OCTOPAMINE SIGNALING FOR FEEDING REGULATION IN DROSOPHILA LARVAE

by

TING ZHANG

(Under the Direction of Ping Shen)

ABSTRACT

As a basic instinct in living organisms, feeding behavior is essential across evolution and species. The ability to be attracted to palatable food enables animals to gain energy efficiently. Using *Drosophila melanogaster* (fruit fly) as the model organism, and feeding assays to measure larval feeding rate on palatable food, I have demonstrated that octopamine (OA), the insect equivalent of norepinephrine, is acutely required for the fly larva's hunger-elicited feeding response to palatable food. The inhibition of octopaminergic neurons blocked the hunger-elicited feeding rate increase in fly larvae. Oral treatment with OA also promotes the feeding response to palatable food of fly early third instar larvae. Using laser ablation, I was also able to map the octopaminergic neurons involved in feeding regulating functions to the subesophageal ganglion (SOG), which is known as the feeding control center of insects. Two clusters of octopaminergic neurons in the SOG, which are ventral unpaired medium (VUM) neurons VUM1 and VUM2, respectively, regulate feeding response to palatable food antagonistically. The VUM1 octopaminergic neurons inhibit feeding when the animals are fed, while the VUM2 octopaminergic neurons promote hunger-elicited feeding activity. OAMB and OCTß3R are the antagonistic receptors required for the two functions, respectively. In addition, I have demonstrated that the Pvr/drk/Ras pathway regulates OA function in the feeding response. Drk

activity in the *tdc2-Gal4* neurons positively regulated larval feeding rate in the palatable food. Expression of a dominant negative form of Ras in *tdc2-Gal4* neurons blocked drk^{cDNA} function in the food response. PDGF- and VEGF-related Receptor (Pvr) is the receptor receiving extracellular signals to activate this pathway, and PDGF- and VEGF-related factor 2 (Pvf2) may be the extracellular signal. My work is the first study to unveil and characterize the function of OA in *Drosophila melanogaster* feeding behavior, and provided molecular and genetic evidence for the neural circuits underlying the complex preferred-food response of fly larvae, which may suggest a conserved pathway in the mammalian system.

INDEX WORDS: Octopamine; octopamine receptor; Oamb; Octß3R; Neuropeptide F; feeding circuits; food motivation; PDGF and VEGF-related receptor; drk; Pvf2

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DEDICATION

This dissertation is dedicated to my husband Fei, my parents Hongcai and Fengqin.

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TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 HOMEOSTATIC AND HEDONIC CONTROL OF FEEDING ACTIVITIES

According to a report from the centers for disease control and prevention (CDC), there are approximately 72.5 million obese adults in the United States in 2007-2008 (Sherry et al., 2010). A recent estimate suggests that the annual medical cost for obesity rose to 168.4 billion dollars (Cawley and Meyerhoefer, 2012). The increasing social and economic costs of such diseases have made research into their underlying mechanisms a critical area of focus in the field of neuroscience.

In vertebrates, palatable foods trigger reward-related feeding responses such as approaching and gathering high quality food, and appetitive learning. These responses allow animals to obtain sufficient energy to satisfy metabolic needs while budgeting the energy expenditure for feeding efficiently.

Investigations on vertebrate homeostatic feeding have focused on the feeding center in the hypothalamus. The arcuate nucleus (ArcN) of the hypothalamus receives circulating energy balance signals such as leptin and ghrelin (Figure 1.1). Leptin and ghrelin are satiety and hunger signals from the adipose tissues and the stomach, respectively (Horvath et al., 2001). The ArcN contains neurons that release orexigenic peptides such as neuropeptide Y (NPY) and Agouti-related peptide (AgRP), as well as neurons that release anorexigenic peptides such as cocaine and amphetamine regulated transcript (CART) and α-melanocyte-stimulating hormone (α-MSH).

Leptin inhibits the release of NPY/AgRP, while ghrelin promotes that. The melanocortin 4 receptor (MC4R) neurons in the paraventricular nucleus (PVN), whose excitation inhibits food intake, are inhibited by NPY/AgRP neurons and excited by CART/α-MSH neurons (Kenny, 2011). The hypothalamus regulates food intake via NPY/AgRP, CART/α-MSH and MC4R activity (Carlson, 1994; Kenny, 2011). Many feeding-related hormones and neurotransmitters regulate feeding behavior via the regulation of neuronal activities in the ArcN. For example, insulin reduces the expression of transcriptional factor Foxo1 in hypothalamus. Foxo1 decreases the expression of proopiomelanocortin (POMC), a precursor of α-MSH, and promotes AgRP/NPY expression. By reducing Foxo1 expression, insulin regulates the ArcN to inhibit food intake (Kim et al., 2006). Norepinephrine (NE) has been known to regulate feeding for decades (Goldman et al., 1985; Leibowitz et al., 1984), and several recent studies also suggest that the food intake change along with NE treatment may be a result of NE-triggered hypothalamic NPY alteration (Kalra et al., 1998; Smialowska et al., 1997). The ArcN is important for the homeostatic feeding, which is to fulfill the energy need for metabolism, because it is regulated by signals about internal state such as leptin and stomach distention.

In contrast to homeostatic feeding, the hedonic aspects of feeding have only recently begun to attract the interests of researchers (Saper et al., 2002). How food palatability and reward systems assign reinforcing prosperities to food and how homeostatic and hedonic systems cooperate to regulate food intake have become new hot research topics. Gustatory signals from the oral cavity relay information to the nucleus tractus solitarius (NTS) in the brainstem then amygdala (Rolls, 2006). In vertebrates, the nucleus accumbens (NAc) in the striatum plays a role as the pleasure center (Cardinal et al., 2002), and is innervated by neurons from amygdala (Kenny, 2011; Rolls, 2006), responds to the afferent sensory signals from food (Fallon et al.,

2007), and sends signals to modulate the hypothalamic activity (Kelley et al., 2005) (Figure 1.1). The hypothalamus then releases NPY and AgRP to stimulate food ingestion. The perception of food adjusts the striatal release of reward-related neurotransmitters such as dopamine (DA) (Fallon et al., 2007; Hajnal and Norgren, 2004) and opioid peptides (Kelley et al., 2005), which in turn alter the pattern of feeding behavior. DA transmission in the NAc is involved in reward-related learning (Ljungberg et al., 1992) and motivation (Baldo et al., 2003), while opioid peptides regulates the hedonic evaluation of food (Levine and Billington, 2004).

Remarkably, these feeding pathways have been found to be highly conserved, and *Drosophila* melanogaster has been found to be an excellent model system for studying feeding behaviors (Melcher et al., 2007; Wu et al., 2005a; Wu et al., 2005b). Feeding-related peptides such as insulin-like peptides, neuromedin-U related peptides and NPY are conserved from insects to mammals (Melcher et al., 2007; Wu and Brown, 2006; Wu et al., 2003). NPF, a *Drosophila* homolog of NPY, promotes larva feeding under stressful conditions, such as bitter or hard non-palatable food or at low temperature (Lingo et al., 2007; Wu et al., 2003; Wu et al., 2005a; Wu et al., 2005b). In mammals, insulin inhibits NPY neurons, whereas in *Drosophila* insulin-like peptides (iLPs) regulate NPF-targeting NPFR1 neurons to mediate food intake (Wu et al., 2005b).

The subesophageal ganglion (SOG) is proposed to act as a feeding control center in the central nervous system of insects. For example, in *Drosophila*, the SOG is dense with feeding-related neuronal activity, and is innervated by the axons of gustatory receptor neurons (Gerber and Stocker, 2007), interneurons that project to motor neurons, and several motor neurons that can be activated by palatability cues (Gordon and Scott, 2009). It was proposed that octopaminergic and dopaminergic neurons in the SOG region carry appetitive and punishment

signals, respectively, to the mushroom body (MB) in the anterior fly brain (Kelley et al., 2005; Schleyer et al., 2011) (Figure 1.1). The MB has similar functions as the striatum, the hippocampus, and the prefrontal cortex in mammals, and is required for fly learning, memory (Skoulakis et al., 1993), and decision-making (Zhang et al., 2007). The functional similarity between SOG and hypothalamus, and between MB and striatum, suggests that the SOG/MB circuit in insects may function similarly to the corticostriatal-hypothalamic circuit for hedonic feeding in mammals. Also, octopamine (OA), NE and DA are similar in structure (Figure 1.2), suggesting that OA may adopt similar mechanisms as NE and DA in mammals to regulate the insect feeding activities. With the various genetic tools and short developmental cycles, studying OA in *Drosophila* will help to illuminate the underlying mechanisms of the homeostatic and hedonic feeding control in invertebrates as well as in vertebrates.

1.2 OCTOPAMINERGIC SIGNALING SYSTEM

The endogenous biogenic amine octopamine (OA) was originally discovered in Octopoda in 1948 (Erspamer, 1948). As the invertebrate homolog of NE, OA has been studied intensely for decades, and it has been known for its feeding regulating function across phyla (Cohen et al., 2002; Elliott and Vehovszky, 2000; Long and Murdock, 1983). OA also plays important roles in the control of foraging (Barron et al., 2007b), food ingestion (Long and Murdock, 1983), appetitive learning (Schroll et al., 2006; Schwaerzel et al., 2003), circadian cycles (Crocker and Sehgal, 2008; Crocker et al., 2010), locomotion regulation (Saraswati et al., 2004), and aggressive behavior (Zhou and Rao, 2008).

1.2.1 OA Synthesis and Localization in Invertebrates

OA is synthesized from tyramine by the enzyme of tyramine ß-hydroxylase (TßH), and tyramine is synthesized from tyrosine by tyrosine decarboxylase (TDC) (Figure 1.2). As a result, researchers are able to manipulate OA signaling by modulating TDC/TßH expression or by regulating the neuronal activities of TDC/TßH neurons. OA is very close to some other tyrosine derivatives in chemical structures, such as DA and NE (Figure 1.2), suggesting that they may share similar functions.

In early years, OA was localized in different tissues using the enzymatic-isotopic method (Molinoff et al., 1969; Saavedra, 1974) and chromatographic system (Macfarlane et al., 1990). The majority of OA was found in the nervous tissues and salivary glands. In the late 1980's, specific OA antiserum was raised, which enabled the mapping of OA-immunoreactive (IR) neurons (Konings et al., 1988). Similar to NE, OA is synthesized in only a few neurons of the insect central nervous system (CNS), while their arborizations are widespread in the brain and body (Roeder, 1999, 2005). For example, in *Drosophila* larvae, OA immunohistochemistry found the soma of octopaminergic neurons only in the feeding control center SOG, and locomotion control center the thoracic and abdominal ganglia. However, these octopaminergic neurons innervate broadly, projecting to MB, protocerebrum, optical neuropils, and every segment of body wall muscles (Monastirioti et al., 1995). In *Drosophila* adults, additional OA-IR neurons were found in the dorsal and lateral protocerebrum (Monastirioti et al., 1995). In other arthropods, like honey bees, cockroaches, blow flies, and lobster, OA-IR neurons have also been found in the SOG and thoracic/abdominal ganglia (Roeder, 1999; Sinakevitch et al., 2005; Sinakevitch and Strausfeld, 2006). In *Drosophila* larvae, *tdc2-Gal4* neurons in the SOG regions

are all octopaminergic (Honjo and Furukubo-Tokunaga, 2009), making the manipulation of the subesophageal octopaminergic neurons convenient.

1.2.2 Adrenergic Receptors and Octopaminergic Receptors

NE and OA are almost identical in chemical structure except that the former has one more hydroxyl group in 3-position of phenolic ring than the latter (Figure 1.2). Their receptors are homologous in sequence and have similar pharmacological properties as well (Evans and Maqueira, 2005; Roeder, 2005). Almost all octopaminergic receptors are adrenergic-like G-protein coupled receptors with seven transmembrane domains.

In mammals, NE has two types of receptors, α and ß, which were defined according to the rank-order potency of activation by adrenergic receptor agonists (Ahlquist, 1948). In the subsequent studies, with the development of biotechnology, researchers discovered that at the cellular level, different groups of adrenergic receptors adopted different second-messenger pathways. The activation of α1-adrenergic receptors increases intracellular Ca^{2+} level (Piascik and Perez, 2001) and inositol phosphate formation (Williams et al., 1998), while the activation of α2-adrenergic receptors inhibit cyclic adenosine monophosphate (cAMP) signaling (Williams et al., 1998), and ß-adrenergic receptor activation stimulates intracellular cAMP formation (Williams et al., 1998).

Early studies showed that in vertebrates, pharmacological treatments with NE, NE agonists, or NE antagonists changed animal feeding behaviors. In rodents, the activation of α2 adrenergic receptor stimulates foods intake (Leibowitz, 1988), while the activation of α1 (Morien et al., 1993) and ß (Ramos et al., 2005) adrenergic receptor inhibits food intake. NE is mainly produced in the dorsal vagal complex and the locus coeruleus. These areas send efferent

projections to the spinal cord and afferent to the cortex, the thalamus, and the hypothalamus, which contains nuclei that act as the mammalian feeding center (Schwartz et al., 2000). In the hypothalamic paraventricular nucleus (PVN), α1 and α2 adrenergic receptors are organized in an antagonistic pattern (Ramos et al., 2005). It has been demonstrated that injecting NE to the rat PVN stimulates feeding behavior and increases food intake by activating α2 adrenergic receptors (Leibowitz, 1988).

Comparably, the invertebrate octopaminergic receptors have similar second-messenger pathways upon activation. Many OA receptors, which increase intracellular cAMP upon activation, are considered as ß-adrenergic like receptors, such as *Lymnea* OAR1 (Gerhardt et al., 1997a). In contrast, just like α2-adrenergic receptors, some OA receptors inhibit the activity of adenylate cyclase. For example, the activation of the OAR in *Heliothis* inhibits the cAMP signaling *in vivo* (von Nickisch-Rosenegk et al., 1996). There are also OA receptors increase intracellular Ca^{2+} signaling as α1-adrenergic receptors, including the *Drosophila* OAMB (Han et al., 1998). Interestingly, the *Lymnea* OAR2 is a GPCR-bound chloride channel, which hyperpolarizes the neuron upon activation (Gerhardt et al., 1997b).

In *Drosophila,* there are four different OA receptors identified so far, including OAMB, OA2, OCTß2R and OCTß3R. OAMB increases both cAMP and Ca^{2+} signaling (Han et al., 1998). Although BLAST shows that OAMB is more homologous to α-adrenergic receptors in sequence, functionally it contains the characteristics of both α- and ß- adrenergic receptors. The OA2, OCTß2R and OCTß3R are ß-adrenergic like receptors, which increase the cAMP formation upon activation (Maqueira et al., 2005). Similarity in adrenergic receptors and octopaminergic receptors suggests that they may have similar functions in the regulation of

behaviors, which makes it possible to study NE function in mammals using the information from OA function in the invertebrates.

1.2.3 OA Functions in Feeding Regulation

The invertebrate octopaminergic system has been known to regulate food intake for decades. Treating blowflies or cockroach with OA or its various agonist increases the food intake of animals, and the injection of OA antagonists, such as phentolamine, reduces their food intake compared to the controls (Cohen et al., 2002; Long and Murdock, 1983). In insects, the hemolymph injection or the oral treatment of OA to animals induces hyperphagia. However, the molecular mechanism and neural circuits underlying OA function in feeding activity is still under investigation. Interestingly, an analysis on honeybee dance style revealed that OA signaling modulated the food value-encoding module in the dancing language (Barron et al., 2007b), suggesting that OA might reinforce the appetitive signals during feeding activities.

Early pharmacological studies applied OA to the entire body of the insects, rather than to the CNS tissue alone. One limitation is that the brain manipulation of insects may lead to fetal damage, making behavioral assays impractical. In addition, pharmacological treatment with OA or agonist to the brain may diffuse the drugs to other tissue (Barron et al., 2007a), which limits the conclusion that can be drawn from this specific approach. As biotechnology develops, recent studies localized OA activity to the CNS. OA activity is altered in response to both food deprivation and food stimuli. For instance, a honeybee study revealed that several neurosecretory cells in the pars intercerebralis showed OA-immunoreactivity when the animal was food-deprived (Kreissl et al., 1994). Another honeybee study showed that octopaminergic neurons in the SOG responded to the stimulation of the antennae with sucrose with a burst of action

potentials (Schroter et al., 2007). OA activity in the CNS, especially in the SOG, responds to food stimuli.

