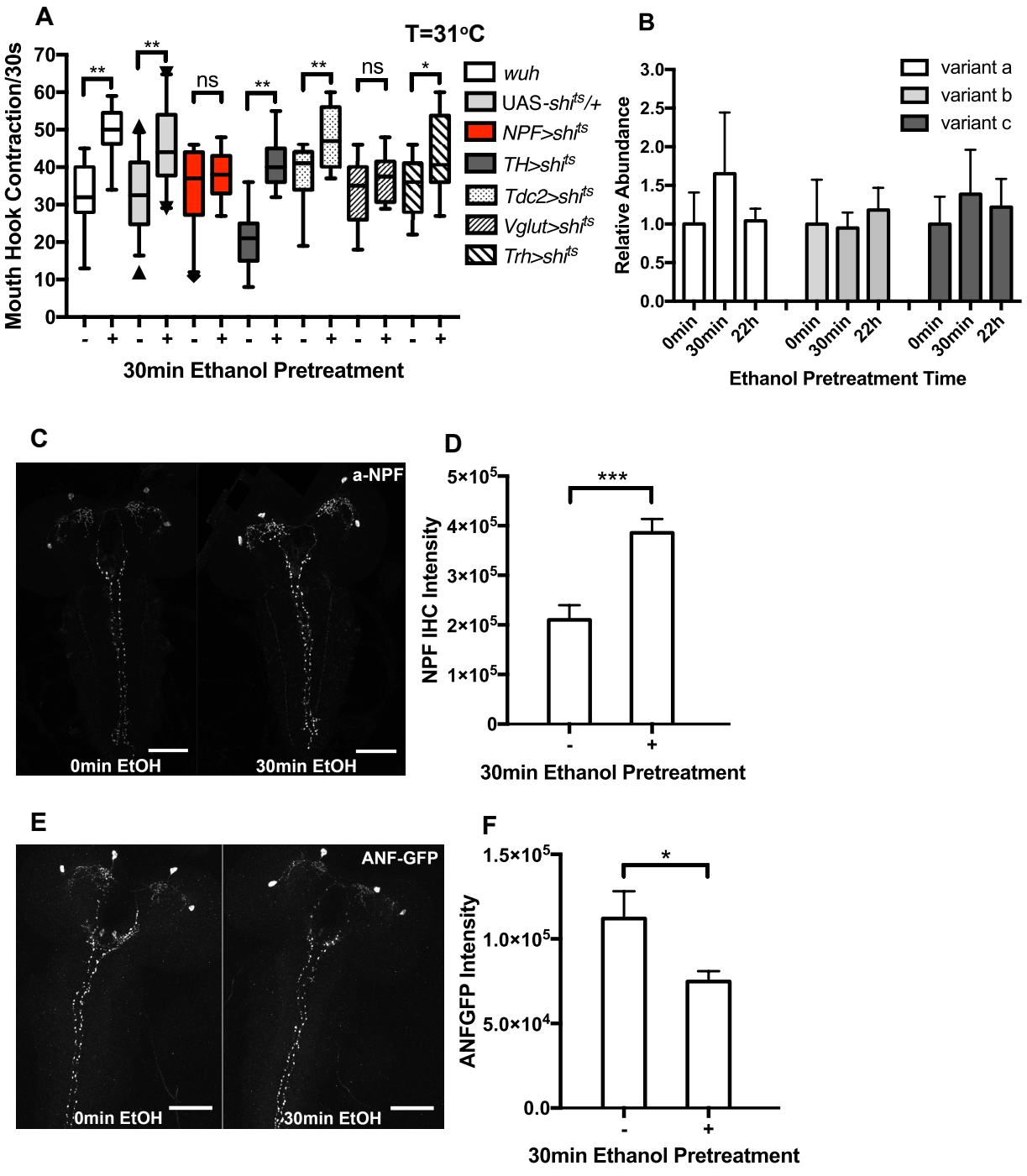

A
T=31°C
Mouth Hook Contraction/30s
70
60
50
40
30
20
10
0
**
**
ns
**
**
ns
*
wuh
UAS-shi^ts/+
NPF>shi^ts
TH>shi^ts
Tdc2>shi^ts
Vglut>shi^ts
Trh>shi^ts
- + - + - + - + - + - + - +
30min Ethanol Pretreatment
B
Relative Abundance
3.0
2.5
2.0
1.5
1.0
0.5
0.0
variant a
variant b
variant c
0min
30min
22h
0min
30min
22h
0min
30min
22h
Ethanol Pretreatment Time
C
a-NPF
0min EtOH
30min EtOH
D
NPF IHC Intensity
5×10^5
4×10^5
3×10^5
2×10^5
1×10^5
0
***
- +
30min Ethanol Pretreatment
E
ANF-GFP
0min EtOH
30min EtOH
F
ANFGFP Intensity
1.5×10^5
1.0×10^5
5.0×10^4
0.0
*
- +
30min Ethanol Pretreatment

Figure 4.3 *Feeding rate stimulating effect of ethanol is mediated through NPF neurons.* (A) Inhibition of various subsets of neurons by temperature sensitive shibire (n≥10). Feeding response was carried out on 10% liquid glucose media. (B) NPF transcription level in CNS was not significantly altered after 30 min ethanol ingestion (n=3). (C) NPF expression level in representative whole mount larval CNS tissues. Left: no ethanol pretreatment; right: 30 min ethanol pretreatment. Scale bar=50μm. (D) Quantification of immunohistochemical staining intensity (n≥12). (E) ANF-GFP expression driven by *NPF-Gal4*. Left: no ethanol pretreatment; right: 30min ethanol pretreatment. Scale bar=50μm. (F) Quantification of GFP intensity (n≥16). Statistical analysis was performed using Student's-t test. *P<0.05, **P<0.01, **P<0.001.

