CONSERVED NEURAL MECHANISMS FOR REGULATION OF FOOD INTAKE IN FLY AND RODENT MODELS

(Under the Direction of Ping Shen)

ABSTRACT

Obesity and excess body fat are the result of caloric intake that exceeds metabolic need, and their development is correlated with insufficient exercise and overeating. Evolutionary pressures tend to promote biological mechanisms which enhance an organism’s ability to obtain nutrients and minimize excessive energy utilization. However, the worldwide trend toward positive energy balance has led to an increased prevalence of obesity. It is a quiet killer, predisposing individuals to a variety of diseases including cardiovascular and metabolic disorders, increasing rates of morbidity and mortality (1). The financial consequences of obesity in terms of medical expenses and loss of productivity has deleterious social costs (2).

In order to understand the mechanisms that promote overeating and obesity at the behavioral level, it is informative to make use of invertebrate and mammalian models of feeding behavior. Using a Drosophila larvae model system, we have demonstrated that norepinephrine-like octopamine (OA) neurons in the fly are acutely required for homeostatic food intake regulation, and selectively enhance intake of sweet, palatable foods. The feeding regulatory effects of OA neurons were determined to dependent on Octβ3R and OAMB receptors, respectively. In addition, we have demonstrated that OA neurons are regulated by a vascular endothelial growth factor receptor (dVegfr, or Pvr), whose activity is required for OA dependent feeding rate increases (3). Based on the homology of the fly OA and norepinephrine systems in mammals,
and the similarity of fly dVegfr to mammalian VEGFR2, we hypothesized that VEGF/VEGR2 may have a homologous role mammalian food intake regulation. To investigate this possibility, young rats were treated intracerebroventricually (i.c.v.) with a single dose of VEGFR2 Tyrosine Kinase Inhibitor V (VTKI-V). Treated rats displayed decreased food intake and weight gain. These effects are selectively manifested in different feeding regimens. Animals treated with VTKI-V showed markedly attenuated consumption of palatable food that is sweet and fatty. Meal pattern analysis reveals that this is achieved by consumption of smaller, shorter meals. Overall, our results suggest that VTKI-V treatment may interfere with two separate VEGFR-mediated mechanisms: one which promotes overconsumption of palatable food, and the other mediates body weight gain.

INDEX WORDS: Octopamine, norepinephrine, neuropeptide Y, neuropeptide F, Oamb, Octβ3R, vascular endothelial growth factor receptor (VEGFR), subesophageal ganglion, food intake, appetitive motivation, hyperphagia, palatable diet, Drosophila melanogaster, rodents.
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by

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DEDICATION

This work is dedicated to Meredith, Trip, Joyce, and Drew, my wonderful family, and to Ana and Peter, for helping me get through it.

“Always you have been told that work is a curse and labour a misfortune.

But I say to you that when you work you fulfil a part of earth's furthest dream, assigned to you when that dream was born,

And in keeping yourself with labour you are in truth loving life,

And to love life through labour is to be intimate with life's inmost secret.

You have been told also life is darkness, and in your weariness you echo what was said by the weary.

And I say that life is indeed darkness save when there is urge,

And all urge is blind save when there is knowledge,

And all knowledge is vain save when there is work,

And all work is empty save when there is love;

Work is love made visible.”

~Khalil Gibran, “On Work”
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CHAPTER 1: INTRODUCTION

You can tell a lot about a fellow’s character by the way he eats jellybeans ~ Ronald Reagan.

The developed world is in the midst of an epidemic of obesity. A great deal of research has been performed in the study of food intake in both human and rodent models, providing evidence of environmental, genetic, epigenetic, endocrine, and neural mechanism which may predispose individuals to weight gain and obesity and contribute to its manifestation and persistence. However, in the case of humans, it is abundantly clear that the obesogenic environment of the developed world plays an enormous role as it limits energy expenditure and provides easy access to cheap, tasty, high energy foods. The specter of starvation has been a vastly greater threat to human survival over the course of our evolution, thus mechanisms for preventing overeating during abundance have been subjected to less pruning. There is a great interest and need for thorough characterization of biological systems involved in the prevention of palatable food overconsumption in order to combat and prevent obesity.