Beside pharmacological and biochemical method for testing OA activities in feeding regulation, genetic tools in animal models such as *Drosophila* make it possible to manipulate neuronal activity *in vivo*. For example, there are two tyrosine decarboxylases (the enzyme for OA precursor synthesis, Figure 1.2) coding gene in *Drosophila*, named *Tdc1* and *Tdc2*. The gene of *Tdc1* is expressed in non-neuronal tissues, and *Tdc2* is exclusively expressed in the central nervous system (Cole et al., 2005), making the manipulation of octopaminergic neurons possible with *tdc2-Gal4* via the GAL4/UAS system. In the SOG of *Drosophila*, there are three clusters of octopaminergic neurons (Monastirioti et al., 1995), and all of them are *tdc2-Gal4* neurons (Honjo and Furukubo-Tokunaga, 2009). There are also constructs such as UAS-*Kir2.1* and UAS-*NaChBac*, which inhibits and activates neurons, respectively. Previous research in our lab demonstrated that a set of feeding behavior assays can be used to quantify the *Drosophila* feeding activities (Wu et al., 2003). As a result, with *Drosophila* as a model, OA neuronal function in feeding regulation can be investigated with less difficulty.

1.3 PDGF/VEGF SIGNALING PATHWAY

Platelet-Derived Growth Factor (PDGF) was first identified as a mitogen for the fibroblasts released by the α-granules of platelets in human (Kaplan et al., 1979). Researchers later revealed that PDGF was synthesized in various cell types and it targeted different cells, from muscle cell to neurons (Heldin and Westermark, 1999; Sasahara et al., 1991; Schatteman et al., 1992). Vascular Endothelial Growth Factor (VEGF) was first discovered as a tumor-secreted vascular permeability factor in guinea pigs (Senger et al., 1983). VEGF was later characterized

as having angiogenic property, and was purified from bovine pituitary folliculostellate cells (Ferrara and Henzel, 1989; Leung et al., 1989).

PDGF and VEGF are closely related in terms of structure, with both containing PDGF/VEGF homology domains with a conserved pattern of cysteine-rich motifs (Fredriksson et al., 2004; Joukov et al., 1997; Murray-Rust et al., 1993). PDGF is critical in embryonic and CNS development, vascular regulation, and wound healing, and VEGF is required in blood vessel development and growth (Dunn et al., 2000; Ferrara et al., 2003). They have recently become targets of intense study due to their angiogenic roles in tumor formation (Dunn et al., 2000; Shibuya, 2001). In *Drosophila*, PDGF- and VEGF-related signaling has similar functions as is observed in mammals. The *Drosophila* PDGF- and VEGF-related Receptor (Pvr) is known to regulate cell migration, hemocyte proliferation and survival, and glial cell survival during axon guidance and wound healing (Bruckner et al., 2004; Duchek et al., 2001; Munier et al., 2002; Wu et al., 2009). Given the high level of conservation between the mammalian PDGF/VEGF and invertebrate signaling pathways, the *Drosophila* model provides a genetically tractable system for further study of this family.

1.3.1 PDGF/VEGF related ligands and receptors

PDGF and VEGF both signal through receptor tyrosine kinases (RTK). Induced by ligand binding, the monomers of RTKs dimerize and autophosphorylate specific intracellular tyrosine residues. These phosphorylated tyrosine residues recruit Src homology 2 (SH2) domain-containing signal transducers to the receptor, such as p85, phospholipase C-γ, Grb2/Sos, etc, which activate the phosphoinositide 3-kinase (PI3-kinase) pathway, the protein kinase C pathway, and Ras pathway, respectively (Marshall, 1995).

So far, there are four types of PDGF ligands identified in human and other mammals, including PDGF-A, PDGF-B, PDGF-C and PDGF-D (Marshall, 1995; Reigstad et al., 2005). They combine to generate either homo- or hetero- dimeric molecules (i.e. PDGF-AA, -AB, -BB, -CC and –DD) to activate PDGF receptors. There are also two types of PDGF receptors (PDGFR), dimerizing to either homo- or hetero- complex upon activation (PDGFR-αα, -αß and -ßß) in different cell types (Marshall, 1995).

The mammalian VEGF family consists of five different members, VEGF-A, -B, -C, -D and Placenta growth factor (PLGF), which form homodimers or the VEGF-A/PlGF heterdimers to activate VEGF receptors (VEGFR) (Olsson et al., 2006). Three different types of VEGFR, VEGFR-1, -2 and -3 form homo- or hetero- dimeric molecules as PDGFR do, which are VEGFR-11, -12, -22, -23 and -33 upon ligand binding (Olsson et al., 2006). Different combinations of receptor subunits play distinctive roles. For example, VEGFR1 is required in macrophage migration (Hiratsuka et al., 1998), and VEGFR3 regulates lymphatic system (Makinen et al., 2001). Intriguingly, several anticancer drugs, such as Sorafenib and Sunitinib inhibit both VEGFR and PDGFR (Jain et al., 2006), suggesting the similar pharmacological characters between these two.

The PDGF/VEGF signaling is conserved from invertebrates to the vertebrates. For example, the PDGF- and VEGF-related factor 1 (Pvf1) from *C. elegans* is able to activate mammalian VEGFR and induce angiogenesis (Tarsitano et al., 2006). In *Drosophila*, three proteins were identified with PDGF/VEGF homology domain, including Pvf1, Pvf2 and Pvf3 (Cho et al., 2002; Duchek et al., 2001; Heino et al., 2001). In contrast to mammalian PDGF/VEGF receptors, there is only one Pvr in *Drosophila*, which has four different isoforms

created by alternative splicing. Hence, *Drosophila* may provide a simple model to study the PDGF/VEGF family functions.

1.3.2 PDGF/VEGF functions in the nervous system

In mammals, PDGF receptors have been identified on neurons and glial cells, and they have been demonstrate to have important roles in the development and maintenance of the nervous system (Hart et al., 1989; Heldin and Westermark, 1999; Pietz et al., 1996; Schatteman et al., 1992; Smits et al., 1991). For example, PDGF promoted the proliferation of oligodendrocyte-type-2 astrocyte progenitor cells (Hart et al., 1989), and it also protects the rat dopaminergic neurons from the neurotoxin 6-hydroxydopamine (Pietz et al., 1996).

VEGF also has neurotrophic and neuroprotective effects. For instance, VEGF is able to induce axonal outgrowth (Sondell et al., 1999), and it showed protective effect on cultured hippocampal neurons against glutamate-induced toxicity (Matsuzaki et al., 2001). In addition, recent research has revealed that it also played an important role in neurogenesis. In adult rats, the VEGF signaling regulates exercise- and environmental enrichment-induced hippocampal neurogenesis (Cao et al., 2004; Jin et al., 2002), and consequently it is required in learning and memory. Mammalian PDGF/VEGF signaling function in neuronal plasticity suggests a potential role that this signaling pathway may modify animal behaviors in response to the change of environment.

PDGF/VEGF in the invertebrates share conserved functions and signaling pathways with the vertebrates. In *Drosophila,* Pvf/Pvr signaling pathway is known to regulate border cell migration in ovaries (Duchek et al., 2001), hemocyte proliferation (Munier et al., 2002), and axonal growth due to its gliatrophic role (Learte et al., 2008). However, whether Pvr regulates

neuronal activity and animal behavior is unspecified. In my research, I provide several pieces of evidences showing that Pvr may regulate the octopaminergic circuit in *Drosophila*, and consequently it controls the hunger-driven feeding response to enriched food. *Drosophila* may serve as a model to characterize the role of PDGF/VEGF in the neuronal plasticity regulation.

Figure 1.1 A Simplified Schematic View of Mammalian and Insect Brain Circuits Involved in Food Intake.

The mammalian pathway is modified from Kelley et al. (Kelley et al., 2005), and the insect pathway is summarized based on the information we have so far. In mammals, stimuli from food and hunger state control feeding via the hypothalamus, whereas the similar organ in insect has not been defined yet, which may be part of subesophageal ganglion region. The arrows represent neural signal transmissions, and dashed arrows indicate these signal tracts are not proved yet.

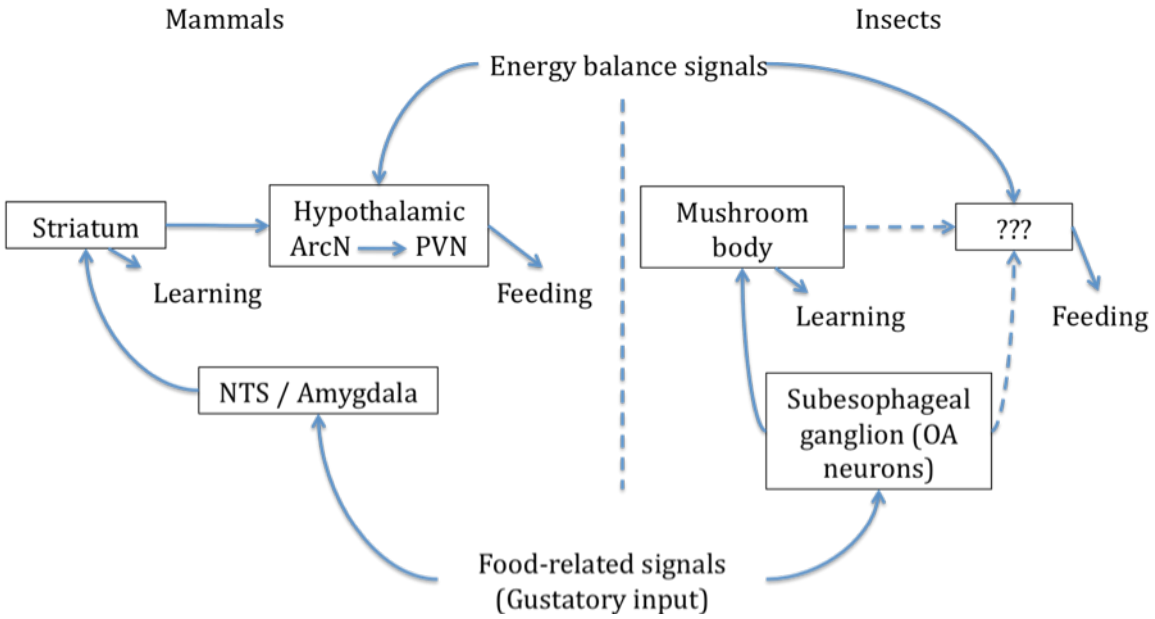
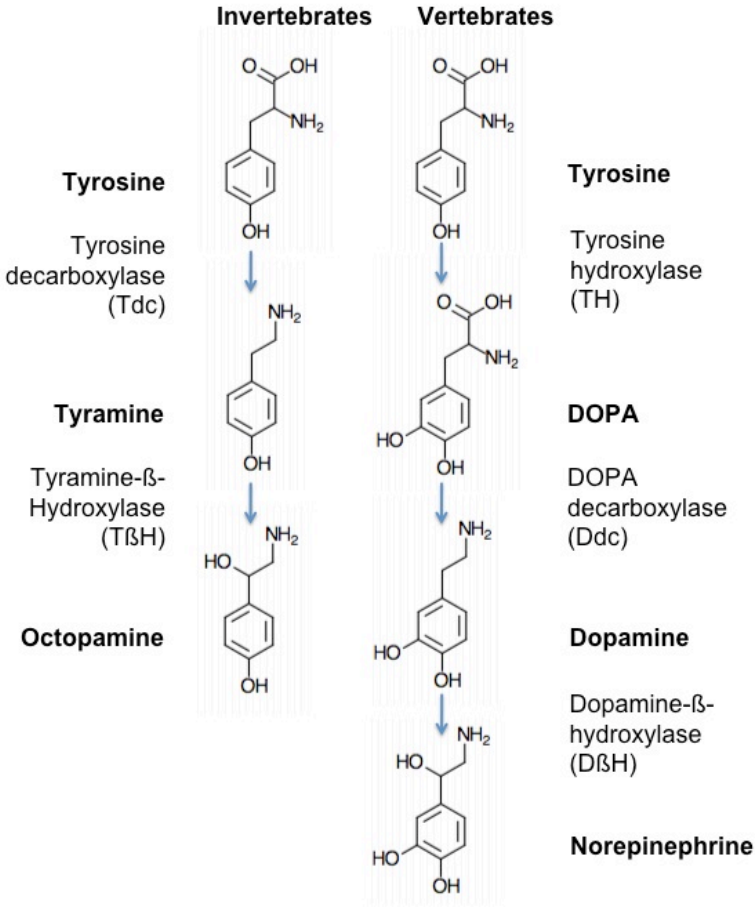

Mammals
Insects
Energy balance signals
Striatum
Hypothalamic
ArcN → PVN
Learning
Feeding
NTS / Amygdala
Mushroom
body
???
Learning
Feeding
Subesophageal
ganglion (OA
neurons)
Food-related signals
(Gustatory input)

Figure 1.2 The Major Biosynthesis Pathways for OA in Invertebrates and NE in Vertebrates

The picture was adapted from Roeder (Roeder, 1999) and Cooper et al. (Cooper et al., 2003). OA is synthesized from tyramine (TA) via tyramine ß-hydroxylase (TßH), which is synthesized from tyrosine via tyrosine decarboxylase. NE is a tyrosine derivative as well, whereas NE is synthesized from a different pathway. NE is synthesized from DA via dopamine ß-hydroxylase (DßH), which is synthesized from DOPA via DOPA decarboxylase, and DOPA is from tyrosine. These tyrosine derivatives, OA, DA and NE are close in chemical structures.


Invertebrates
Vertebrates
Tyrosine
Tyrosine decarboxylase (Tdc)
Tyramine
Tyramine-ß-Hydroxylase (TßH)
Octopamine
Tyrosine
Tyrosine hydroxylase (TH)
DOPA
DOPA decarboxylase (Ddc)
Dopamine
Dopamine-ß-hydroxylase (DßH)
Norepinephrine

CHAPTER 2

OCTOPAMINE AND NEUROPEPTIDE F PATHWAYS DIFFERENTIALY REGULATE FEEDING RESPONSE TO FOOD IN *DROSOPHILA*

2.1 INTRODUCTION

Animals display different feeding responses to a broad range of food sources that vary with respect to nutritional quality, taste and foraging cost. However, underlying control mechanisms for food motivation are complex and remain largely underexplored. For example, in hungry animals, food motivation can be enhanced through modification of diverse neural systems including those responsible for receiving and processing sensory properties and assigning reward and motivational significance of food stimuli (Berridge, 2009; Root et al., 2011; Verhagen et al., 2009).

Animals, including humans, have an inborn tendency to prefer readily accessible palatable foods and avoid those requiring high energetic costs. Our previous studies suggest that genetically tractable *Drosophila* larvae offer a useful model for studying adaptive feeding responses to natural rewards (Wu et al., 2003; Wu et al., 2005b). Larvae fed *ad libitum* tend to prefer liquid sugar media but decline foods that are hard to access or have aversive taste. However, as food deprivation is prolonged, larvae display exuberant feeding responses to both preferred and unpreferred food sources.

We previously developed a behavioral paradigm to quantitatively evaluate a hungry larva's willingness to work for food (Wu et al., 2005a). We have shown that larvae become

increasingly persistent in extracting sugar solution embedded in solid agar with their mouth hooks as food deprivation is prolonged. An evolutionarily conserved signaling cascade, involving neuropeptide F (NPF, the fly counterpart of human neuropeptide Y, NPY) and insulin-like peptides (dILPs), has been identified as regulating the motivation of hungry larvae to approach less-accessible solid food (Wu et al., 2005a). The NPF system selectively integrates motivational state (hunger) with persistence to pulverize solid food, while the insulin-like pathway modulates NPF receptor (NPFR1) signaling activity through transduction of hunger signals to NPFR1 neurons (Wu et al., 2005a; Wu et al., 2005b).

The observation that the conserved NPY-like system selectively motivates food acquisition that requires high energetic cost has led us to postulate that fly larvae may employ another conserved neural mechanism to regulate acquisition of readily accessible palatable food. In this work, we provide evidence that support this hypothesis. We show that an OA-dependent circuit mechanism controls larval motivation to selectively acquire readily accessible sugar media.

2.2 RESULTS

2.2.1 Hunger-Evoked Food Motivation of Fly Larvae

Drosophila larvae fed *ad libitum* display a basal level of feeding response to liquid food, which can be quantified by counting the number of larval mouth hook contractions (MHC) during a 30-sec test period. As food deprivation is prolonged, larval feeding response becomes increasingly more intense (Figure 2.1A and Figure 2.1C). For example, 150-min fasting caused an approximately 70% increase in the rate of MHC. To estimate the hunger effect on the peak speed of MHC, we compared the frequencies of MHC between fed and fasted larvae that were

engaged in feeding throughout a 3-second test period. We found that 150 min deprivation only caused a small increase (11%) in the peak rate of MHC (Figure 2.1B). On the other hand, fasted larvae exhibited a large increase of persistence in feeding activity, as evidenced by the shorter time intervals between bites (reflecting increased meal sizes; Figure 1D, E). Therefore, fasted larvae, like hungry mammals, display increased motivation in feeding activity.