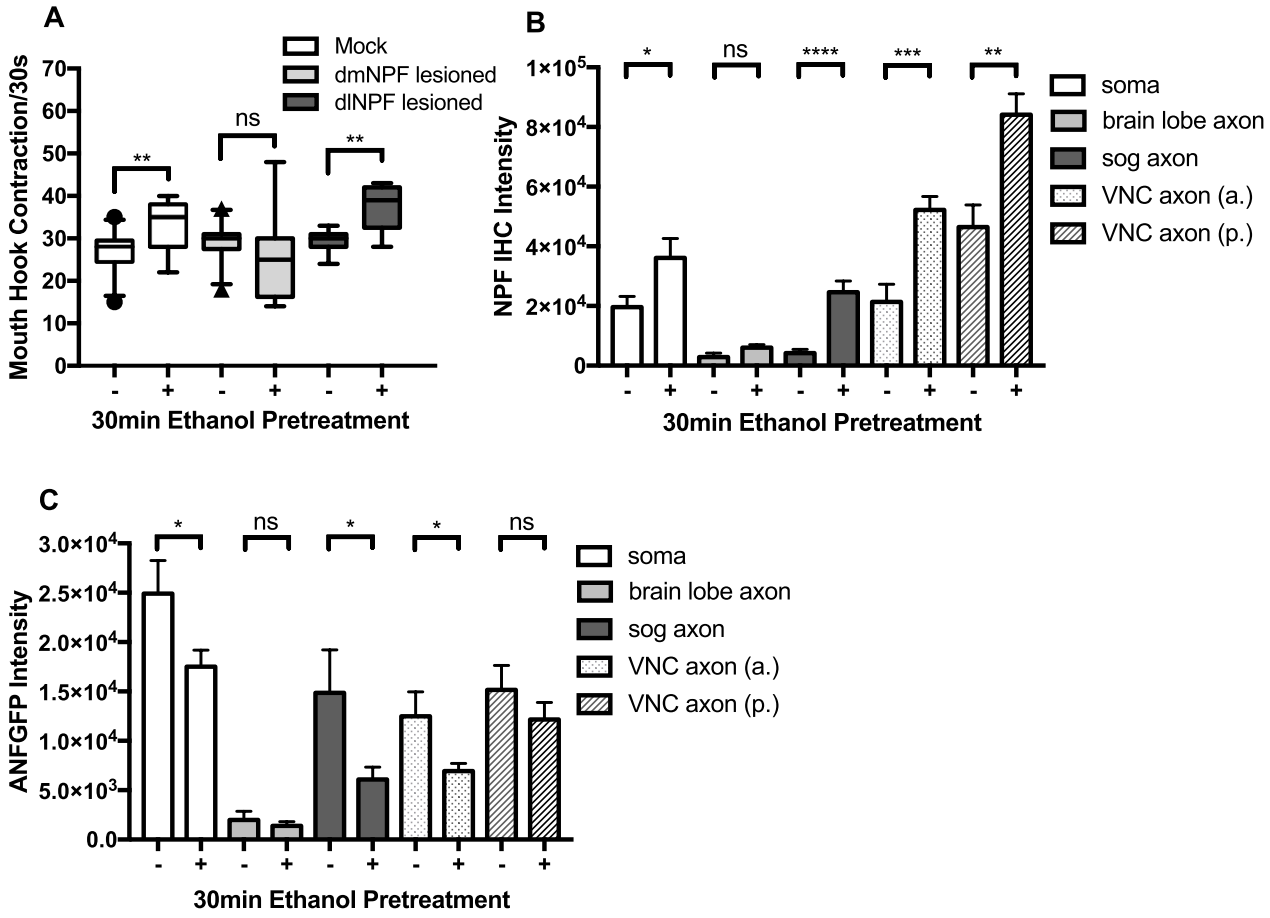

A
Mouth Hook Contraction/30s
Mock
dmNPF lesioned
dlNPF lesioned
**
ns
**
30min Ethanol Pretreatment
B
NPF IHC Intensity
*
ns
****
***
**
soma
brain lobe axon
sog axon
VNC axon (a.)
VNC axon (p.)
30min Ethanol Pretreatment
C
ANFGFP Intensity
*
ns
*
*
ns
soma
brain lobe axon
sog axon
VNC axon (a.)
VNC axon (p.)
30min Ethanol Pretreatment

Figure 4.4 *Ethanol mediates feeding rate increase by activating dmNPF neurons.* (A) Laser ablation of single NPF neuron. Feeding response after ethanol ingestion was tested on 10% glucose media (n≥8). (B) Quantification of NPF expression level in different regions of dmNPF neuron (n≥12). (C) Quantification of ANF-GFP expression level in different regions of dmNPF neuron (n≥15). Statistical analysis was performed using Student's-t test. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