Due to the difficulty of controlling the many factors involved in the development of obesity, animal models, particularly mice and rats and more recently the fruit fly, have served as reasonable surrogate models. Earlier studies making use of broad physical, pharmacological, and electrolytic techniques for disruption of neural circuit components identified key regions of the hypothalamus and hindbrain as critical regulators of energy homeostasis. Such work revealed the
presence of homeostatic control centers within the brain which operate to match short term and long term food intake with metabolic output such that body weight remains stable over time despite variations in food availability and quality. In addition, it is now increasingly recognized that the brainstem and several cortico-limbic systems coordinating cognitive, hedonic, and emotional neural processes also play important roles in food selection and ingestion (4).

While these studies provide important evidence of the identity of the anatomical location of neural substrates promoting or preventing food intake and weight gain, they were limited in their capacity to elucidate circuit level connectivity and physiologically relevant circuit activity. In recent decades, the advent of genetic tools for targeting and manipulating neural circuits at the cellular level has allowed for a greater spatial and temporal control over circuit dynamics. While our map of feeding neurocircuitry remains far from complete, it has become clear that the division of food intake regulation into “homeostatic” and “hedonic” categories is an oversimplification. Both homeostatic and hedonic circuits appear to adaptively respond to palatable food consumption. Furthermore, there is a considerable amount of cross-talk between hypothalamic, cortical, and limbic structures, and both are responsive to circulating nutritive and endocrine factors(4). The predisposition of individuals to overeating and obesity can theoretically develop from any pathological disruption or maladaptation of this complex system to alterations of internal and external factors.

*Summary of Primary Findings*

This introduction will provide an outline of the major theories of how short and long term body weight and food intake are controlled, followed by an overview of the main neurotransmitter systems which are investigated in the experiments. The main chapters will describe the
characterization of an invertebrate neural circuit required for control of homeostatic control of palatable food intake. The circuit relies on signaling by norepinephrine-like octopamine (OA) neurons in the larval subesophageal ganglion, which acts as an integration center between olfactory and gustatory inputs and the expression of ingestive motor patterns. The activity of separate subsets of these neurons are required for the efficient re-establishment of energy balance following food deprivation, and for the suppression of palatable food consumption during satiety. These selective roles are linked to separate downstream pathways mediated Octβ3R and Oamb/NPF, respectively. Furthermore, we have identified an upstream regulatory pathway mediated by a VEGF/VEGFR/Ras-like signaling molecules which is required in OA neurons for appropriate modulation of palatable food intake. This finding prompted an investigation into the role of the VEGFR receptor in the rodent brain, which demonstrated that inhibition of this receptor results in reduced body weight gain and prevention of diet induced hyperphagia by reducing the caloric size of meals. The relevance of these findings to current knowledge regarding the relevant systems is presented in the discussion.

Motivated Behaviors

In order for a species to survive, the individual of the species must be able to execute adaptive motivated behaviors such that its internal needs are resolved with its external environment. This requires that individuals engage in goal-oriented behaviors that allow it to obtain resources necessary for its survival, such as food, water, and sexual partners, as well as to avoid potentially harmful foods or predators (5). “Drives” are excitatory pressures exerted by homeostatic disturbances (biological, emotional, social, and/or cognitive) which have the power to compel an individual to coordinate their behaviors in order to obtain a particular goal (6). Motivated
behaviors are those that are guided by internal drives in order obtain internal or external goals. As they are guided by internal forces, such behaviors present an inherent difficulty as objects of study: the underlying internal variables driving motivated behaviors, such as thirst or hunger, are not directly observable or measurable. Thus, in order to attempt to identify and characterize the biological mechanism(s) underlying motivated behaviors, scientists must focus on the observable phenomena that result from internal states (7). The three broad classes of motivated behaviors that are essential for the survival of an organism and/or species are ingestion, defense, and reproduction.