2.2.2 Role of *tdc2-Gal4* Neurons in Food Motivation

Our previous study of the NPF system suggests that the incentives for hungry larvae to seek various rewards from food may be regulated by distinct neural mechanisms (Wu et al., 2003; Wu et al., 2005a). The fly OA system has been implicated in reward-mediated learning and memory (Kitamoto, 2001; Schwaerzel et al., 2003). We tested the OA neuronal circuit for its potential role in differential regulation of food motivation by using the *tdc2-Gal4* driver that directs reporter expression in central neurons producing OA and/or tyramine (TA) (Cole et al., 2005). Expression of an inwardly rectifying potassium channel protein (Kir2.1; Baines et al., 2001) directed by *tdc2-Gal4* completely abolished hunger-induced exuberance to approach liquid food (Figure 2.2A). Importantly, fasted *tdc2-Gal4/*UAS-*Kir2.1* larvae showed normal hunger-driven feeding response to less-accessible solid sugar food, opposite to the behavioral phenotypes of fasted *npf-Gal4/*UAS-*Kir2.1* larvae expressing UAS-*Kir2.1* in NPF neurons (Figure 2.2A, B, C).

We also transiently inhibited the neurotransmission of *tdc2-Gal4* neurons by expressing UAS-shi^{ts1}, which encodes a temperature-sensitive, semidominant-negative form of dynamin (Kitamoto, 2001). At the restrictive temperature of 30 ^{o}C, *tdc2-Gal4*/UAS-shi^{ts1} larvae failed to display hunger-driven feeding response to liquid food (Figure 2.3A), but their solid food

procurement activity was normal (Figure 2.3B). These findings suggest that *tdc2-Gal4* neurons are acutely required to regulate hunger-motivated seeking of readily accessible sugar media, and the foraging decisions to seek palatable food requiring low or high energetic cost by hungry larvae are independently regulated by distinct neural systems.

2.2.3 Behavioral Effects of Stimulating *tdc2-Gal4* Neurons

To better understand the functional significance of *tdc2-Gal4* neurons in foraging decisions, we stimulated *tdc2-Gal4* neurons in fed larvae by expressing a UAS-*NaChBac* construct that encodes a bacterial sodium channel (Nitabach et al., 2006). We found that fed *tdc2-Gal4*/UAS-*NaChBac* larvae displayed increased persistence to approach liquid sugar food (Figure 2.4A and 2.4C). Moreover, the enhancing effect of *tdc2-Gal4* neurons on larval MHC is sugar dependent. For example, normal fed larvae exhibited similar basal levels of feeding activity immediately after transfer from growth media to feeding assay media with or without sugar. However, fed *tdc2-Gal4*/UAS-*NaChBac* larvae displayed significantly increased feeding activities in liquid media containing 0.5-10% of glucose, but failed to do so in the sugar-free medium. As expected, fed *tdc2-Gal4*/UAS-*NaChBac* larvae again showed no detectable changes in feeding response to 10% glucose-containing solid food (Figure 2.4B). Therefore, stimulation of *tdc2-Gal4* neurons selectively distorted the foraging decision of *tdc2-Gal4*/UAS-*NaChBac* larvae, resulting in overeating of liquid sugar media.

2.2.4 Selective Regulation of Feeding of Liquid Sugar Media by OA

To test whether OA is directly responsible for the observed phenotypes of *tdc2-Gal4*/UAS-*Kir2.1* and *tdc2-Gal4*/UAS-*NaChBac* larvae, we first examined feeding response to

liquid food in *tβh*^{nM18} larvae carrying a null mutation in the *tyramine β hydroxylase* gene essential for OA synthesis (Monastirioti et al., 1996). We found that blocking of OA synthesis completely abolished hunger-driven feeding response in liquid food, phenocopying the *tdc2-Gal4*/UAS-*Kir2.1* larvae (Figure 2.5A). Moreover, Pre-feeding normal fed larvae with food containing 10 mM of OA also led to a detectable increase in feeding activity (Figure 2.5B). In addition, the same OA treatment of fasted *tdc2-Gal4*/UAS-*Kir2.1* larvae largely restored hunger-driven feeding response to liquid food (Figure 2.5C). These findings indicate that OA signaling underlies the activity of *tdc2-Gal4* neurons in regulation of selective food acquisition.

2.2.5 Differential Regulation of Feeding by OA and NPF

We wondered how simultaneous increases of OA and NPF signaling levels might affect food motivation in fed larvae. To this end, OA was introduced orally to fed *elav-Gal4*/UAS-*npf*^{cDNA} larvae that overexpress NPF. We found that fed *elav-Gal4*/UAS-*npf*^{cDNA} larvae treated with OA behaved similarly to OA-treated fed control larvae in liquid food (Figure 2.6A). However, in solid food, OA-treated *elav-Gal4*/UAS-*npf*^{cDNA} behaved similarly to untreated *elav-Gal4*/UAS-*npf* ^{cDNA} larvae (Figure 2.6B). These results suggest that the OA-mediated circuit mechanism for seeking readily accessible palatable food is functionally independent from that regulated by NPF.

2.3 DISCUSSION

2.3.1 Role of the OA System in Foraging Behaviors of Insects

We have provided genetic and pharmacological evidence for the critical role of OA in the regulation of motivated acquisition of liquid sugar food. OA has been reported to mediate

diverse neurobiological functions including appetitive memory formation and modulation of the dance of honeybee foragers to communicate floral or sucrose rewards (Barron et al., 2007b; Schroll et al., 2006; Schwaerzel et al., 2003). Together, these findings suggest that in insects, OA has been recruited to different neural circuits that promote diverse behaviors beneficial for obtaining high-quality food.

2.3.2 A Complex Neural Network for Differential Regulation of Food Motivation

The incentive to approach food is heavily influenced by the nutritional quality, taste and the energy costs for foraging. Our findings suggest that *Drosophila* larvae have evolved a complex neural network to execute acquisitions of various food sources. In hungry fly larvae, OA neurons appear to mediate a reward/motivation circuit specialized for promoting persistent seeking of readily available liquid food. This OA circuit functions in parallel to the previously characterized NPF/NPFR1-mediated mechanism that drives exuberant feeding of unpreferred solid food (Wu et al., 2003; Wu et al., 2005a). Since food deprivation triggers simultaneous activation of both circuits, hungry larvae become capable of adaptively responding to diverse energy sources of high or low quality. It remains to be determined how OA signaling promotes persistent feeding response to liquid sugar food in hungry larvae. One possible scenario is like midbrain dopamine (DA) neurons that (Matsumoto and Hikosaka, 2009; Schultz, 1998; Tobler et al., 2005), increased activity of OA neurons in the SOG may upregulate a neural circuit for motivated food seeking that may be conditionally activated by gustatory cues associated with rich palatable food.

2.4 EXPERIMENTAL PROCEDURES

2.4.1 Fly Strains, Media and Larval Growth

The fly rearing and the egg collections were performed as previously described (Shen and Cai, 2001). After a 2.5 synchronized egg collection, eggs were kept in a 12-hour light/dark cycle 25˚C incubator. Larvae were transferred to a fresh apple juice plate with yeast paste at the age of 48~52 hour (<80 larvae per plate).

The fly lines used include: *tdc2-Gal4* (Cole et al., 2005), *npf-Gal4*, UAS-npf^{cDNA} (Wu et al., 2003), UAS-*Kir2.1* (Baines et al., 2001), UAS-shi^{ts1} (Kitamoto, 2001), $tßh^{nM18}$ (Monastirioti et al., 1996), UAS-*dTrpA1* (Hamada et al., 2008), UAS-*NaChBac* (Bloomington Drosophila Stock Center at Indiana University).

2.4.2 Behavioral Assays

The rate of larval food intake was quantified by following a previously published protocol with slightly modification (Wu et al., 2005a; Zhang et al., 2010). Liquid glucose agar was prepared by mixing 45ml ddH_2O, 5g D-Glucose (Fisher Chemical) and 6g agar powder (US Biological). Solid glucose agar was prepared by melting a mixture of 90ml ddH_2O, 10g D-Glucose (Fisher Chemical) and 2.3g agar powder (US Biological). The feeding assay was performed in a 35mm Petri Dish containing 1g of glucose-agar paste or 2 ml of solid glucose agar. Solidified glucose agar was further cut into eight blocks as described (Zhang et al., 2010), and allowed to dry in a 70% humidity chamber overnight. 10 ~ 20 early third instar larvae were transferred to the center of the assay plate, and then each plate was videotaped for 2 minutes. The number of mouth hook contraction per 30 seconds was scored and analyzed. The dynamic patterns of larval mouth hook contraction were generated with a computer program using the

MatLab software (MatLab Inc.). All assays were analyzed under blind conditions. At least 3 separate trials were conducted for each test. Statistical analyses were performed using One-way ANOVA followed by Student-Newman-Keuls or by Dunn's test.

For heat shock treatment, larvae were transferred to 30˚C water (food deprivation) or 30˚C yeast paste followed by rinsing with 30˚C water (no food deprivation). The total heat shock time was 20 min before videotaping.

Oral administration of OA was performed by feeding larvae with yeast paste or water containing 10mM OA for 60 minutes before the feeding assay. The stock solution contained 500mM OA in water.

2.4.3 Quantification of Food Intake

Ten to twenty early third-instar larvae (74-76hr AEL) were fed with dyed food for 3 minutes, washed with ice water and then immobilized with boiling water. The dyed liquid food was composed of 2.5 g glucose, 3g agar and 21.5 ml of water. 0.5 ml black food dye was added to 5.4g of liquid food before the feeding assay. Larvae were aligned in a drop of water on the microscope slide, and covered with a coverslip. The slide was placed under a dissection scope (Leica), at the magnificence of 22 fold. Light was adjusted to minimize shadow. The light level was set as 8.5 and the exposure time was 683 ms (Q capture). Larvae were rolled to find the maximal area of gut containing the dyed food and imaged. The color intensity of the larval gut was imaged and quantified by calculating the pixel number using ImageJ.

2.5 ACKNOWLEDGEMENT

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Figure 2.1 Quantification of Hunger-Driven Feeding Responses to Liquid Sugar Food

The liquid food contains 10% glucose agar paste. Unless stated otherwise, synchronized early third instar larvae (74-76 AEL) were used for all behavioral tests. At least 12 larvae were tested for each time point. (A) The rate of larval mouth hook contraction is linearly increased as food deprivation prolongs up to150 min; the correlation coefficiency R = 0.99. (B) The rates of MHC of fed and fasted larvae during the 3 seconds of undisrupted mealtime showed only a small difference (ca. 10%). (C) The initial 3 min food intake gradually increased as food deprivation was prolonged (One way ANOVA followed by Dunn's test, $P<0.05$); the correlation coefficiency R = 0.95. (D) Each dot represents one bite. Three typical feeding patterns sampled from 8 fed or 150 min-deprived larvae are shown. (E) The box plot showing variations of time intervals between bites during a 30 sec period, which are larger in fed animals. Each box represents one individual larva. The inset shows the standard deviation of intervals from eight individual larvae. Statistical analyses were performed using One-way ANOVA followed by Dunn's test; $P<0.05$. Error bars represent the standard error of mean (SEM) in this and all other figures.

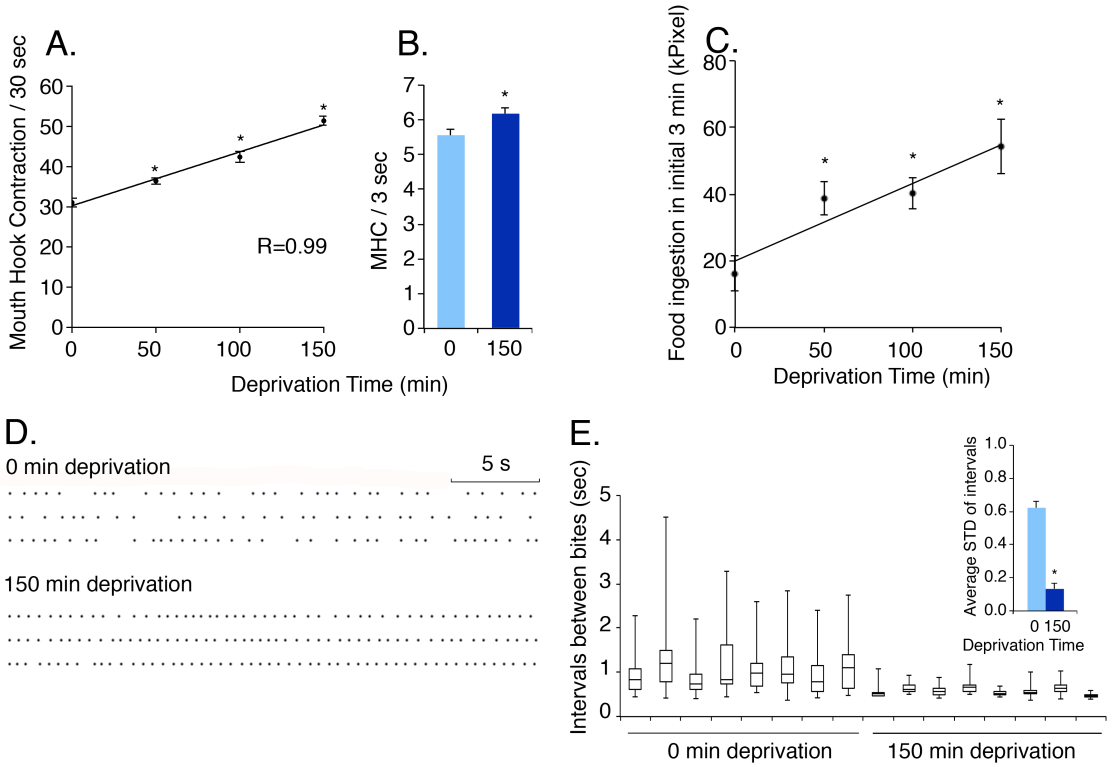
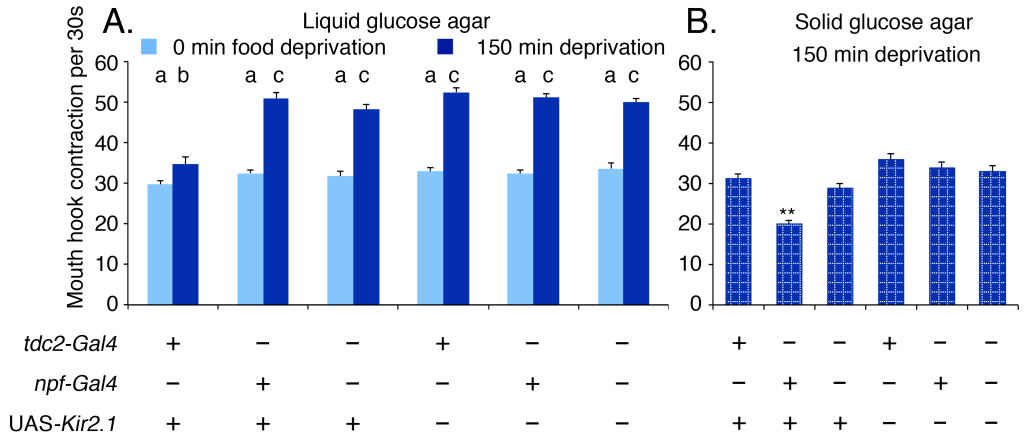

A.
Mouth Hook Contraction / 30 sec
R=0.99
Deprivation Time (min)
B.
MHC / 3 sec
C.
Food ingestion in initial 3 min (kPixel)
Deprivation Time (min)
D.
0 min deprivation
5 s
150 min deprivation
E.
Intervals between bites (sec)
Average STD of intervals
Deprivation Time
0 min deprivation
150 min deprivation

Figure 2.2 Differential Effects of *tdc2-Gal4* and *npf-Gal4* Neurons on Hunger-Driven Food Acquisition

(A) Inhibition of *tdc2-Gal4* not *npf-Gal4* neuronal activity using UAS-*Kir2.1* blocked hunger-induced feeding rate increase in liquid food ($P<0.001$). Columns with identical letters indicate differences are statistically insignificant. (B) In contrast, inhibition of *npf-Gal4* not *tdc2-Gal4* neurons attenuated hunger-induced feeding rate increase in solid food, which contains 10% glucose agar block; $P<0.01$. (C) The dynamic patterns of mouth hook contractions (MHC) of *npf-Gal4/*UA*S-Kir2.1, tdc2-Gal4/* UAS-*Kir2.1* and wild type larvae. The larvae were fasted for 150 min, and feeding assay was performed on 10% glucose agar blocks (solid food). Unless stated otherwise, statistical analyses were performed using One-way ANOVA followed by Student-Newman-Keuls test in all figures.