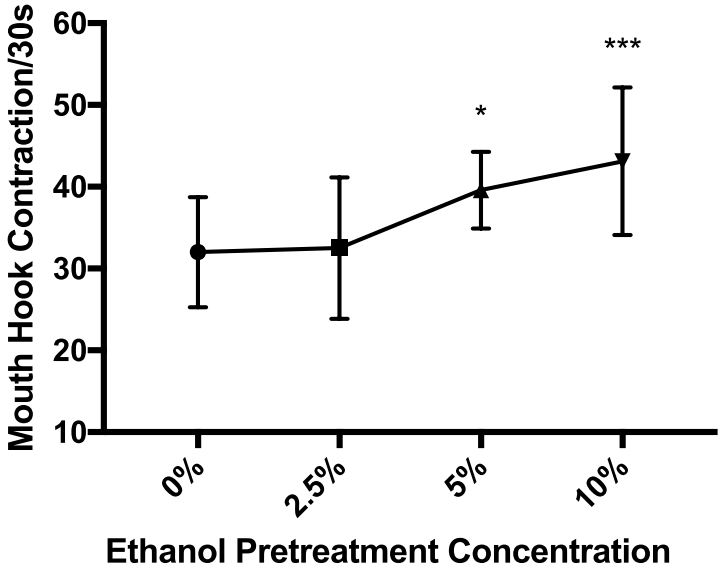
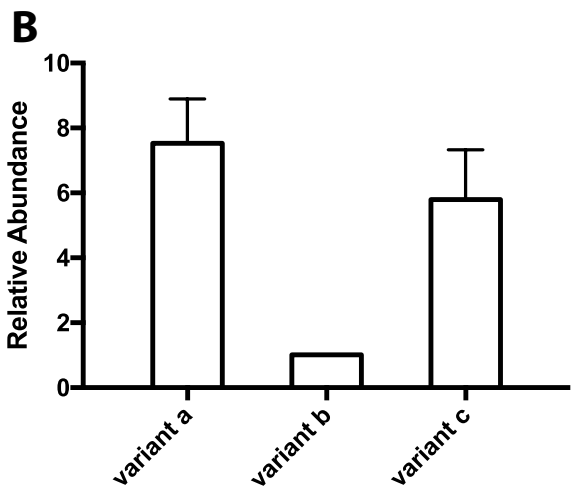

Mouth Hook Contraction/30s
60
50
40
30
20
10
*
***
0%
2.5%
5%
10%
Ethanol Pretreatment Concentration

Figure 4.S1 *Feeding rate quantification after exposure to different concentrations of ethanol.* 30 min pretreatment of ethanol using different ethanol concentrations ($n \geq 10$). Statistical analysis was performed using One-way ANOVA followed by Dunnett's multiple comparisons test. *P<0.05, ***P<0.001.


A
forward primer 6 (61 .. 82)
reverse primer 5 (169 .. 188)
100
200
300
400
500
exon 2
NPF CDS
Exon 4
Exon 5
npf variant a
569 bp
forward primer 5 (8 .. 31)
reverse primer (118 .. 137)
100
200
300
400
500
NPF CDS
exon 1
Exon 4
Exon 5
npf variant b
518 bp
forward primer 7 (55 .. 75)
reverse primer (179 .. 198)
100
300
400
500
exon 2
NPF CDS
Exon 3
Exon 4
Exon 5
npf variant c
579 bp


B
Relative Abundance
10
8
6
4
2
0
variant a
variant b
variant c

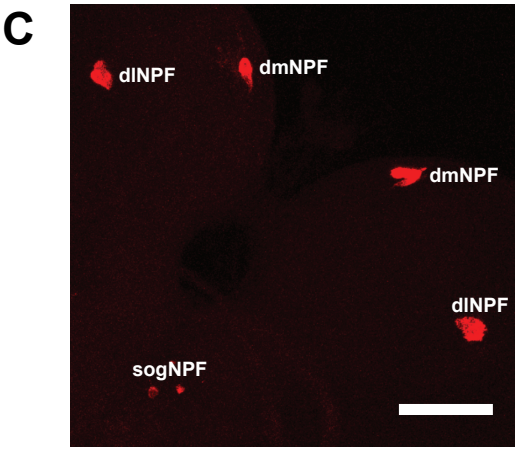
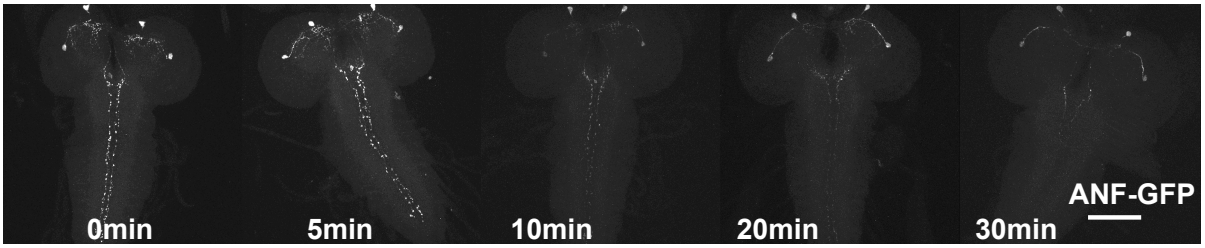