The Meal
Like other classes of motivated behaviors, ingestive behaviors occur in cycles or phases. Though the character and sequence of the behaviors performed are highly individualized and species specific, the main phases are similar. Ingestive behaviors begin with an initiation phase, followed by procurement, consumption and termination, and one full cycle of these phases is referred to as a meal (8). The meal initiation phase begins with the physiological deficit signals encoding internal energy status, which are integrated with sensory input. Together this information is centrally processed to encode the incentive value of the goal object, in this case palatable foodstuffs. The output of this processing provides a value for the “drive” associated with feeding, and this is the basis for selection of an appropriate motor program to execute in order to obtain the goal object, which begins the “procurement” phase. When food is located via sensory mechanisms, the consummatory phase begins, which involves the execution of stereotypical rhythmic motor programs such as chewing and swallowing such that the individual is able to consume the selected food. During this phase, the animal structures its meal (in terms of
duration, quantity ingested, etc.) via dynamic integration of sensory and endocrine inputs which communicate with central mechanisms regulating motor output. As the consummatory phase continues, internal sensory feedback signals arise that either sustain ingestive behaviors or promote termination of consumption. Termination occurs either when satiation is achieved or at any time during this sequence if a second motivated behavior becomes more imperative or appropriate (8).

Like other animals, humans and other mammals eat in an episodic fashion, and these episodes are referred to as “meals” or “snacks” (9, 10). Once a meal is initiated, it tends to continue until a sensation of “fullness” or “satiation” is attained, which is followed by a non-feeding period referred to as satiety (11, 12). During the satiety phase, the drive to eat is very low, and this drive must build up again in order to initiate the next feeding episode (13). The internal force providing the drive to search, chose, and ingest food is referred to as “appetite” (13).

The cycle of ingestive behaviors is also demonstrated in invertebrate species, such as *Drosophila*. During both larval and adult stages, flies execute a number of distinct, highly stereotyped, and quantifiable behaviors relating to meal initiation, procurement, ingestion, and termination. For example, the proboscis extension reflex in adult flies can be stimulated by the application of sweet compounds to the chemosensory bristles of the leg, which is used in assays of meal initiation and food preference (14). In the larval stages, actively foraging larvae feed steadily when provided with soft, sweet media. Larval food ingestion rate and feeding preferences can be assessed by the use of counting the rate of bites or measuring the intake of dyed feeding media (3, 15, 16), while their persistence in continuing to feed on a particular media can be used as a readout of foraging and procurement (15). Using assays such as these,
researchers have investigated mechanisms underlying both homeostatic and hedonic food intake regulation in the fly which share homology with those found in vertebrates.

**Short and Long Term Regulation of Body Weight and Food Intake in Mammals and Flies**

Energy homeostasis is a balance between energy intake, expenditure, and storage, which is achieved by continual dialogue between multiple brain and body communication networks. The concept was first proposed 60 years ago by Kennedy (17), who described energy homeostasis as a system whereby circulating signals provide the brain information regarding available energy stores, and in response the brain makes corrective adjustments to food intake and energy expenditure. When a negative energy balance occurs within an organism, nutrient deprivation signals from the periphery are relayed to brain where several overlapping circuits are triggered to implement foraging, appetitive, and ingestive behaviors to obtain nutrients and to simultaneously transition to an energy conservation mode via autonomic, endocrine, and peripheral cellular mechanisms (18). In mammals, the hypothalamus and dorsal vagal complex of the hindbrain are two neural regions that are critical in regulating these responses. These neurons project to areas important to homeostatic maintenance via behavioral, physiological, and endocrine effects (19-21). The disregulation of these pathways has been demonstrated in mice experiencing diet induced obesity (20, 22), ob/ob mice (23), and in diabetic patients (24-26).