A.
Liquid glucose agar
0 min food deprivation
150 min deprivation
Mouth hook contraction per 30s
a b
a c
a c
a c
a c
a c
B.
Solid glucose agar
150 min deprivation
**
tdc2-Gal4
npf-Gal4
UAS-Kir2.1

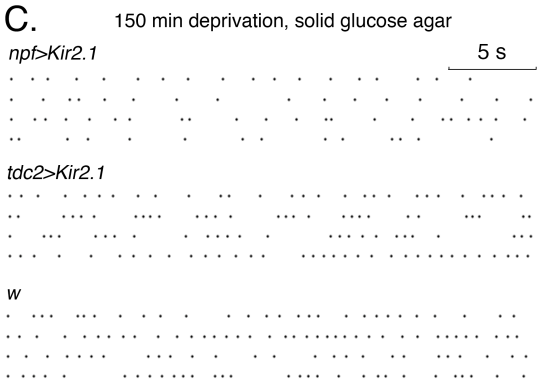

C.
150 min deprivation, solid glucose agar
npf>Kir2.1
5 s
tdc2>Kir2.1
w

Figure 2.3 Acute Inhibition of *tdc2-Gal4* Neuronal Activity Effects on Hunger-Elicited Feeding

(A) Transient inhibition of neurotransmission by *tdc2-Gal4* neurons by expressing UAS-shi^{ts1} at 30˚C abolished the hunger-elicited feeding response to liquid food; $P<0.001$. (B) The same larvae showed no significant difference from the controls in solid food response.

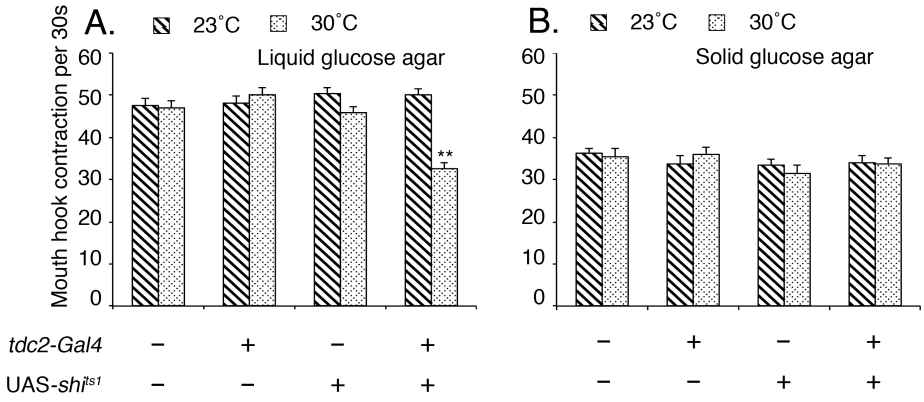

A.
23˚C
30˚C
Liquid glucose agar
Mouth hook contraction per 30s
60
50
40
30
20
10
0
**
tdc2-Gal4 − + − +
UAS-shi^ts1 − − + +
B.
23˚C
30˚C
Solid glucose agar
60
50
40
30
20
10
0
− + − +
− − + +

Figure 2.4 Acute Activation of *tdc2-Gal4* Neuronal Activity Sugar Dependent Effects on Hunger-Elicited Feeding

(A) Stimulation of *tdc2-Gal4* neurons by expressing UAS-*NaChBac* increased feeding response to liquid sugar but not sugar-free media; $P<0.001$. (B) Stimulation of *tdc2-Gal4* neurons failed to increase feeding response to solid sugar food. (C) The dynamic patterns of MHC of fed and fasted fed *tdc2-Gal4/* UAS-*NaChBac* and wild type larvae in 10% glucose agar paste (liquid food).

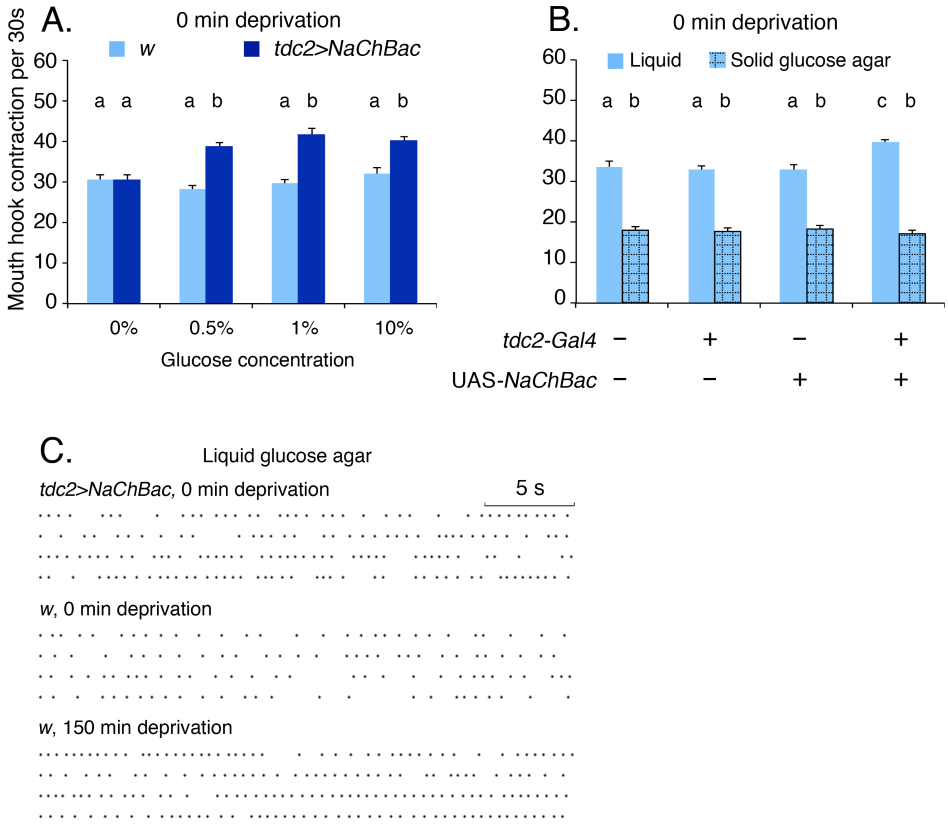

A.
0 min deprivation
w
tdc2>NaChBac
Mouth hook contraction per 30s
60
50
40
30
20
10
0
a a
a b
a b
a b
0%
0.5%
1%
10%
Glucose concentration
B.
0 min deprivation
Liquid
Solid glucose agar
a b
a b
a b
c b
tdc2-Gal4 − + − +
UAS-NaChBac − − + +
C.
Liquid glucose agar
tdc2>NaChBac, 0 min deprivation
5 s
w, 0 min deprivation
w, 150 min deprivation

Figure 2.5 Characterization of the Role of the OA System in Food Motivation

(A) The null mutant *tβh*^{nM18} is deficient in hunger-driven feeding response; One-way ANOVA followed by Dunn's test; $P<0.05$. (B) Prefeeding of fed larvae with 10 mM OA-containing growth media for one hour increased feeding response to liquid food; $P<0.001$. (C) Oral OA treatment significantly restored the hunger-driven feeding response of fasted *tdc2-Gal4/*UAS-*Kir2.1* larvae; $P<0.001$.

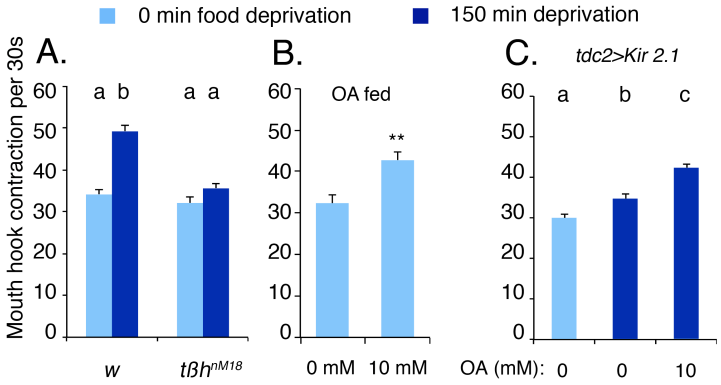
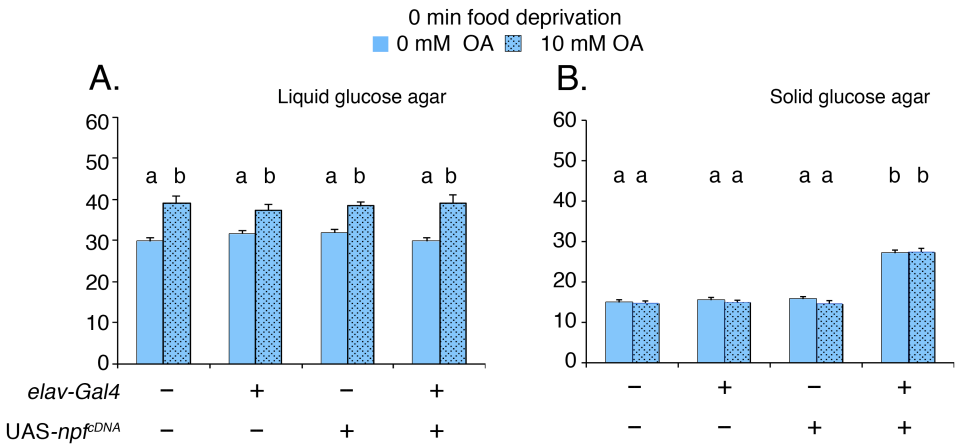

0 min food deprivation
150 min deprivation
Mouth hook contraction per 30s
A.
a b
a a
60
50
40
30
20
10
0
w
tβh^nM18
B.
OA fed
**
0 mM
10 mM
C.
tdc2>Kir 2.1
a
b
c
OA (mM): 0
0
10

Figure 2.6 Differential Regulations of OA and NPF on Hunger-Driven Food Acquisition

(A) Overexpression of NPF failed to further increase feeding response to liquid food by OA-treated fed larvae. (B) Fed NPF-overexpressing larvae showed elevated feeding response to solid food ($P < 0.001$), which was not further enhanced by OA treatment.


0 min food deprivation
0 mM OA
10 mM OA
A.
Liquid glucose agar
B.
Solid glucose agar
60
50
40
30
20
10
0
a b
a b
a b
a b
a a
a a
a a
b b
elav-Gal4 − + − +
UAS-npf^cDNA − − + +

CHAPTER 3

ANTAGONISTIC OCTOPAMINE PATHWAYS FOR FEEDING RESPONSE TO READILY ACCESSIBLE FOOD IN *DROSOPHILA*

3.1 INTRODUCTION

Easy access to energy-rich palatable food makes it difficult to resist food temptation. *Drosophila* larvae are surrounded by rich palatable food most of their life, raising the question how these animals modulate food-seeking motivation in tune with physiological needs. In mammals, the norepinephrine (NE) is known to regulate feeding antagonistically. Activation of α1 and ß adrenergic receptor inhibits feeding, and activation of α2 adrenergic receptor promotes feeding (Kanzler et al., 2011; Leibowitz, 1988; Morien et al., 1993; Ramos et al., 2005). However, the specific neural circuits remain unexplored.

In the previous chapter I described that octopamine (OA), the insect NE equivalent, promotes feeding activity specifically in readily accessible rich food. Here, we report two opposing food-related activities of an OA-mediated neural circuit that act in concert to differentially control larval motivation specific for acquiring readily accessible sugar media. Using genetic and targeted lesion analyses, we show that prevention of overeating in fed larvae involves a subprogram requiring a small subset of OA neurons and α–adrenergic-like receptor Oamb, while exuberant feeding by fasted larvae requires a subprogram involving a separate subset of OA neurons and β-adrenergic-like receptor Octβ3R. These results provide fresh mechanistic insights into how brain mechanisms differentially organize motivated seeking

behaviors in responses to various kinds of food sources under different energy states. Our findings may also have important implications in therapeutic intervention of overeating and drug use disorders.

3.2 RESULTS

3.2.1 Differential Regulations of Feeding by Two OA Receptors

Four different OA receptors have been identified in *Drosophila* (Han et al., 1998; Maqueira et al., 2005). A mifepristone-inducible pan-neural *GS-elav-Gal4* driver was used to perform dsRNA-mediated conditional knockdown of individual OA receptors in the larval nervous system (Figure 3.1A). The *Octβ3R* gene encodes a *β*-adrenergic-like OA receptor (Maqueira et al., 2005). We found that expression of the dsRNA of *Octβ3R* but not other OA receptors in the larval nervous system attenuated hunger-driven feeding response to liquid food. Unlike in *tdc2-Gal4*/UAS-*Kir2.1* larvae, oral introduction of OA failed to rescue the defect of feeding response in fasted GS-*elav-Gal4*/UAS-*Octβ3R*^{dsRNA} larvae (Figure 3.1A and B). Moreover, *Octβ3R*^{MB04794} larvae containing a transposable element that disrupts *Octβ3R* are also deficient in the hunger-driven feeding response (Figure 3.1C). These results suggest that Octβ3R mediates a subprogram that promotes exuberant feeding of liquid sugar media in fasted larvae. However, dsRNA-mediated knockdown of other OA receptor genes, *Oamb*, *oa2*, and *Octβ2R* had no detectable effects on hunger-driven feeding (Figure 3.1A; Han et al., 1998; Hoff et al., 2011; Maqueira et al., 2005).

Intriguingly, fed larvae expressing UAS-*Oamb*$^{dsRNA\text{-}1}$ or UAS-*Oamb*$^{dsRNA\text{-}2}$, driven by *GS-elav-Gal4*, showed significantly increased feeding response to liquid food (Figure 3.2A and B), suggesting that Oamb mediates another subprogram that prevents overeating of readily

available palatable food in fed larvae. However, the knockdown of the other three OA receptors failed to trigger the increase of feeding (Figure 3.2A). Therefore, the control of food motivation in different energy states by the OA-mediated circuit mechanism appears to require the concerted activities of two positive and negative regulatory subprograms.

3.2.2 Functional Mapping of *tdc2-Gal4* Neurons

Tdc2-Gal4 is expressed in multiple clusters of OA neurons in the larval subesophageal ganglia (SOG; Figure 3.3A), which receives sensory inputs from gustatory receptor neurons (Scott et al., 2001; Stocker, 2008; Stocker and Schorderet, 1981; Honjo and Furukubo-Tokunaga, 2009). Anatomical mapping of *tdc2-Gal4* neurons in adults shows three cluster of octopaminergic neurons in the SOG as well, and at the single-cell resolution it revealed that the ventral unpaired median (VUM) neurons in the mandibular compartment and maxillary compartment of SOG appear to project to several common areas of the protocerebrum, while those in the labial compartment of SOG mostly project to the ventral nerve cord (Busch et al., 2009; Busch and Tanimoto, 2010). These mandibular, maxillary and labial VUM octopaminergic neurons in adults correspond to the larval VUM1, VUM2 and VUM3 neurons (Figure 3.3B).

To determine whether the OA neurons in the SOG are involved in larval food motivation, we generated targeted lesions in the subsets of *Tdc2-Gal4* neurons using the laser beam (Xu et al., 2008) (Figure 3.3 B, C and D). Targeted lesions in five VUM1 plus four VPM1 neurons or five VUM1 neurons alone caused a significant increase in the feeding activity of fed larvae (Figure 3.4A), but this increased feeding activity was blocked when five VUM1 and six VUM2 neurons were both ablated (Figure 3.4A). In fasted larvae, targeted lesions in six VUM2 neurons or both VUM1 and VUM2 neurons attenuated hunger-elicited increases of feeding response

(Figure 3.4B), suggesting that proper control of food motivation under fed and fasted conditions requires the negative and positive regulatory activities from VUM1 and VUM2 neurons, respectively. Lesions in all OA neurons in the posterior compartment (5 VUM3 plus 2 VPM3) had no effects on larval feeding response (Figure 3.4A and B). In addition, temperature-induced excitation of *tdc2-Gal4* neurons by expressing UAS-*dTrpA1* triggered increased feeding response to liquid sugar media in fed larvae. However, this *dTrpA1*-stimulated effect was completely abolished by targeted lesions in VUM2 and VPM2 neurons (Figure 3.4C). Together, our findings also raised the possibility that VUM1 neurons may exert the negative regulatory effect in fed larvae by suppressing the positive regulatory effect of VUM2 neurons.

3.3 DISCUSSION

3.3.1 Two Opposing OA Activities in Regulation of Food Motivation

We have provided evidence, at both molecular and neuronal levels, that the OA-mediated feeding circuit exerts two opposing effects on food motivation. When surrounded by liquid sugar media, the OA circuit is essential to prevent fed animals from overeating. Since functional knockdown of *Oamb* activity or targeted lesions in VUM1 neurons caused excessive feeding response, it is possible that VUM1 neurons and α adrenergic-like receptor Oamb may together define an inhibitory subprogram within the OA feeding circuit (Figure 3.5A). We also obtained parallel evidence that functional knockdown of *Octβ3R* activity or targeted lesions in VUM2 neurons attenuated hunger-induced increases of feeding response, suggesting that VUM2 neurons and β adrenergic-like receptor Octβ3R may define a subprogram that enhances feeding in fasted larvae. Further experiments will be needed to determine whether VUM1 and VUM2 neuronal activities are selectively mediated by the OA receptors of Oamb and Octβ3R.