C
dlNPF
dmNPF
dmNPF
dlNPF
sogNPF

Figure 4.S2 *Ethanol failed to alter NPF mRNA level in the CNS.* (A) NPF mRNA variants and primer design. (B) Relative abundance of three NPF mRNA variants (n=3). Variant b levels were set at 1. Variant a and c levels were normalized to variant b. (C) Visualization of NPF mRNA expression in larval CNS by *in situ* hybridization. Scale bar=25 μm.

A


0min
5min
10min
20min
30min
ANF-GFP

B

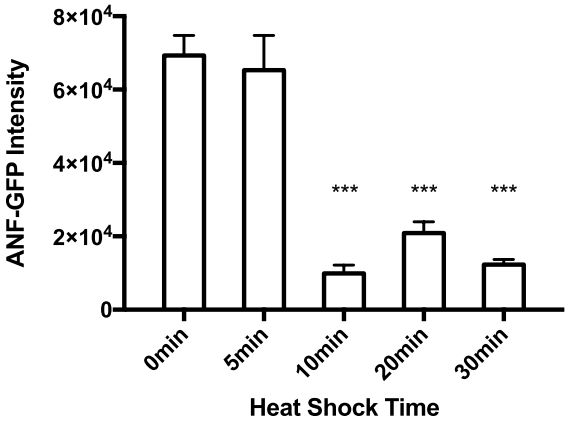

ANF-GFP Intensity
8×10^4
6×10^4
4×10^4
2×10^4
0
***
***
***
0min
5min
10min
20min
30min
Heat Shock Time

Figure 4.S3 *ANF-GFP as an indicator for NPF release activity.* (A) ANF-GFP expression level in representative CNS tissues after a series of heat shock events. Scale bar=50μm. (B) Quantification of total GFP intensity in CNS tissues (n≥4). Statistical analysis was performed using One-way ANOVA followed by Tukey's multiple comparisons test. GFP intensity under each heat shock time was compared with the non-heat shock control group. ***P<0.001.

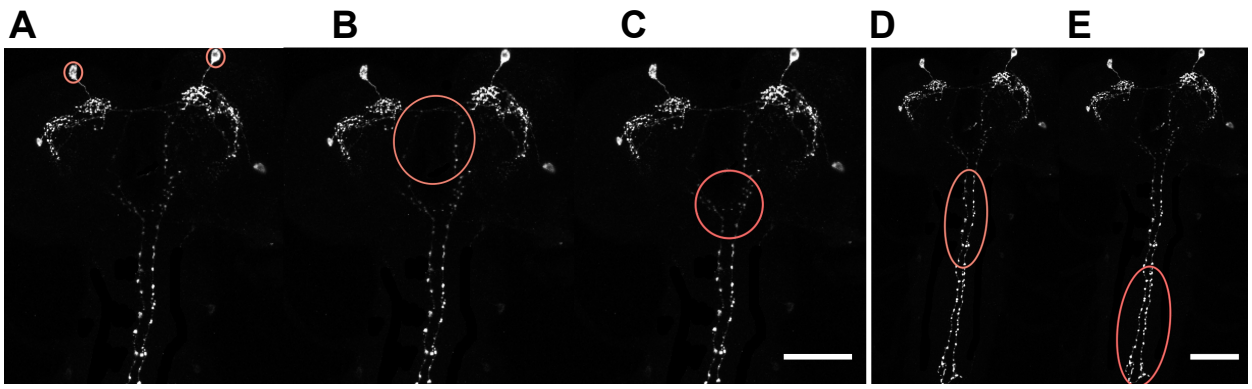

A
B
C
D
E

Figure 4.S4 *Region illustration of dmNPF neuron.* (A) Soma. (B) Axon in the brain lobe. (C) SOG region. Scale bar=50 μm. (D) Anterior VNC axon region. (E) Posterior VNC axon region. Scale bar=50 μm.