**Mammalian Systems**

In the mammal homeostatic system, afferent projections from peripheral organs as well as humoral signals, peptides, converge on neurons in the hypothalamus, hindbrain, and other areas which form an interwoven yet distributed system of neurocircuits (27). For each of its regulatory
functions, the hypothalamus senses environmental or body signals and uses this information to organize an appropriate response, and subsequently to command other brain regions to implement that response. Ultimately, the autonomic nervous system is recruited to implement the appropriate responses via descending autonomic projections originating the in the hypothalamus and brainstem (28). In regards to the regulation of food intake, nutrient deprivation signals are sent to the hypothalamus to implement foraging, appetitive, and ingestive behaviors to obtain nutrients and to simultaneously transition to an energy conservation mode via autonomic, endocrine, and peripheral cellular mechanisms (18). Specialized metabolic sensors within these regions receive and integrate these inputs and respond by coordinating various downstream systems involved in food intake, metabolism, and energy storage. One such signal is leptin, a hormone secreted by adipocytes into plasma at levels that are proportional to body fat stores (29), enters the brain in proportion to its level in plasma (30), and acts on key neurons that regulate energy balance (31, 32). The pancreatic hormone insulin is another example, as it also circulates at levels proportional to body fat (33) and directly acts on neural targets to reduce food intake (34), while reduced neuronal insulin signaling results in a mild expansion of fat mass (35). Leptin and insulin are thus both implicated in this adiposity negative feedback control system, although the feeding effect of leptin is quantitatively much greater than that of insulin (36). Both leptin and insulin cross the blood brain barrier and interact with neurons in the arcuate nucleus of the hypothalamus (ARC) which produce and secrete neuropeptide Y (NPY), agouti-related peptide (AgRP), and proopiomelanocortin (POMC) (37-40).

Early studies based on gross anatomical lesions or neuropharmacological manipulations identified key areas in the brain mediate hyper or hypophagic behaviors, including the PVN, LHA, ARC, and hindbrain adrenergic bundles. The ARC is located in the periventricular region
of the hypothalamus, where the blood-brain barrier is relatively weak, allowing access to circulating signals relating to energy balance. This location allow the ARC to act as a relay center through which information regarding internal energy status and external energy options are integrated. The ARC receives strong intrahypothalamic inputs from other peri- and paraventricular nuclei and LHA, and sensory inputs (olfactory, taste, visual), but in regards to food intake, the most important inputs are hormonal (27). Leptin from adipocytes provides input to the ARC regarding long term energy status, acting centrally as a potent inhibitor of food intake. Direct administration of leptin to the brain reduces food intake and body weight gain (41, 42), while reduced or impaired leptin signaling promotes hyperphagia and weight gain (43, 44). In addition, the neurons in the ARC are receptive to ghrelin produced by the enteroendocrine cells of the stomach in response to fasting. Ghrelin promotes food intake, and therefore acts opposite to leptin and insulin (28). The ARC also receives input regarding immediately available energy stores via neurons which are responsive to insulin, glucose, free fatty acids, and other metabolites circulating in the plasma (27). Though other regions of the brain and hypothalamus may also be sensitive to these signals, ARC projections to the paraventricular nucleus (PVN) and lateral hypothalamic area (LHA) are key to transmitting their effects on energy balance and food seeking behavior (28).

In terms of food intake regulation, the ARC contains two major populations of neurons. The first are neurons that release orexigenic peptides, such as those expressing neuropeptide Y (NPY) and agouti-related protein (AgRP), which project the PVN and LHA in the periventricular zone (27). These neurons express both the leptin and ghrelin receptors, which act to inhibit or activate the release of NPY/AgRP, respectively. The second major group are those that release anorexigenic peptides including those expressing pro-opiomelanocortin (POMC) and cocaine- and
amphetamine regulated transcript (CART). Melanocortins, such as α-melanocyte stimulating hormone (α-MSH), are processed from the POMC gene. CART and α-MSH are both anorexigenic peptides, and their expression levels positively correlate with circulating levels of leptin (45), while POMC neuron activity is inhibited by activation of the ghrelin receptor (46). NPY producing neurons in the ARC project to the paraventricular nucleus (PVN) and lateral hypothalamic area (LHA) (47, 48) where NPY binding to its Y1 receptors triggers increases in food intake and reduction in energy expenditure (49). POMC neurons also project to the PVN and LHA where they release alpha-melanocyte-stimulating hormone (α-MSH) (50), which acts via melanocortin-4 receptor neurons to decrease intake and increase expenditure (51). These neurons also respond to AgRP released from ARC NPY/AgRP neurons, which acts as an antagonist of the functions of α-MSH signaling through melanocortin receptors (38). Together, leptin and insulin signaling in the ARC acts as a negative feedback system which prevents against weight gain and obesity. Increases in adipose tissue results in an increase in leptin and insulin levels, which then inhibit orexigenic NPY/AgRP neurons while activating the anorexogenic pathway mediated by POMC neurons (40).