Two distinct OA receptors are required for OA-mediated feeding response to liquid sugar food. Oamb inhibits overeating in fed larvae while Octβ3R stimulates feeding in fasted larvae. It will be interesting to determine whether the Oamb- and Octβ3R-mediated signaling pathways are functionally conserved among insects.

3.3.2 Functional Relationship between VUM1 and VUM2 Neurons

We have shown that targeted lesions in VUM1 neurons alone increased feeding response in fed larvae, but this effect was blocked by double lesions in both VUM1 and VUM2 neurons. These findings suggest that the activity of VUM2 neurons may be suppressed by a VUM1 neurons/Oamb-dependent mechanism under well-nourished conditions. Consistent with this notion, oral introduction of OA or activation of *tdc2-Gal4* neurons with NaChBac or dTrpA1 led to increased feeding response while targeted lesions in VUM2 neurons of fed *tdc2-Gal4*/UAS-*dTrpA 1* larvae completely blocked such overfeeding. The anatomical data also show that VUM1 and VUM2 neurons project to many common areas of the larval brain. Future work will be needed to determine whether VUM1 neurons inhibit directly or indirectly the activity of VUM2 neurons.

3.3.3 Functional Parallels between OA and Norepinephrine (NE) Systems

NE, the vertebrate counterpart of OA, has been shown to promote ingestion of carbohydrate-rich food at the beginning of a natural feeding cycle (Leibowitz et al., 1985; Stanley et al., 1989). This feeding activity of NE resides in the paraventricular nucleus (PVN) of the feeding control center. It has also been shown that in the PVN, α1 and α2 adrenergic receptors are organized in an antagonistic pattern (Ramos et al., 2005); Activation of α1

adrenergic receptor inhibits food intake (Morien et al., 1993), while activation of the α2 receptor stimulates food intake (Leibowitz, 1988). Therefore, the OA system in insects and the NE system in mammals both require a pair of antagonistic receptor activities for regulation of food intake. Another functional parallel is that activation of both OAMB and α1 receptors, which are inhibitory to food intake in flies and rodents, leads to increased intracellular Ca^{2+} signaling (Han et al., 1998; Piascik and Perez, 2001), suggesting that molecular mechanisms underlying the regulation of feeding response by OA and NE systems may be conserved. The activity of NE in PVN has been shown to antagonize that of 5-HT, which suppresses intake of carbohydrate-rich food (Leibowitz and Alexander, 1998). In *Drosophila*, 5-HT is also known to suppress feeding response (Neckameyer, 2010). In conclusion, our studies suggest that *Drosophila* larvae offer an excellent opportunity to gain comprehensive insights into the neurobiology and evolution of motivational mechanisms for acquiring natural rewards as well as pathophysiology of compulsive overeating disorders.

3.4 EXPERIMENTAL PROCEDURES

3.4.1 Fly Strains, Media and Larval Growth

See 2.4.1 for fly rearing and the egg collections. The fly lines used include: *tdc2-Gal4* (Cole et al., 2005), *GS-Elav-Gal4* (Osterwalder et al., 2001), UAS-*dTrpA 1* (Hamada et al., 2008), UAS-$Octß2R^{dsRNA}$, UAS-$Octß3R^{dsRNA}$, UAS-*LacZ-NZ*, UAS-*GFP.nls* (Bloomington Drosophila Stock Center at Indiana University), UAS-$Oamb^{dsRNA\text{-}1}$ (#2861) and UAS-$Oamb^{dsRNA\text{-}2}$ (#106511) UAS-$oa2^{dsRNA}$ (#47896) were obtained from the Vienna Drosophila RNAi Center (Dietzl et al., 2007).

3.4.2 Immunohistochemistry

Larval brains from 74 ~ 76 hour after-egg-laying larvae were dissected for immunostaining, which was performed as previously described (Xu et al., 2008) by using anti-ßGal (Promega) 1:1000, and Alexa 488-conjugated anti-mouse IgG 1:2000, with Phalloidin (Invitrogen) 1:500.

3.4.3 Behavioral Assays

See 2.4.2 for the procedures for the fly larval behavioral assays.

To perform mifepristone-induced expression of *GS-elav-Gal4*, larvae were fed with inactivated yeast paste containing 1mM mifepristone (RU486; Cayman Chemical). Yeast was inactivated by mixing 5g yeast powder with 10ml boiling water. The stock solution contained 20mM mifepristone in 80% ethanol. After cooling to room temperature, the inactivated yeast paste and mifepristone stock solution were mixed at the 20:1 ratio (v/v). 0.5 ml of RU486-containing yeast paste were spread on the surface of apple juice plates, which were air dried for 24 hours before use to evaporate ethanol and excess water. Up to 20 larvae in middle second-instar stage were transferred to RU486- or ethanol- containing yeast plates one day before assay. Induction of *GS-elav* expression was verified using the UAS-*mCD8-GFP* reporter.

3.4.4 Laser ablation

The 337-nm nitrogen laser unit (Micro Point®, Model 337-USAS) was calibrated and adjusted as previously described (Xu et al., 2008). *Tdc2-Gal4* neurons were labeled with UAS-*GFP.nls* expressing a nuclear GFP. Laser ablation of *tdc-Gal4* neurons in the subesophageal ganglion (SOG) was performed in early second-instar larvae (48 hr *a*fter *e*gg *l*aying, AEL). At

the age of 48 hours, 6 to 9 larvae were rinsed briefly and transferred to 150µl ddH_2O on a microscope slide in a 90mm Petri Dish, which serves as an anesthetization chamber. 250µl ether was added to this chamber. After three minutes, the anesthetized larvae were used for laser ablation. Two bursts of 15 shots were fired at a rate of 3 shots per second. After the treatment, larvae from both experimental and control groups were allowed to recover on fresh food plates. The mortality rates at the stage of third instar larvae for mock treated and experimental groups were not significantly different.

3.5 ACKNOWLEDGEMENT

We thank Drs. Jay Hirsh and John Tower for generously providing fly lines. Dr. Yonghua Wang's assistance in the laser ablation and guidance for the imagine technique is gratefully acknowledged. We also thank the TRiP at Harvard Medical School (NIH/NIGMS R01-GM084947) and Vienna Drosophila RNAi Center for supplying transgenic RNAi fly stocks. This work is supported by the grant from US National Institutes of Health (DK058348) to P.S.

Figure 3.1 Excitation of Food Motivation by Octß3R

(A) Conditional knockdown of *Octβ3R* but not other octopaminergic receptors in the nervous system attenuated hunger-driven feeding of liquid food. $P < 0.001$. (B) OA treatment failed to rescue the neural knockdown of *Octβ3R* triggered feeding attenuation ($P>0.14$). (C) $Octβ3R^{MB04794}$ larvae also showed attenuated hunger-driven feeding of liquid food. $P< 0.05$. The $Octβ3R^{MB04794}$ allele has a P-element insertion in the second intron of the *Octβ3R* gene.

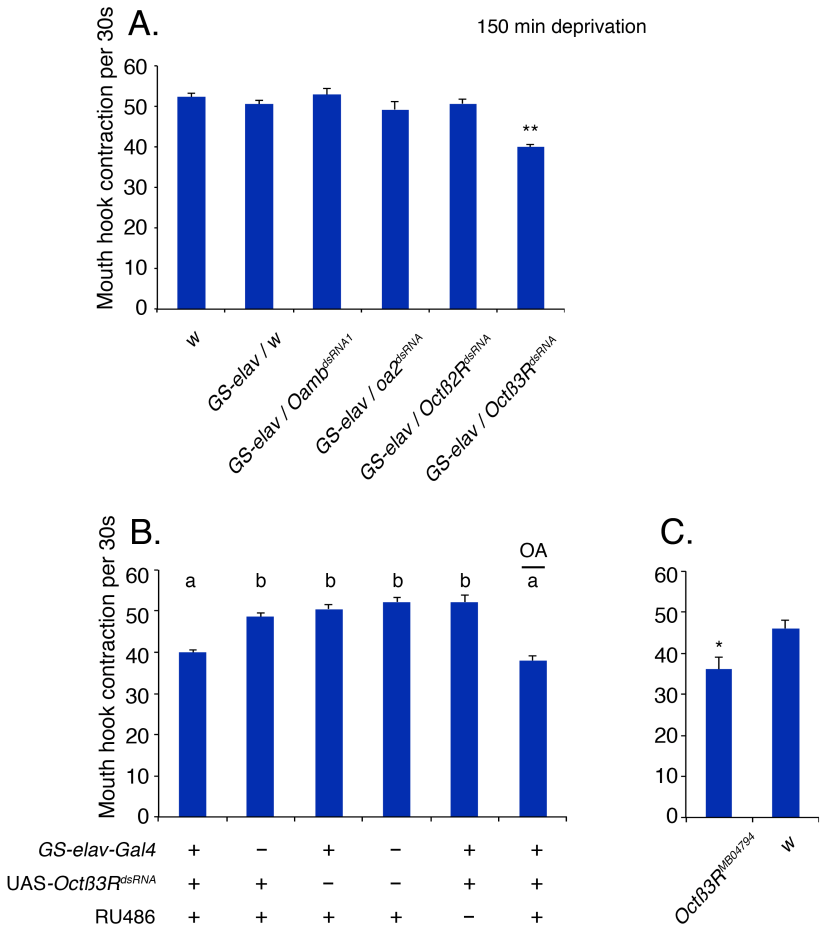

A.
150 min deprivation
Mouth hook contraction per 30s
60
50
40
30
20
10
0
**
w
GS-elav / w
GS-elav / Oamb dsRNA1
GS-elav / oa2 dsRNA
GS-elav / Octβ2R dsRNA
GS-elav / Octβ3R dsRNA
B.
OA
a
b
b
b
b
a
GS-elav-Gal4 + − + − + +
UAS-Octβ3R dsRNA + + − − + +
RU486 + + + + − +
C.
*
Octβ3R MB04794
w

Figure 3.2 Inhibition of Food Motivation by Oamb

(A) Conditional knockdown of *Oamb* but not other octopaminergic receptors in the nervous system promoted feeding response to liquid food in fed larvae. $P<0.01$. (B) Expression of two different *Oamb* dsRNA sequences triggered increased feeding response to liquid food in fed larvae. $P< 0.01$.

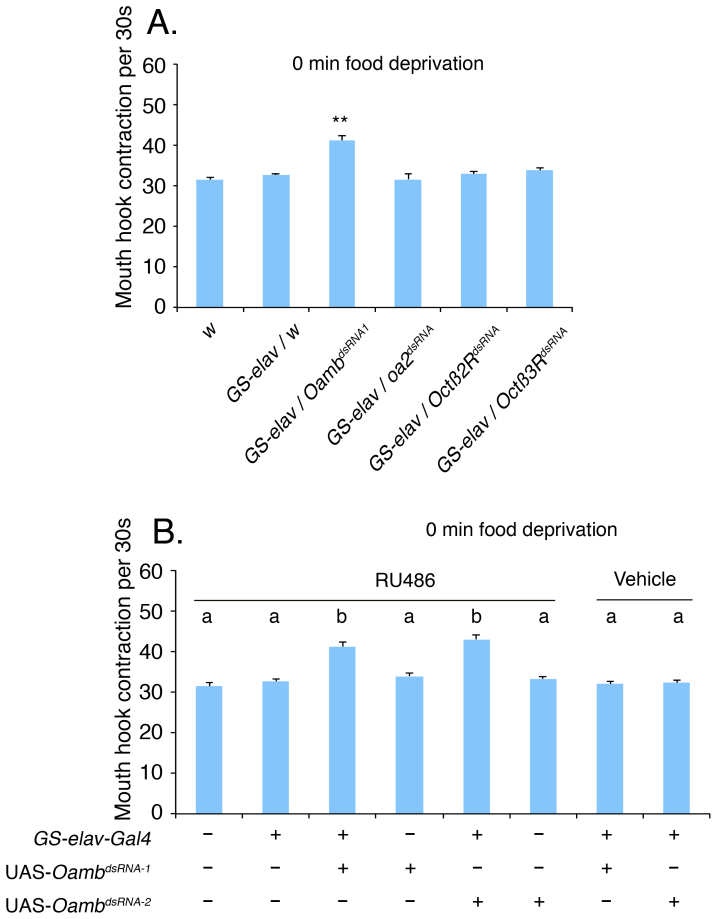

A.
0 min food deprivation
Mouth hook contraction per 30s
60
50
40
30
20
10
0
**
w
GS-elav / w
GS-elav / Oamb dsRNA1
GS-elav / oa2 dsRNA
GS-elav / Octβ2R dsRNA
GS-elav / Octβ3R dsRNA
B.
0 min food deprivation
RU486
Vehicle
a a b a b a a a
GS-elav-Gal4 − + + − + − + +
UAS-Oamb dsRNA-1 − − + + − − + −
UAS-Oamb dsRNA-2 − − − − + + − +

Figure 3.3 Laser Ablation of *tdc2-Gal4* Neurons

(A) Expression of nuclear LacZ in the *tdc2-Gal4* neurons from the brain lobes and ventral nerve cord of second-instar larvae (*tdc2-Gal4/*UAS-*lacZ-NZ*). The rectangular box indicates the subesophageal ganglia (SOG) region. There are 9, 7 and 6 octopaminergic *tdc2-Gal4* neurons including ventral unpaired and paired medial (VUM and VPM) neurons in each of the three sub-segments of the SOG. Arrows indicate three thoracic segments. The tissue is counterstained with phalloidin. Scale bar = 50μm. (B) Expression of nuclear GFP in the brain lobes and ventral nerve cord in the early second instar larvae of *tdc2-Gal4/* UAS-*GFP.nls*. The rectangular box indicates the larval SOG region. (C, D) An example of laser-induced lesions showing GFP fluorescence in the nuclei of targeted VUM1 and VPM1 neurons but not neighboring neurons was greatly diminished after the laser treatment (arrows). The laser beams were focused onto the nucleus of a target neuron, and two bursts of 15 shots were fired at a rate of 3 shots per second. (Xu et al., 2008).

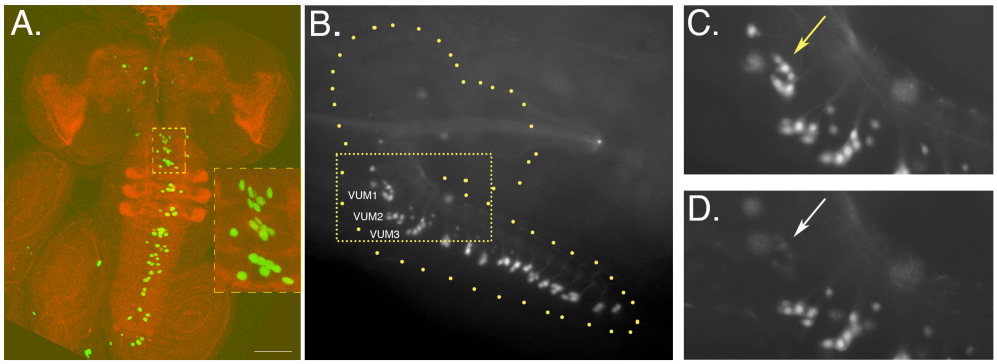

A.
B.
VUM1
VUM2
VUM3
C.
D.

Figure 3.4 Functional Mapping of *tdc2-Gal4* Neurons by Targeted Laser Ablation

(A, B) Functional analysis of feeding-related activities of OA neurons in the SOG in fed and fasted larvae by generating targeted lesions using focused laser beams ($P < 0.01$). (C) *tdc2-Gal4*/UAS-*dTrpA 1* larvae showed abnormally high feeding response, which was blocked by targeted lesions in VUM2 and VPM2 neurons. $P<0.01$.