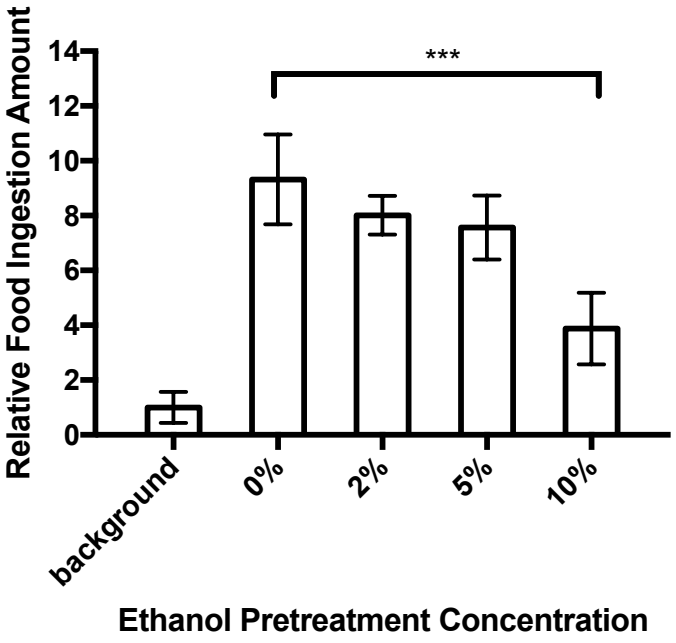

Relative Food Ingestion Amount
14
12
10
8
6
4
2
0
***
background
0%
2%
5%
10%
Ethanol Pretreatment Concentration

Figure 4.S5 *Quantification of food ingestion amount after exposure to different concentrations of ethanol.* 30 min 10% ethanol pre-feeding resulted in food ingestion amount decrease when tested on 10% liquid glucose media (n≥3). Background food dye intensity was set at 1. Ingested food dye amount under various ethanol pretreatment concentrations were normalized to the background level. Statistical analysis was performed using One-way ANOVA followed by Tukey's multiple comparisons test. ***P<0.001.

CHAPTER 5

DISCUSSION AND GENERAL CONCLUSIONS

In the previous chapters, we have characterized three different types of interfering inputs for feeding behaviors in *Drosophila* larvae including body energy level, natural food reward, and the drug ethanol. The corresponding neurocircuits have been identified with multiple tools such as genetic modulations, optical electrophysiology, imaging, and laser lesioning. Fly larvae make feeding decisions according to current body energy level, with OA receptor Oamb to inhibit overfeeding in a satiated situation and Octβ3R to promote feeding during food deprivation (chapter 3). The baseline feeding in satiated larvae is not saturated and can be elevated when food is highly palatable. The food reward can be influenced by multi-modal sensory systems such as olfaction and gustation. We have dissected a complete CNS circuit that is required for this reward-induced overeating: from the DL2 dopamine neurons that receive the olfactory reward signal from the peripheral, to the dmNPF neurons, and to the sog0NPFR1 neurons that project to the peripheral enteric systems (chapter 2). Ethanol hijacks the dmNPF reward pathway to interfere with the feeding behavior by activating NPF synthesis and release. It is likely that ethanol only affects the motivation to feed as it elevated feeding motility without changing the amount of food intake.