Numerous short term hormonal and nutrient related signals also potently influence feeding, such as gut peptides involved in the perception of satiety and thus promote termination of individual meals. The decision to initiate a meal is influenced by many external factors, while the amount eaten (meal size) is primarily determined by internal signals (36). The major internal signals affecting meal size are peptides, such as cholecystokinin (CCK) and GLP-1 which are secreted from gastrointestinal tract in response to food ingestion (52, 53). Satiety information is conveyed by these peptides as well as by neural signals generated by gastric distention, to the CNS through afferent fibers of the vagus nerve that project from the gut to the nucleus of the solitary tract.
(NTS) in the caudal hindbrain to trigger short lived food intake inhibition (36) at the level of the meal.

Long term regulation of food intake is dependent in part on adiposity negative feedback systems which reduce food intake in part by increasing brain responsiveness to satiety signals (54), such as leptin and insulin, while weight loss lowers the plasma levels of these same hormones, thereby increasing meal size by reducing the satiating effect of food (36). For example, leptin enhances the brain’s response to satiety signals (55), which decreases meal size (56); conversely, low levels of leptin signaling reduces the responsiveness of central circuits to CCK, which leads to an increase in meal size (54). This interaction between leptin and satiety signals is dependent on activation of leptin receptors in the hindbrain (NTS), and hypothalamic neurons (ARC nucleus) which project directly or indirectly to the NTS (40). Ultimately, this interaction enables food consumption during individual meals to be adjusted to compensate for changes in body fat mass (36).

**Drosophila Systems**

The fly fat body is homologous to adipose tissue in mammals, acting as a storage compartment for lipids and as a regulator of lipid homeostasis. Carbohydrate and lipid homeostasis in the fly are regulated by insulin and adipokinetic hormone (AKH) which performs a similar function as mammalian glucagon (57). Insulin producing cells (IPCs) are located in the median neurosecretory region of the brain, and act in a manner similar to that of pancreatic beta cells, while AKH is produced by corpora cardiac (CC) cells in the neuroendocrine ring gland, which correspondingly act as pancreatic alpha cells (57). Like their mammalian counterparts, insulin is upregulated following food ingestion and acts to promote uptake of sugar from circulating
hemolymph for storage as glycogen and fats, while AKH is secreted during starvation and breaks down glycogen and fats (58). IPCs produce several isoforms of insulin-like peptides (dILPs; isoforms dILP2, dILP3 and dILP5 are produced by IPCs) (59, 60). dILPs are secreted when nutrient levels are high, and ablation of IPCs or dILP expression in these cells disrupts carbohydrate homeostasis (61), body size regulation (62, 63), and feeding behavior (16). The fly fat body responds to nutrient deprivation triggers mobilization of fats by remotely stimulating AKH release from CC cells, which is then secreted into hemolymph to target peripheral energy stores (58).

Like mammals, adult flies and larvae must determine when to eat, what to eat, and how much to eat, and these decisions are determined by the coordinated actions of peripheral signals of indicating the levels of internal energy stores, as well as external cues of nutrient availability. The decision to initiate a meal is largely determined by the detection of nutritive compounds in the environment by gustatory receptor neurons (64, 65), while detection of noxious stimuli such as aversive taste (66) or temperature (67) elicit rejection behaviors. Once a feeding period is initiated, the rate of ingestion and duration are dynamically modulated based on internal metabolic status via circulating hormones from neuroendocrine cells and fat body tissue such as the Drosophila insulin-like peptides (16, 61), adipokininetic hormone (58), and leptin homolog Unpaired 2 act as coding mechanisms for the availability of carbohydrate and lipid stores (57). Post-ingestive feedback from the gut following a meal may also inhibit feeding via the recurrent nerve or medial abdominal nerve (68), acting similarly to gut distention signals in mammals.