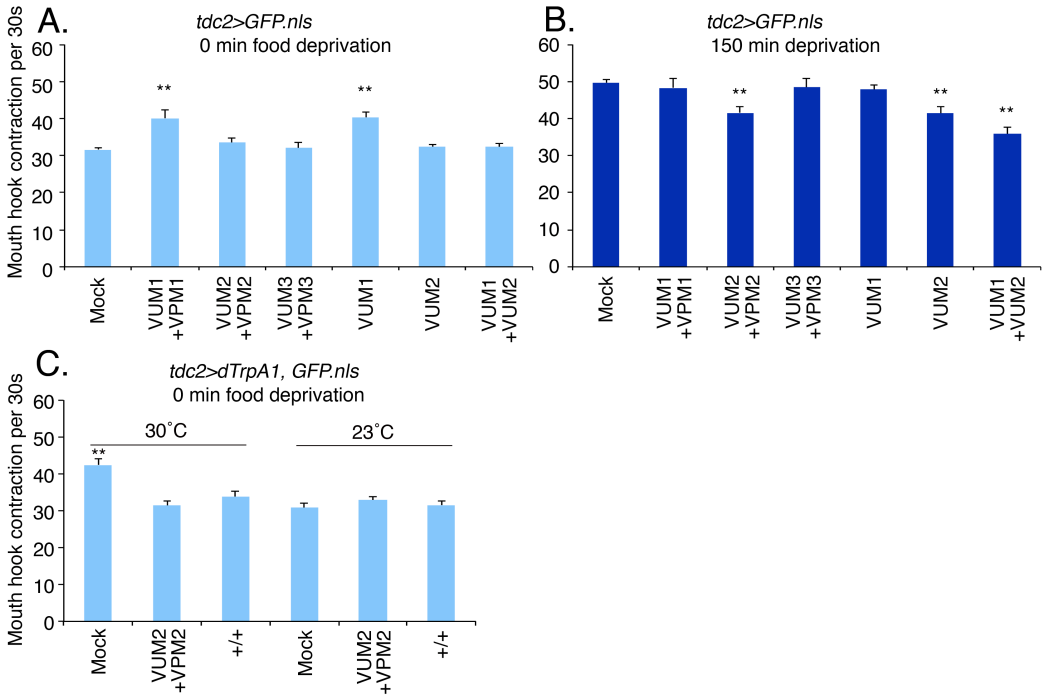
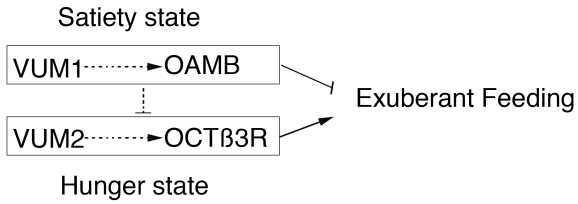

A.
tdc2>GFP.nls
0 min food deprivation
Mouth hook contraction per 30s
60
50
40
30
20
10
0
**
**
Mock
VUM1 +VPM1
VUM2 +VPM2
VUM3 +VPM3
VUM1
VUM2
VUM1 +VUM2
B.
tdc2>GFP.nls
150 min deprivation
60
50
40
30
20
10
0
**
**
**
Mock
VUM1 +VPM1
VUM2 +VPM2
VUM3 +VPM3
VUM1
VUM2
VUM1 +VUM2
C.
tdc2>dTrpA1, GFP.nls
0 min food deprivation
Mouth hook contraction per 30s
30˚C
23˚C
60
50
40
30
20
10
0
**
Mock
VUM2 +VPM2
+/+
Mock
VUM2 +VPM2
+/+

Figure 3.5 A Working Model for the Antagonistic OA Circuits for Regulation of Feeding Activity

(A) A schematic presentation of a working hypothesis proposing two subprograms in the OA circuit for regulation of feeding in fed and fasted larvae.

A. OA circuit for feeding regulation



CHAPTER 4

A PDGF AND VEGF-RELATED RECEPTOR SIGNALNG PATHWAY FOR FOOD RESPONSE IN *DROSOPHILA*

4.1 INTRODUCTION

Downstream of receptor kinases (drk), the mammalian Grb2 homolog, is an adaptor protein that specifically recruits the guanine nucleotide exchange factor son of sevenless (Sos) to phosphorylated receptor tyrosine kinase (RTK) to activate Ras pathway (Olivier et al., 1993). Drk is required in the *Drosophila* mushroom body for both learning and memory stability (Moressis et al., 2009), but its function in other forms of behavioral neuroplasiticity remains largely uncharacterized. Here, we demonstrate that *drk* activity in *tdc2-Gal4* neurons is required in the feeding response to readily accessible food. This observation guided us to search for and find the essential RTK for hunger regulation of food motivation, which is the *PDGF/VEGF-receptor related* (*Pvr*).

In mammals, the receptors of PDGF and VEGF are homologous and have similar pharmacological characters (Jain et al., 2006). PDGF signaling is able to induce glial cell proliferation and has neuroprotective effect (Hart et al., 1989; Heldin and Westermark, 1999; Pietz et al., 1996; Schatteman et al., 1992; Smits et al., 1991). VEGF promotes axonal outgrowth (Sondell et al., 1999), and displays neuroprotective effect against glutamate-induced toxicity (Matsuzaki et al., 2001), and also promotes adult neurogenesis (Cao et al., 2004; Jin et al., 2002). Mammalian PDGF/VEGF signaling function in neuronal plasticity suggests a potential mechanism, by which this signaling pathway may modify animal behaviors in response to the

change of environment. However, multiple ligands and receptors make the study of PDGF/VEGF signaling in mammals difficult and complicated. In contrast, there is only one Pvr in *Drosophila*, which can simplify the pathway to characterize the role of PDGF/VEGF in the neuronal plasticity regulation. In *Drosophila,* the Pvr signaling pathway regulates cell migration (Duchek et al., 2001), hemocyte proliferation (Munier et al., 2002), and axonal growth (Learte et al., 2008). Here, our study is the first to demonstrate that Pvr activity is required for the hunger-driven feeding response to the readily accessible food, which may suggest a novel PDGF/VEGF function in mammals and help develop new treatment and therapy for obesity and eating disorders.

4.2 RESULTS

4.2.1 Downstream Receptor Kinase (Drk) in Hunger-Evoked Food Motivation

From a previous genetic screen, we isolated a candidate gene *drk*, the fly homolog of human *Grb2* (Stern et al., 1993), whose mutations affect larval feeding response to liquid but not solid food (Figure 4.1A). For example, under fed conditions, larvae trans-heterozygous for three independent loss-of-function *drk* alleles $drk^{\Delta P24}/drk^{R1}$, $drk^{\Delta P24}/drk^{10626}$ (Olivier et al., 1993; Simon et al., 1993) showed basal levels of feeding activity similar to wild type larvae. However, after 150-min deprivation, the mutant larvae exhibited significantly attenuated feeding response to liquid food (Figure 4.1A). Functional interference of *drk* activity through expression of *drk* double-stranded RNA (dsRNA) (Moressis et al., 2009), driven by pan-neural *elav-Gal4*, lead to significantly reduced hunger-driven feeding response to liquid food, suggesting that the neural activity of *drk* is responsible for the observed behavioral phenotypes. Conversely, overexpression of *drk* cDNA in fed *elav-Gal4*/UAS-drk^{cDNA} larvae caused excessive feeding

response (Figure 4.1B). Importantly, both fasted *elav-Gal4*/UAS- drk^{dsRNA} and fed *elav-Gal4*/UAS- drk^{cDNA} showed normal responses to solid food (Figure 4.1C).

4.2.2 Regulation of *tdc2-Gal4* Neuronal Signaling by *drk* and *Pvr*

Since loss of the neural activity of *drk* or OA signaling led to similar feeding behavioral defects, we wondered whether *drk* regulates OA neuronal signaling. Indeed, larvae expressing UAS-drk^{dsRNA} in *tdc2-Gal4* but not *npf-Gal4* neurons displayed attenuated hunger-driven feeding response to liquid food, but showed normal food response under fed conditions (Figure 4.2A). Moreover, oral administration of OA to *tdc2-Gal4*/UAS-drk^{dsRNA} larvae largely restored their deficiency in food motivation (Figure 4.2B). In addition, both fasted *tdc2-Gal4* /UAS- drk^{dsRNA} and fed *tdc2-Gal4*/UAS- drk^{cDNA} showed normal responses to solid food (Figure 4.2C). These findings suggest that *drk* regulates liquid food response through its modulation of OA neuronal signaling.

The fact that Drk is a SH2/SH3 adaptor protein that directly binds to activated receptor tyrosine kinase (RTK) strongly implicates the involvement of a yet unknown RTK in modulation of OA neuronal activity by external stimuli. At least fourteen RTK genes have been identified in the *Drosophila* genome (Beiman et al., 1996; Duchek et al., 2001; Dura et al., 1995; Loren et al., 2001; Oates et al., 1998; Pulido et al., 1992; Shilo, 1992). To search for the RTK or RTKs that physiologically regulates OA neurons, we first expressed an array of dsRNA sequences specific to each of the fourteen known RTK genes in *tdc2-Gal4* neurons (Table 4.1). This initial screening led to the identification of three candidates, *PDGF- and VEGF-receptor related* (*Pvr)*, *Eph receptor tyrosine kinase (Eph)* and *heartless* (*htl)* (Table 4.1).

To assess whether the effects of three RTKs on *tdc2-Gal4* neurons are physiological, we conditionally knocked down the individual activity of *Pvr*, *Eph* or *htl* using the *GS-elav-Gal4* driver. Second-instar larvae were fed with yeast paste containing 1mM mifepristone for 22 hours to induce dsRNA expression before the test. We found that only *GS-elav-Gal4*/UAS-Pvr^{dsRNA} larvae showed significantly attenuated hunger-driven response to liquid food (Figure 4.3A). Similarly, *GS-elav*/UAS-drk^{dsRNA} larvae also showed significantly attenuated hunger-driven response to liquid food. These findings indicate that the *Pvr* pathway has a previously uncharacterized role in the physiological regulation of hunger-driven food motivation.

Moreover, the phenotype of *tdc2-Gal4*/ UAS-Pvr^{DN} larvae expressing a dominant-negative form of Pvr provides further verification of the essential role of Pvr in food motivation (Figure 4.4A) (Duchek et al., 2001), and knockdown of *Pvr* in *tdc2-Gal4* neurons failed to change the feeding activity in solid food which phenocopied knockdown of *drk* (Figure 4.4B). The efficiency of the UAS-Pvr^{dsRNA} were also tested by reverse transcript real-time PCR (Figure 4.4D). We also expressed a dominant-active Pvr (Pvr^{ACT}) in *tdc2-Gal4* neurons of fed larvae. The *tdc2-Gal4*/UAS-Pvr^{ACT} fed larvae display excessive feeding in the liquid food, suggesting that increased Pvr signaling in *tdc2-Gal4* neurons is sufficient to trigger overeating of readily accessible palatable food (Figure 4.4C).

To provide evidence for the functional interaction between *drk* and *Pvr* in *tdc2-Gal4* neurons, we co-expressed UAS-Pvr^{dsRNA} and UAS-drk^{cDNA} under the direction of *tdc2-Gal4*. We found that expression of UAS-drk^{cDNA} in fasted *tdc2-Gal4*/UAS-Pvr^{dsRNA} larvae completely restored the deficiency in feeding response to liquid food (Figure 4.5A). Since Drk signaling is mediated by Ras85D GTPase (Raabe et al., 1995), we coexpressed a dominant-negative form of mammalian Ras protein (UAS-Ras^{DN}; (Lee et al., 1996)) together with UAS-drk^{cDNA} in *tdc2-*

Gal4 neurons. As expected, expression of UAS-Ras^{DN} blocked the excessive food response in fed *tdc2-Gal4*/UAS-drk^{cDNA} larvae (Figure 4.5B). Together, these findings suggest that *drk* and *Ras* function in the *Pvr* pathway to regulate OA neuronal signaling.

4.2.2 A Ligand for Pvr with Putative Feeding Regulation Function

The Drosophila genome encodes three PDGF/VEGF homologs (Pvf1-3) that function as the ligands for Pvr (Duchek et al., 2001). It has been shown that the RNA transcripts of *Pvf2* are enriched in the larval CNS, while the *Pvf1* and *Pvf3* transcripts are either very low or undetectable (http://flyatlas.org/atlas.cgi?name=CG13780-RA). We tested the hunger-driven feeding response of homozygous Pvf1, 2 and 3 mutants carrying transposons that disrupt the gene. We found that similar to Pvr-deficient larvae, $pvf2^{d02444}$ larvae displayed significant attenuation of the hunger-driven feeding response (Figure 4.6A), suggesting that Pvf2 and Pvr define a novel neuropeptide system for OA-mediated hunger regulation of food motivation. In previous studies (Wu et al., 2005a, Wu et al., 2005b), we showed that the fly insulin and NPY-like systems co-regulate hunger-elicited motivation to acquire solid sugar media. Together, these findings suggest that the neural activities of distinct RTK systems differentially regulate the neural processes for making or execution of specific foraging decisions under different food and metabolic conditions.

4.3 DISCUSSION

4.3.1 The Role of *Pvr* in OA neurons

We have identified a novel role of *Pvr* in physiological regulation of motivated feeding in preferred liquid sugar food. The feeding activity of the Pvr signaling pathway requires two

regulatory proteins Drk and Ras, and oral introduction of OA restored the hunger-driven feeding response in *tdc2-Gal4*/ UAS-drk^{dsRNA} larvae. Therefore, these results suggest that the Pvr pathway positively regulates OA release by *tdc2-Gal4* neurons. Among the three identified ligands of Pvr (Cho et al., 2002; Duchek et al., 2001), Pvf2 is enriched in the larval CNS, and appears to regulate the feeding-related activity of the Pvr pathway. It is possible that Pvf2 may transduce a metabolic stimulus to Pvr neurons that signals the energy state of larvae. In the honeybee brain, OA neurons from the SOG have been reported to respond to sugar stimulation (Hammer, 1993; Schroter et al., 2007). Therefore, it would be interesting to test whether the Pvf2/Pvr pathway may be responsible for the transduction of sugar stimuli.

Our previous studies showed that the fly insulin and NPY-like systems co-regulate hunger-elicited motivation to acquire solid sugar media (Wu et al., 2005a; Wu et al., 2005b). We have now provided evidence that the fly PDGF/VEGF- and NE-like systems control larval motivation to acquire liquid sugar media. These findings strongly suggest that the neural activities of different RTK systems play critical roles in making or execution of specific foraging decisions under various food and metabolic conditions. Therefore, further investigation of the mechanistic details of the food-related functions of RTK systems in the *Drosophila* model may provide novel insights into the neurobiology and evolution of the reward and motivation systems as well as pathophysiology of reward seeking-related disorders.

4.4 EXPERIMENTAL PROCEDURES

4.4.1 Fly strains, media and larval growth.

The fly rearing and the egg collections were performed as 2.4.1. The fly lines used include: *tdc2-Gal4* (Cole et al., 2005), *npf-Gal4* (Wu et al., 2003), drk^{R1}, $drk^{\Delta P24}$ (Olivier et al.,

1993), UAS-*drk*^{cDNA} (see below), UAS-*drk*^{dsRNA} (Moressis et al., 2009), *GS-Elav-Gal4* (Osterwalder et al., 2001), UAS-*Pvr*^{DN}, UAS-*Pvr*^{ACT}, *drk*^{10626}, *actin-Gal4*, *elav-Gal4*, UAS-*Ras*^{DN}, *Pvf1*^{MB01242}, *Pvf2*^{d02444}, *Pvf3*^{EY09531}, *Pvf3*^{MI02845}, *Octβ3R*^{MB04794} (Bloomington Drosophila Stock Center at Indiana University), UAS-*Pvr*^{dsRNA} (#105353), UAS-*Eph*^{dsRNA} (#4771), UAS-*htl*^{dsRNA} (#27180), UAS-*Oamb*$^{dsRNA-1}$ (#106511), UAS-*Oamb*$^{dsRNA-2}$ (#2861), UAS-*Alk*^{dsRNA}, UAS-*breathless*^{dsRNA}, UAS-*Nrk*^{dsRNA}, UAS-*Dror*^{dsRNA}, UAS-*Torpedo*^{dsRNA}, UAS-*sevenless*^{dsRNA}, UAS-*off-track*^{dsRNA}, UAS-*Cad96Ca*^{dsRNA}, UAS-*torso*^{dsRNA}, UAS-*Insulin-like receptor*^{dsRNA} and UAS-*derailed*^{dsRNA} (Vienna Drosophila RNAi Center ((Dietzl et al., 2007)).

4.4.2 Behavioral Assay

See 2.4.2 and 3.4.3 for the protocol.

4.4.3 Transgenic Constructs

The *drkcDNA* clone from Berkeley Drosophila Genome Project (Rubin et al., 2000) was digested with *Eco*RI and *Xho*I (New England Biolabs), and the resulting 1558 bp sequence was subcloned into the *Eco*RI and *Xho*l sites of the pUAST vector. The purified UAS-*drk*^{cDNA} construct was injected to *w*^{1118} flies (BestGene Inc.).

4.4.4 Quantification of RNA

Total RNAs from *actin-Gal4/UAS-pvrRNAi* and control third-instar larvae (74 hr AEL) were extracted using Trizol (Invitrogen), and 5μg of each RNA sample was reverse transcribed to cDNA using the SuperScript III First-Strand Synthesis Kit (Invitrogen). The forward and reverse primer sequences are as follows: TGCACCGCGATACGAATG and

CGAATTTCTGCGATGAGTTG. The level of *RpS17* transcripts was used as the endogenous control. The real time PCR reaction was performed using Maxima/ROX SYBR Green qPCR Master Mix (Fermentas) in the 7500 Real Time PCR System (Applied Biosystems).