5.1 COVERGENCY OF ODOR AND TASTE PERCEPTIONS

In chapter 2, we have identified the existence of crossmodal integration in *Drosophila* larvae, in which the combination of odor and taste exhibited a supra-additive behavior response. Such

fusion of different senses was also discovered in mammals. Studies in human revealed that a subthreshold odor stimulus could make a subthreshold tastant detectable (221), or simply showed that rating for flavor could be enhanced by combining taste with the relevant odor (310, 311). Supra-additive regional and cellular responses were also observed and analyzed in mammalian models. Multiple cerebral cortex regions are involved. Functional magnetic resonance imaging (fMRI) showed a response toward an odor and taste mixture in the insula, operculum, orbitofrontal cortex, and the anterior cingulate cortex, with a higher intensity than the sum of individual odor and taste responses (312-315). The nucleus of the solitary tract (NTS, the primary CNS taste center) is another CNS region where odor and taste converge. Electrophysiological studies revealed a group of bimodal responsive neurons in the NTS, and a non-linear integrated activity to mixed odor and taste stimuli (316-318).

The evidence for integration of odor and taste on and a cellular level is still missing in *Drosophila* larvae. The location and cellular mechanisms for odor and taste convergence need to be clarified to explain the neuronal basis for the behavior. The SOG region could be the potential target since it accommodates the first gustatory center, paralleling the NTS in the mammalian brainstem. In addition, the NPF signaling induced by olfactory stimulus in this region is required for developing the hyperphagia behavior. The olfactory information might interact with the gustatory system through the NPFR1 receptors on the axon terminal of Gr43a sugar receptor neurons. Intriguingly, mammalian NPY is known to exert a regulatory role in sensory perceptions (319, 320). It would be interesting to test whether odor and sugar are able to generate a supra-additive cellular effect, which correlates with the behavioral responses, on Drosophila taste neurons or feeding executive neurons. To achieve this goal, membrane potential monitoring by Arclight as well as calcium imaging may serve as useful tools.

5.2 INTEGRATION OF HOMEOSTATIC AND HEDONIC CONTROLS

Although homeostatic and hedonic controls of feeding are relatively separated on neurocircuits level, cross-talk between the two was observed. Perception of food rewards can be affected by the motivational states (321, 322), indicating an integration of homeostatic control on the hedonic system.

Studies in mammals showed that homeostatic hormones directly act on peripheral sensory systems to modulate sensitivities to odor and taste. The 'hunger hormone' ghrelin increases olfactory sensitivity through ghrelin receptors in the glomerulus and olfactory bulbs (323). It also modulates salty and sour taste responsiveness through receptors on taste buds (324). The 'satiety hormones' leptin and insulin reduce peripheral olfactory sensitivity (325-327). Leptin also suppresses taste bud cells and gustatory nerves responses to sweet tastants (328-330). Insulin effect in peripheral gustatory structures was not reported, however, it modulates the sensation of taste by acting on the NTS, the primary CNS taste center in the brain stem (331). Receptors of these hormones are widely expressed in olfactory and gustatory structures, including the olfactory epithelium, olfactory bulbs, taste bud cells, and nucleus of solitary tracts (332-335), suggesting a direct signaling between the homeostatic hormones and the sensory neurons.

Another place integrates homeostatic and hedonic feeding controls locates in the VTA reward center in the CNS. It has been reported that ghrelin is able to activate mesolimbic dopamine release by acting on the ghrelin receptors (336, 337). Both leptin and insulin suppress mesolimbic dopamine levels. Leptin inhibits dopamine neurons firing pattern through leptin receptors (338, 339), while the inhibition effect of insulin is achieved by increasing dopamine reuptake (339-341).

Such integration was also observed in fruit flies. Food deprivation made *Drosophila* larvae less sensitive to aversive sensory cues, including bitter taste (203), cold temperature (342), and solid texture (228). In addition, dopaminergic modulation of sucrose perception was enhanced by hunger (343). We pay special attention to the SOG region since it is where the octopaminergic homeostatic control, olfactory related NPF-ergic pathway and sweet gustatory pathway converge in. It would be interesting to know whether hunger also modulates the reward systems in *Drosophila* larvae.