In previous investigations in our lab, we have developed a behavioral experimental paradigm to evaluate larval food intake and willingness to work for food (66). These studies demonstrated that, like other animals, fly larvae demonstrate adaptive behavioral responses to foods which are
dependent on both their internal satiety status, and external sensory cues of food quality and nutritional value. For example, satiated larvae tend to eat at a steady baseline rate, and prefer soft, sweet foods but decline those that are difficult to masticate or have aversive taste (15, 16, 66). However, these preferences are discarded in a dose dependent fashion as larvae are withheld from food for increasingly long periods. Larvae which have been starved for 2.5 hours double their feeding rate on attractive (16) or aversive (66) foods in an effort to quickly regain energetic equilibrium, returning to their baseline feeding rate and preferences when satiated. The behavior of satiated larvae is similarly responsive to modification by external cues indicating the presence of preferred foods. For example, satiated larvae display a significant increase of their rate of ingestion when they are in the presence of food related odors, such as banana-like amyl acetate (69), or when media has a sweet taste, even if sugar is not present. These observations demonstrate that flies, like mammals, are capable of modulating their behavioral output according to both their internal energy status and environmental cues of nutrient availability in order to maintain energetic homeostasis by maximize consumption of nutritive foods and minimize energy expenditure.

_Hedonic Regulation of Food Intake in Mammals and Flies_

The hedonic aspects of food intake regulation are evident in rodents and humans studies which demonstrate that drugs of abuse and consumption of highly palatable foods converge on a shared pathway within the limbic system to mediate motivated behaviors (70, 71). Most motile animals display active foraging behavior to locate and obtain energy resources in their environment, and will act to avoid stimuli that are harmful. Rewarding stimuli are characterized as having a
reinforcing property, in that almost all motile animals studied will learn to repeat actions that bring about or bring them closer to a rewarding outcome (72, 73).

**Mammalian Systems**

Rewarding stimuli are characterized as having a reinforcing property, in that when they are presented, animals will learn to repeat actions that will obtain them or bring them closer to obtaining them. (73, 74). Natural rewards such as food are able to influence output effector pathways controlling food intake (70). Dopamine (DA) has long been known to act as a key regulator in motor function in mammals, but began to receive attention as a mediator of goal-driven or “rewarding” behavior when it was shown that administration of dopamine receptor antagonists blocked the motivation to respond to food rewards (72, 73, 75), which appeared to be due to a reduction in the reinforcing properties of rewarding stimuli (75). Dopamine is now thought to regulate learning processes that encode stimuli which animals associate with a previously experienced reward (72). Stimulation of midbrain neurons expressing dopamine, such as the VTA and LHA, has a reward reinforcing effect, and recordings from these areas show strong responses following presentation of primary rewards like food and water or conditioned rewards (72). Projections from midbrain dopamine neurons terminate on areas of the nucleus accumbens (NAC) and frontal cortex, and display a tonic baseline firing rate unless subjects are presented with rewarding stimuli, which results in phasic bursts of activation, the intensity of which is thought to be modulated by the prediction of reward value (74).

When food is consumed, gustatory and viscero-sensory pathways are activated which project information to the NTS and amygdala (76). The signals are processed by a system that included the nucleus accumbens, ventral pallidum, and the ventral tegmental area which is located in the
midbrain and projects via the mesolimbic dopamine system back to the nucleus accumbens, the prefrontal cortex, the hippocampus, and the amygdala (4). The mesolimbic dopamine pathway is thought to play a central role in both reward driven and addictive behaviors due to the fact that all common drugs of abuse increase dopamine signaling from terminals in the ventral tegmental area which project to neurons in the nucleus accumbens (77). This increase in dopamine signaling is due to direct activation of dopamine neurons, or indirectly by inhibition of gabaergic interneurons located in the VTA (70, 71). Presentation of highly palatable foods induces release of dopamine into the nucleus accumbens (70), which acts to coordinate several aspects of an animal’s efforts to procure food rewards such as increased arousal, psychomotor activation, and conditioned learning (77).