4.5 ACKNOWLEDGEMENT

We thank Drs. Jay Hirsh, Ernst Hafen, Denise Montell, Pernille Rorth, Efthimios M. C. Skoulakis, Henrike Scholz and Gary Struhl for generously providing fly lines. Dr. Kajari Mondal's contribution in the initial genetic screen of feeding mutants is gratefully acknowledged. We also thank the TRiP at Harvard Medical School (NIH/NIGMS R01-GM084947) and Vienna Drosophila RNAi Center for supplying transgenic RNAi fly stocks. This work is supported by the grant from US National Institutes of Health (DK058348) to P.S.

Figure 4.1 Neural Activity of *drk* in Hunger-Evoked Food Motivation

(A) Previous studies have shown that $drk^{\Delta P24}$ is a null allele, and drk^{10626} and drk^{R1} are hypomorph (Moressis et al., 2009). drk^{10626} has a P-element insertion at the upstream of the *drk* coding sequence. drk^{R1} has a point mutation in the SH2 domain that interferes binding to receptor tyrosine kinase. The trans-heterozygous larvae are deficient in hunger-driven food response. $P<0.05$. (B) Knockdown of *drk* in the nervous system reduced hunger-driven food response ($P<0.02$), while its overexpression increased food response in fed larvae (One-way ANOVA followed by Dunn's test; $P<0.05$). (C) Reduction of *drk* neural activity had no effects on solid food response.

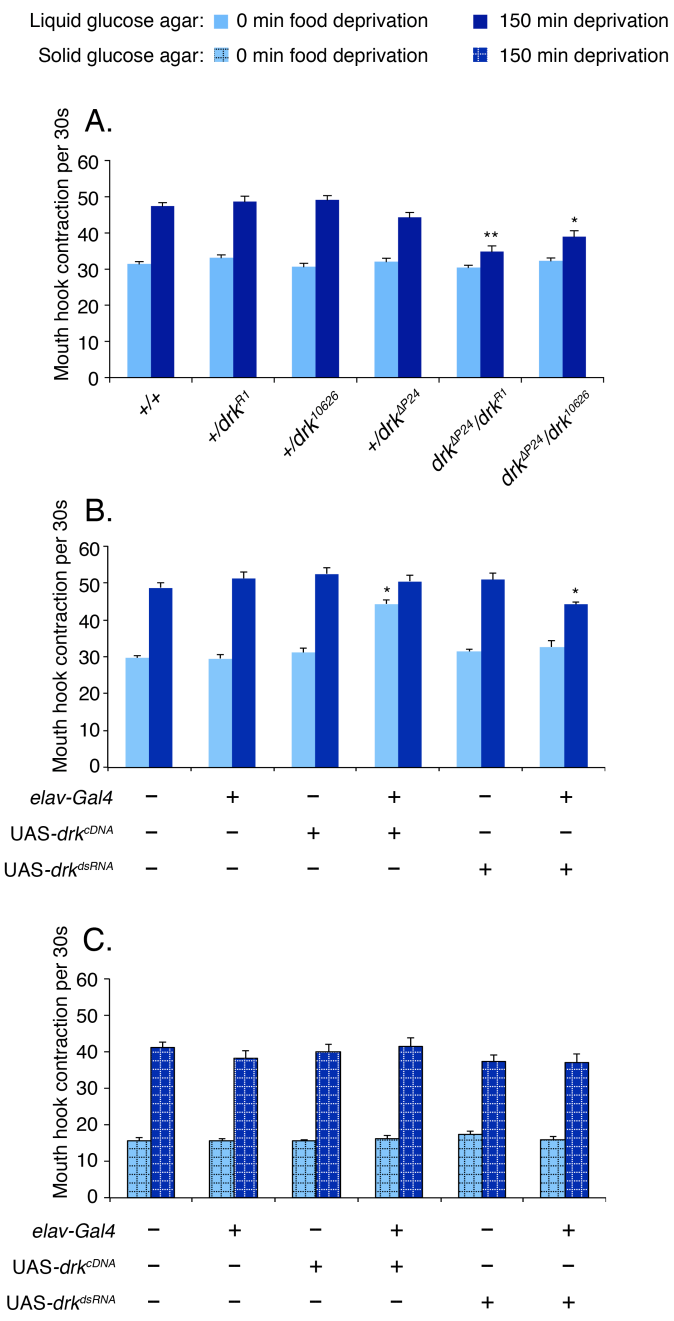

Liquid glucose agar: 0 min food deprivation
150 min deprivation
Solid glucose agar: 0 min food deprivation
150 min deprivation
A.
Mouth hook contraction per 30s
60
50
40
30
20
10
0
**
*
+/+
+/drkR1
+/drk10626
+/drkΔP24
drkΔP24/drkR1
drkΔP24/drk10626
B.
Mouth hook contraction per 30s
*
*
elav-Gal4 − + − + − +
UAS-drkcDNA − − + + − −
UAS-drkdsRNA − − − − + +
C.
Mouth hook contraction per 30s
elav-Gal4 − + − + − +
UAS-drkcDNA − − + + − −
UAS-drkdsRNA − − − − + +

Figure 4.2 *Drk* Activity in *tdc2-Gal4* Neurons for Hunger-Evoked Food Motivation

(A) Knockdown of *drk* activity in *tdc2-Gal4* neurons attenuated hunger-driven food response. One-way ANOVA followed by Dunn's test; $P<0.05$. (B) OA treatment of *tdc2-Gal4*/UAS-drk^{dsRNA} larvae rescued the deficiency of hunger-driven response to liquid food. $P<0.01$. (F) Modulation of *drk* expression in *tdc2-Gal4* neurons had no effects on solid food response.

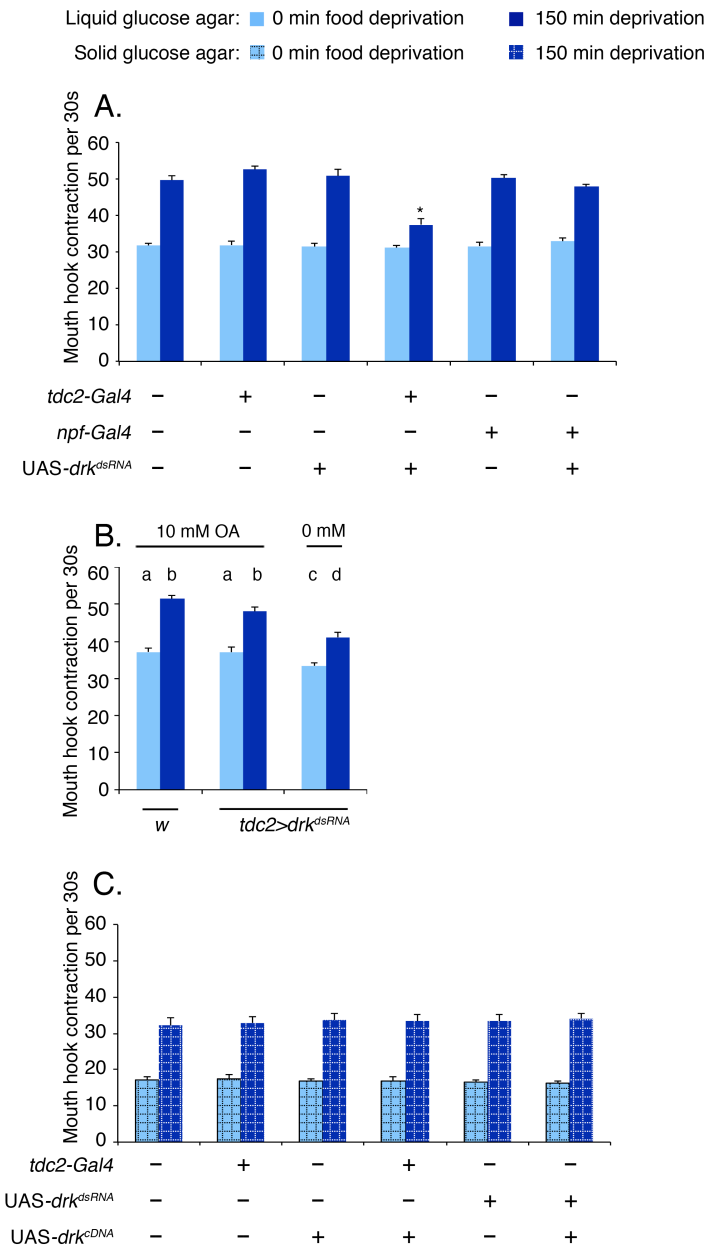

Liquid glucose agar: 0 min food deprivation
150 min deprivation
Solid glucose agar: 0 min food deprivation
150 min deprivation
A.
Mouth hook contraction per 30s
60
50
40
30
20
10
0
*
tdc2-Gal4 − + − + − −
npf-Gal4 − − − − + +
UAS-drk^dsRNA − − + + − +
B.
10 mM OA
0 mM
a b a b c d
Mouth hook contraction per 30s
w
tdc2>drk^dsRNA
C.
Mouth hook contraction per 30s
tdc2-Gal4 − + − + − −
UAS-drk^dsRNA − − − − + +
UAS-drk^cDNA − − + + − +

Table 4.1 Expression of 14 RTK Double-Stranded RNAs (dsRNAs) in *tdc2-Gal4* Neurons

DsRNA of individual RTKs in *tdc2-Gal4* neurons	**Mouth hook contraction per 30s**	
	0 min food deprivation	150 min deprivation
Alk	32.33 ± 1.71	52.40 ± 1.53
breathless	32.56 ± 1.16	49.88 ± 2.04
Nrk	33.38 ± 1.49	48.43 ± 1.21
Dror	32.00 ± 1.32	51.67 ± 2.03
Torpedo(EGFR)	32.80 ± 2.69	50.00 ± 1.30
sevenless	32.88 ± 0.70	52.00 ± 1.73
off-track	31.45 ± 0.69	45.73 ± 1.08
Cad96Ca	30.20 ± 3.04	52.22 ± 1.22
torso	30.20 ± 1.43	49.40 ± 1.72
Insulin-like receptor	34.60 ± 1.92	45.94 ± 1.55
derailed	33.60 ± 1.19	45.65 ± 1.84
Eph receptor	32.80 ± 0.97	40.47 ± 1.15**
heartless	34.64 ± 2.58	42.45 ± 2.44**
Pvr	31.50 ± 1.71	41.70 ± 1.36**
w	31.38 ± 0.61	50.70 ± 0.88
tdc2-Gal4/w	31.07 ± 0.77	49.00 ± 1.43

Statistical analysis was performed using One-way ANOVA followed by Student-Newman-Keuls test. $N \geq 5$, and $P < 0.01$ for **.

Figure 4.3 Identification of the RTK that Acutely Regulates Hunger-Elicited Food Motivation

(A) Conditional expression of the dsRNA of different RTKs was induced by adding RU486 to larval growth media at the stage of second instar. Expression of drk^{dsRNA} and Pvr^{dsRNA} but not Eph^{dsRNA} *or* htl^{dsRNA} reduced hunger-driven food response. Statistic analysis was performed using one-way ANOVA followed by Dunn's test; $P<0.05$.

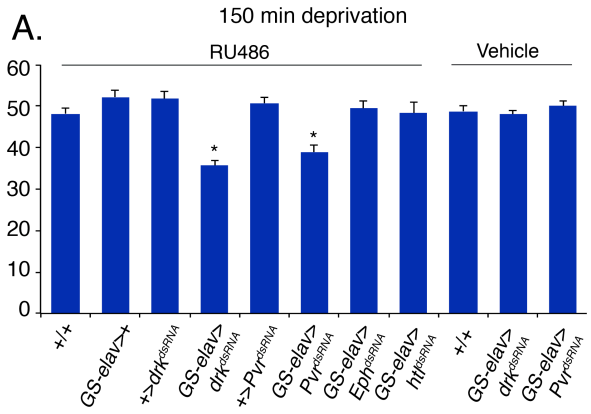

A.
150 min deprivation
RU486
Vehicle
60
50
40
30
20
10
0
*
*
+/+
GS-elav>+
+>drk dsRNA
GS-elav> drk dsRNA
+>Pvr dsRNA
GS-elav> Pvr dsRNA
GS-elav> Eph dsRNA
GS-elav> htt dsRNA
+/+
GS-elav> drk dsRNA
GS-elav> Pvr dsRNA

Figure 4.4 Role of the Pvr in Hunger-Driven Food Motivation

(A, B) Expression of UAS-Pvr^{dsRNA} and UAS-Pvr^{DN} in *tdc2-Gal4* neurons, which encodes a dominant-negative form of Pvr, attenuated hunger-driven feeding response to liquid not solid food. $P< 0.001$. (C) Expression of UAS-Pvr^{ACT}, which encodes a constitutively active Pvr, caused increased feeding in fed larvae. $P< 0.01$. (D) The functional interference by UAS-Pvr^{dsRNA} was confirmed by the quantitative RT-PCR analysis. $N=9$, and $P<0.001$.

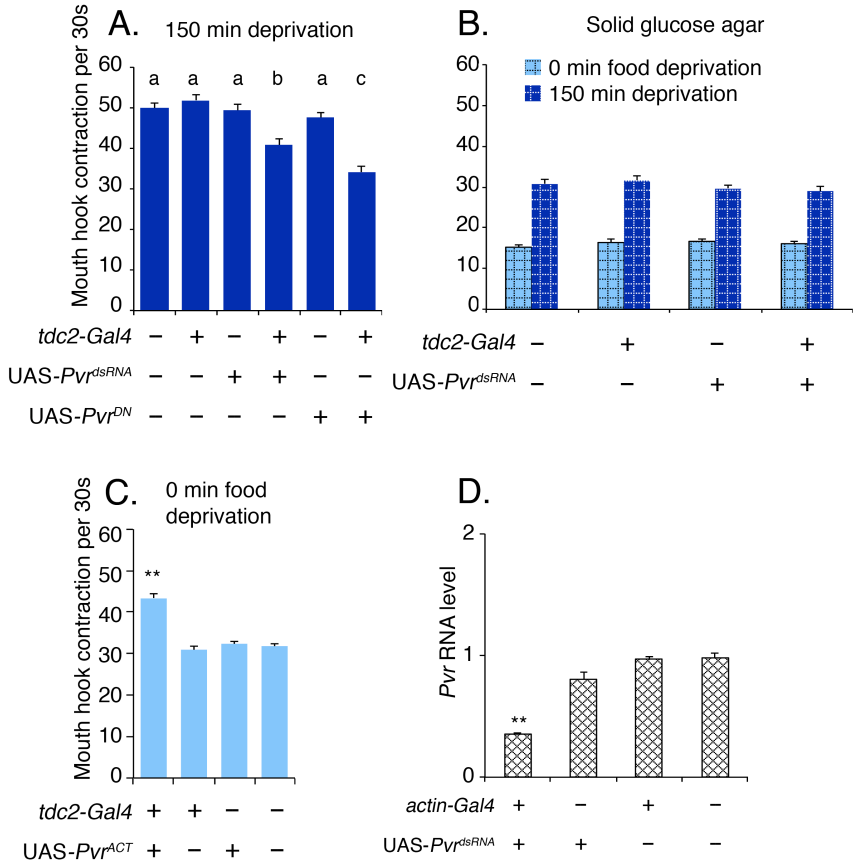

A.
150 min deprivation
Mouth hook contraction per 30s
a a a b a c
tdc2-Gal4 – + – + – +
UAS-Pvr^dsRNA – – + + – –
UAS-Pvr^DN – – – – + +
B.
Solid glucose agar
0 min food deprivation
150 min deprivation
tdc2-Gal4 – + – +
UAS-Pvr^dsRNA – – + +
C.
0 min food deprivation
Mouth hook contraction per 30s
**
tdc2-Gal4 + + – –
UAS-Pvr^ACT + – + –
D.
Pvr RNA level
**
actin-Gal4 + – + –
UAS-Pvr^dsRNA + + – –

Figure 4.5 Identification of the Pvr/drk/Ras Pathway in Hunger-Driven Food Motivation

(A) Fasted larvae expressing both drk^{cDNA} and Pvr^{dsRNA} in *tdc2-Gal4* neurons displayed normal liquid food response. P< 0.001. (B) Fed larvae expressing both drk^{cDNA} and Ras^{DN} in *tdc2-Gal4* neurons also displayed normal liquid food response. P< 0.001.