5.3 DMNPF AS A COMMON REWARD CENTER

Binge drinking and binge eating usually co-occur in the same group of people (344, 345), and similar behaviors and clinical features were observed (346). It suggests that these two impulsive behaviors may share some neurological mechanisms.

The mesolimbic dopamine pathway is assumed to be critical in both disorders (347, 348), though likely to be mediated by different receptors (D4 for alcohol drinking (349) and D1 D2 for feeding). Another shared modulatory component might be targeting the neuropeptide Y receptors, since agonist for Y1 receptor stimulates both feeding and ethanol intake, while the same effect was observed when antagonist for Y2 receptor was applied (350-353). However, NPY regulated feeding and alcohol drinking behaviors seem to be based in different brain regions (amygdala for alcohol drinking and hypothalamus for feeding (354, 355).

Our studies demonstrated that NPF, the invertebrate counterpart of NPY, is involved in regulating both natural food reward-induced and ethanol-induced hyperactivity in feeding. By analyzing on single cell resolution, we further identified two dorsal medial NPF neurons essential for both behaviors, suggesting a potential CNS center for processing multiple types of

rewards. The NPF release from the dmNPF neurons in the SOG region and a cluster of downstream NPF receptor neurons have been found to be critical in natural reward-induced overeating. Whether ethanol-induced feeding rate increase utilizes the same cellular mechanism needs to be characterized. Further studies can be focused on the role of NPF release in specific regions and the downstream NPFR1 neurons modulating ethanol effect. If the same pathway is applied as by the natural food reward, we may argue that the dmNPF/sog0NPFR1 pathway defines feeding motivation, thus stimulating feeding motility, yet a separate pathway is involved in 'liking' the food or generating greater ingestion.

5.4 PHARMACOLOGICAL TREATMENTS OF EATING DISORDERS AND OBESITY

Psychological interventions are commonly used in treating eating disorders (anorexia nervosa, bulimia nervosa, and atypical eating disorders (356)), while pharmacological managements are less documented (357, 358). There are relatively more evidences for the effectiveness of medications in bulimia nervosa. Since a strong correlation was observed between bulimia nervosa and stress (359, 360), antidepressants are applied as the major medications which target on the anti-stress neurotransmitter serotonin and its receptors (361-364).

Pharmacological therapies have also been established to alleviate obesity by hacking food reward and energy homeostasis systems (365). Findings about the involvement of certain neurotransmitters pathways in feeding regulation including opioid peptides (160), serotonin (366), dopamine (164), norepinephrine (157), leptin (10), and NPY (367) inspired the development of multiple pharmacological approaches.

Earlier pharmacological treatments of eating disorders and obesity focused on manipulating the level of neurotransmitters by altering the release or reuptake (368-370). However, these methods

tend to generate side effects because the neurotransmitters have complex roles in multiple systems. Recently, receptors have attracted more and more attention as potential drug targets. Though acting to the same neurotransmitter, different receptors display various affinity and responses to the same drug. This feature potentiates more precisely targeted therapies in treating obesity and eating disorders. Examples are Naltrexone (as an antagonist for the μ-opioid receptor) (371) and Lorcaserin (as an agonist for serotonin 2C receptor) (372, 373).

NPY receptors have been suggested as anti-obesity drug targets (374, 375). Since NPY also exhibits an anxiolytic role (38, 289, 376), the anti-obesity drugs targeting NPY receptors may also influence the feeding behaviors. Because of the highly conserved molecular structure and physiological function between mammalian NPY and *Drosophila* NPF, we believe that a better understanding of the NPF neural circuit and cellular mechanisms in *Drosophila* will provide insights for developing novel pharmacological therapies.

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