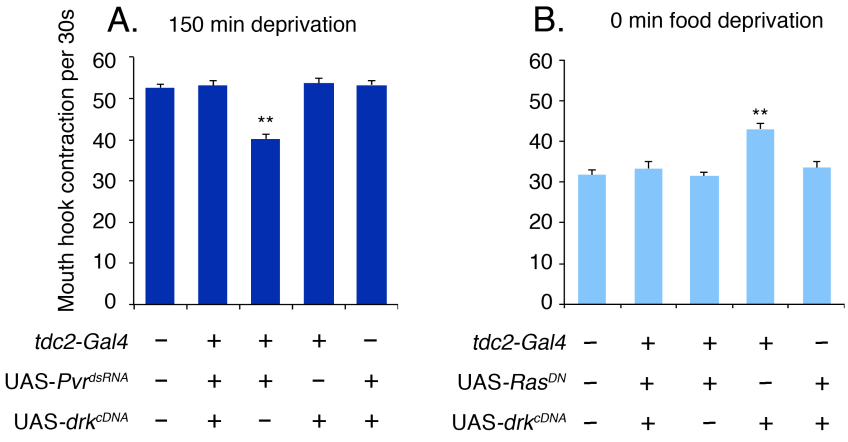
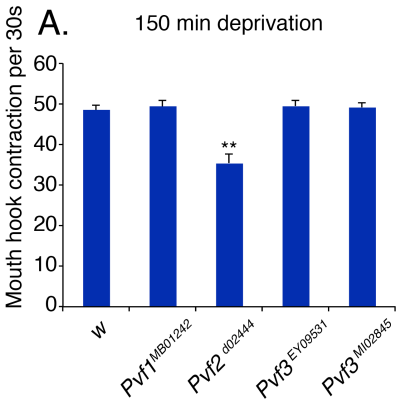

A.
150 min deprivation
Mouth hook contraction per 30s
60
50
40
30
20
10
0
**
tdc2-Gal4 − + + + −
UAS-Pvr^dsRNA − + + − +
UAS-drk^cDNA − + − + +
B.
0 min food deprivation
60
50
40
30
20
10
0
**
tdc2-Gal4 − + + + −
UAS-Ras^DN − + + − +
UAS-drk^cDNA − + − + +

Figure 4.6 The Pvr Ligand Pvf2 as a Putative Effector in Hunger-Driven Food Motivation

(A) Using feeding assay, screening of different mutants in the three Pvr ligand genes found that fasted $Pvf2^{d02444}$ larvae but not the mutations in other ligand genes showed attenuated feeding response. $P < 0.001$.


A.
150 min deprivation
Mouth hook contraction per 30s
60
50
40
30
20
10
0
**
w
Pvf1MB01242
Pvf2d02444
Pvf3EY09531
Pvf3MI02845

CHAPTER 5

CONCLUSIONS AND DISCUSSION

Animals have strong instincts to promote feeding on energy-rich food, while feeding on food that requires high-energy expenditure is inhibited. It remains to be determined how these foods are distinguished, and how the decisions are made. Our system is comprised of two feeding assays for studying these two behaviors separately. The liquid food assay provides a platform to test the feeding behaviors on energy-rich and readily accessible food. In contrast, the solid food is non-readily accessible food, on which the larvae need to invest relatively high energy to extract sugar from the food medium. Intriguingly, we found that NPF and OA signaling independently regulate the non-readily and readily accessible food, respectively. The NPF/NPFR1 circuit disinhibits feeding on the nonpreferred solid food (Wu et al., 2003; Wu et al., 2005a). Comparably, the OA circuit appears to promote feeding on readily accessible liquid food. However, the detailed neuronal mechanisms underlying NPF and OA on feeding regulation remain underexplored. The previous studies in our lab showed that NPFR1 suppressed the sensitivity of nociceptive sensory neurons by attenuating Ca^{2+} influx (Xu et al., 2010). It is possible that NPFR1 also suppresses the sensitivity of other sensory neurons, such as the mechanic sensor for food hardness. In contrast, OA may adopt a similar mechanism as the locus coeruleus NE neurons and midbrain dopamine (DA) neurons (Foote et al., 1980; Matsumoto and Hikosaka, 2009; Schultz, 1998; Tobler et al., 2005), with appetitive gustatory cues increasing the activity of OA neurons in the SOG, and it might in turn activate a neural circuit for motivated food seeking.

In invertebrates, OA activity is required for appetitive responses to food, such as palatable food elicited food intake (Long and Murdock, 1983), appetitive learning behavior (Kitamoto, 2001; Schwaerzel et al., 2003) and the food-value encoding in honeybee dance languages (Barron et al., 2007b). However, how OA functions in the appetitive response is not well characterized, and more information is needed to explore the underlying mechanism. My research provides further evidence suggesting that OA signaling regulates fly larva feeding behaviors specifically for readily available food, and that this behavior is sugar (reward) - dependent. We also propose that two subsets of octopaminergic neurons antagonistically regulate feeding activity in *Drosophila* larvae.

The octopaminergic neurons of insects originate in the ventral nerve cord and the SOG (Monastirioti et al., 1996; Roeder, 1999). The SOG plays a role as the feeding center in insects. In *Drosophila* larvae, all gustatory signals transduce first to the SOG (Gerber and Stocker, 2007). As a result, it is plausible that the octopaminergic neurons may receive appetitive signal inputs at the SOG. This was supported by findings in a honeybee study, in which the SOG octopaminergic neurons responded to sucrose stimulation with a burst of action potentials *in vitro* (Schroter et al., 2007). *Drosophila* SOG octopaminergic neurons project to different brain regions, including the mushroom body (MB) (Busch et al., 2009; Busch and Tanimoto, 2010). The MB controls learning and memory in *Drosophila*, and OA is known to be required for appetitive learning (Schroll et al., 2006; Schwaerzel et al., 2003). As a result, OA circuit was presumed to carry appetitive signals to MB (Gerber and Stocker, 2007). The *Drosophila* octopaminergic receptor Oamb enriches in the mushroom body, and Oamb acts to increase intracellular cAMP level upon binding OA (Han et al., 1998). In addition, cAMP signaling is essential to enable learning behavior (Honjo and Furukubo-Tokunaga, 2005). Consequently, Oamb has been considered as a

putative candidate for controlling appetitive learning (Evans and Maqueira, 2005; Kim et al., 2007; Lee et al., 2003). However, so far, no research has been published to support the idea that Oamb regulates appetitive learning. In fact, one PhD dissertation described that the knockdown of Oamb in the MB or projection neurons failed to inhibit appetitive learning (Thum, 2006). Together with my observations, in which Oamb inhibits but Octß3R promotes feeding response to rich food, it suggests that it may be Octß3R rather than Oamb that receive the appetitive signals from octopaminergic neurons, and Octß3R may be a putative appetitive-associate learning regulator.

OA may regulate the reward system by modulating other neurotransmitters involved in reward signaling, as NE does in mammalian systems. In mammals, DA in the nucleus accumbens (NAc) is required for the reward response (Taylor and Robbins, 1986; Wise, 2004). When the rats are fed with palatable food, both DA and DA metabolite levels increase in the NAc area (Fallon et al., 2007), whereas NE levels are reduced together with NE metabolite 3-methoxy-4-hydrophenylglycol (MHPG) level increases (Fallon et al., 2007). This suggests that DA but not NE activity in the NAc increases in response to food. Interestingly, a recent study by Ventura et al. suggested that NE in the medial prefrontal cortex (mpFC) is required for the DA increase in the NAc in response to rewards (Ventura et al., 2007). Compared with sham treated control group, the depletion of NE with 6-OHDA in the mpFC prevents DA from increasing in the NAc in response to palatable foods or even cocaine (Ventura et al., 2007). However, these studies depleted NE by killing noradrenergic neurons with 6-OHDA. Although the authors used GBR 12909 to prevent dopamine transporters from uptaking 6-OHDA (Ventura et al., 2007), neurons with both dopamine transporters and NE transporters still can die from 6-OHDA uptake. Correspondingly, some dopaminergic neurons should be killed at the same time if DA co-

localizes and is co-released with NE, which is possible since NE is synthesized from DA (Figure 1.2), and should be co-localized in dopaminergic neurons. NE might be required for DA activity regulation in the reward system, but further investigations are needed to examine this hypothesis.

NE in the PVN seems to function to regulate food intake. However, some relatively recent studies argued that this regulation in food intake might be due to a modulation of leptin and NPY. Leptin is a protein secreted from adipocytes that inhibits feeding (Caro et al., 1996), whereas NPY is a neuropeptide that stimulates feeding (Kalra, 1997). One rat study reported that NPY mRNA level increases and leptin mRNA decreases in response to NE depletion in the ventral noradrenergic bundle in dark phase compared to saline treated group (Kalra et al., 1998). Also, rats receiving an intracerebroventricular (i.c.v.) injection of α1 adrenergic receptor agonist show reduced cerebrocortical NPY-immunoreactivity (Chang et al., 1996), which is consistent with findings that show that the activation of α1 adrenergic receptor reduces food intake (Morien et al., 1993). Some antiobesity drugs use NE reuptake inhibitors to suppress feeding (Valassi et al., 2008), and this NE satiety effect might act via the activation of α1 adrenergic receptor to reduce NPY activity. Consequently, the NE-regulated feeding may be at least partially due to the modulation of NPY and leptin. In addition, leptin reduces NE release from the PVN (Kutlu et al., 2010) and NPY increases it (Hastings et al., 1997), which might form a feedback loop for NPY and leptin regulation. In contrast, my data suggests that OA and NPF are independent in *Drosophila* feeding regulation, which may be divergent from NE/NPY interaction in the mammalian system.

The OA circuit may carry the taste-driven reward signals from food in insects. In arthropods, researchers suggest that OA is required for reward responses, but DA is only required for punishment (Barron et al., 2010; Honjo and Furukubo-Tokunaga, 2009; Schwaerzel

et al., 2003), because the inhibition of dopaminergic neurons specifically defects aversive learning but not appetitive. In *Drosophila*, classical olfactory learning and memory studies adopt Pavlovian training procedures (Tully and Quinn, 1985). Researchers trained flies to associate an odor with sugar or electrical shock stimulus in appetitive and aversive associated learning, respectively, followed by a test of memory retrieval (Schwaerzel et al., 2003; Tully and Quinn, 1985). In insects, OA is required for the appetitive but not the aversive learning behavior in the acquisition step, while the aversive-associated acquisition requires DA instead (Schwaerzel et al., 2003). Comparably, several vertebrate studies also show that DA responds to aversive stimuli as well (Fallon et al., 2007), which is consistent with DA function in insects. Knockout of *tβh* (Figure 1.2), which eliminates OA thoroughly in *Drosophila* (Monastirioti et al., 1996), prevents the appetitive-associated rather than aversive-associated learning ability without causing defects in the sensory acuity of sugar. Both the manipulation of octopaminergic neurons and OA treatment alter the appetitive learning ability in *Drosophila* (Honjo and Furukubo-Tokunaga, 2009; Kitamoto, 2001; Schwaerzel et al., 2003). In contrast, the blocking of *Th-Gal4* neurons (Figure 1.2), which depletes synaptic DA, inhibits the aversive learning but not the appetitive learning (Honjo and Furukubo-Tokunaga, 2009). Therefore, OA is necessary for appetitive memory acquisition rather than memory retrieval in *Drosophila*, and DA is necessary for aversive acquisition. More interestingly, light-induced *tdc2-Gal4* neuron excitation with UAS-*ChR2* during training is capable of substituting unconditional stimuli such as sucrose stimulation, allowing flies to pass the memory test (Schroll et al., 2006), suggesting that the reward signal is carried by the octopaminergic circuit. A few recent studies suggested that DA regulates both aversive and appetitive responses in insect as well as in mammals. For example, the excitation of a specific DA neuron in the *Drosophila* SOG is essential in the proboscis extension responses to

sucrose solution (Marella et al., 2012). It has not been determined that whether OA modulates DA circuit to regulate the reward signal transduction.

These studies demonstrate that reward stimuli increase OA activity, and that this activity regulates appetitive reinforcement of *Drosophila* in the learning process. They also indicate that OA is released when fruit flies are in an appetitive state. Furthermore, the light-induced learning assay suggests that OA is sufficient to activate the reward circuits. Although the octopaminergic neurons are not required for reward stimulus perception, they are necessary for reward stimulus cognition.

Another piece of evidence for the reward-response function of OA comes from honeybees. One unique characteristic of honeybees is that honeybee foragers employ dance to exchange information about the food source quality (Seeley and Towne, 1992; Von Frisch, 1967), which quantifies honeybee's desire for foods. Their dance components, such as dance likelihood, vigor, and duration, report the food resource value. Moreover, during pollen collection, the foragers collect rather than ingest food (Barron et al., 2007b), which functionally separates reward stimulus from internal states. The OA-treated foragers perform dances indicating more profitable food compared to the non-OA-treated foragers when reporting the same food source, and the evaluation is dose dependent (Barron et al., 2007b). Similarly, in blowflies OA injection reduces the threshold of proboscis extension response (PER) to sucrose solution (Long and Murdock, 1983). Therefore, insect evaluation of food quality is positively correlated with OA level, further supporting the role of OA in reward cognition. Insects with elevated OA level display increased sensitivity to the reward cues in food, which is consistent with my finding that the excitation of octopaminergic neurons promotes larva feeding on sugar liquid food medium.

In my research on *Drosophila* feeding behaviors, it is possible that OA signaling activity in VUM2 neurons serves to evaluate the reward value from the food quality, and carry the signals to an upper neuron circuit to activate the motivated food seeking behavior. Further studies are needed to seek for the target neurons, which may possibly be DA neurons according to the findings in the mammalian systems. It is also not characterized whether the palatable food cues stimulate OA to synthesize or to release, and whether the amount of OA released or synthesized is correlated to reward quality or intensity. With *Drosophila* as a model, we can inhibit or activate OA neurons with effectors such as Kir2.1 and NaChBac, respectively, and then we can quantify DA level with immunostaining. With the dual binary transcriptional systems GAL4/UAS and LexA/LexAOP, the effectors and the reporters can be expressed independently in DA and OA neurons, which will test the effect of one neurotransmitter manipulation on the other one. There are many reporters available in *Drosophila* that are able to indicate Ca^{2+} flux, cAMP activity or gene transcriptional level, such as G-camp (Nakai et al., 2001) and Epac-camps (Vincent et al., 2008). In addition, these reporters can be detected *in vivo*, which enables the measurement of live cell activities in different parameters. The OA/DA interaction in *Drosophila* may help to understand the NE/DA interaction in vertebrates.

Animals display feeding responses to tasty, energy-rich, and readily accessible food. However, the neural circuits and molecular mechanisms remain uncharacterized. With the solid and liquid food assays, my work has genetically dissected the complicated neuronal pathways regulating feeding behaviors. We have demonstrated that OA plays dual roles in the modulation of feeding response to rich food in *Drosophila* larvae. It may activate different receptors, depending on the internal state of the fly larvae. On the one hand, even when surrounded by rich and tasty food, OA may prevent well-fed larvae from overeating by activating Oamb. On the

other hand, when the larvae are deprived from food, OA may activate Octß3R to promote the larvae to approach to the rich food. My work also identified two subsets of octopaminergic neurons, VUM1 and VUM2, which differentially regulate feeding activities in rich food. The octopaminergic VUM1 neurons seem to function in satiated larvae to inhibit the feeding response to rich food, whereas the octopaminergic VUM2 neurons may function in the energy-deprived state to promote feeding in rich readily accessible food. Compared to VUM1 ablation, when both VUM1 and VUM2 are ablated, fed larvae feeding activity was attenuated to the normal base line of the naïve group, suggesting that VUM1 may inhibit the activity of octopaminergic signaling from VUM2. Although the interactions between Oamb and Octß3R, VUM1 and VUM2, and VUM1/Oamb and VUM2/Octß3R remain to be determined, we identified the novel circuits for antagonistically regulating feeding activity by OA.

Our lab previously demonstrated that the *Drosophila* insulin-like peptide and NPY-like systems co-regulate hunger-driven motivation to approach non-readily accessible food (Wu et al., 2005a; Wu et al., 2005b). We have now showed that the *Drosophila* PDGF/VEGF- and NE-like systems mediate motivation to approach readily accessible food, suggesting that Pvr signaling may modulate OA activity in *Drosophila.* The details of the mechanism underlying how Pvr modulates octopaminergic neurons is underexplored, and it may be due to Pvr function on neural plasticity as is seen with VEGF in mammals. Our lab's unpublished research also showed that the i.c.v. injection of PDGFR and VEGFR inhibitors reduced the food intake of rats, showing a conserved feeding-regulated function of PDGF and VEGF signaling across phyla. My data suggested that it might be Pvf2 that activates Pvr to promote feeding in response to food deprivation. Consequently, it is possible that Pvf2 might serve as a signal peptide that responds to the change of internal state. Further investigation of the feeding related functions of OA and

RTK signaling in *Drosophila* may facilitate the exploration of the mechanistic details about the motivation and reward systems for reward-seeking behaviors as well as the finding of new solutions for overeating disorders.

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