Drosophila Systems

In vertebrates, the mesolimbic dopamine pathway has long been considered the major system underlying reward driven learning and motivated behavior. In Drosophila, however, OA has historically been thought to be the major neurotransmitter required for coordination of reward learning and behavioral responses to reward while dopamine has been strongly connected to aversive learning (78-82). This connection is based primarily studies involving odor learning paradigms where adult flies learn to associate food related odor cues with unconditioned stimuli that are either appetitive (sucrose) or aversive (electric shock) (83, 84). Using appetitive and aversive learning assays in adult flies, it was shown that OA injection into the mushroom body (MB) or antennal lobe (AL) could substitute for sucrose presentation or pairing in conditioned learning paradigms (85). The MB acts as a protocerebral higher brain center similar to the striatum, hippocampus, and prefrontal cortex in mammals, and is best characterized for its roles
in olfactory processing, learning and memory (86), decision making (87), and associative learning (88). In addition, flies lacking OA due to a mutation in the tyramine-beta-hydroxylase enzyme showed no impairment in aversive tests involving the pairing of a novel odor with a shock, but did not learn to associate a sugar reward with odor (78). However, recent studies have revealed that both DA and OA are required signals for reward learning, and that DA is sufficient for both short and long term memory encoding even in flies lacking OA (89). Emerging evidence now indicates that the role OA in learning may be restricted to its ability to code for the gustatory detection of “sweet” tastes (90). In addition, the NPY-like neuropeptide F (NPF) has been characterized as a major modulator of DA dependent reward learning and olfactory coding in flies (88), as well as in olfactory coding for food intake regulation (69).

*The role of norepinephrine in mammalian food intake regulation*

Norepinephrine is a catecholamine and signals through two classes of G protein coupled receptors which are designated α and β based on their rank order potency of agonists (91) and were further divided into subcategories including α1, α2, β1, β2 based on binding and functional characteristics (92). All of the subtypes bind NE and epinephrine with varying affinities. α1 receptors excite neurons by increasing IP3, Ca2+, and DAG and α2 receptors inhibit neurons by decreasing cAMP. β1, β2, and β3 receptors both act to increase cyclic AMP (92).

The central effects of NE on feeding are antagonistically mediated in part by α1 and α2 receptors (93), which display differences in function, spatial and temporal expression patterns, and profiles of drug responsiveness (94). Their roles in the regulation homeostasis are primarily linked to their expression within the nucleus of the tractus solitarius (NTS), dorsal motor nucleus of the vagus (DMV), and the hypothalamic paraventricular nucleus (PVN) and ventral medial nucleus.
(VMH). NTS cells in the brainstem receive visceral and gustatory sensory input from the facial, glossopharyngeal, and sensory vagal nerves. Information from the NTS and hypothalamus are sent to the DMV where they are integrated to regulate various visceral organ secreto-motor functions such as pancreatic hormone secretion, gastric motility, and gastric acid secretion. The interconnected medullary noradrenergic cell groups A1, A2, and A5 innervate the NTS and DMV, and α1- and α2-noradrenergic receptors excite and inhibit these neurons respectively, thus medullary noradrenergic pathways are thought to be important in processing and integrating sensory and motor vagal information, which may allow them to modulate autonomic reflexes or information coming from the hypothalamus (95).

Thus NE plays a role in in the regulation of homeostatic energy balance by mediating hypoglycemic responses, and in coupling signals regarding nutrient levels to appropriate behaviors. Glucose availability is critical to brain function and thus glucose sensitive neurons are stimulated during hypoglycemia to activate glucoprivic counter-regulatory mechanisms (96), such as activation of the sympathoadrenal system and hypothalamo-pituitary axis (97, 98). In the hindbrain, glucose sensing neurons are found in the substantia nigra (99, 100), the NTS (101, 102), DVM (103), the area postrema (104), and NE neurons in the locus coeruleus (105, 106). Thus NE efferents from the locus coeruleus and brainstem are both responsive to glucose levels, and also modulate the glucose sensitive NTS and DMV neurons. These efferents project to several areas, but their primary effects on feeding behavior are mediated by terminals in the PVN and VMH hypothalamic nuclei, areas which are known to receive and integrate inputs from metabolic and neuroendocrine systems regarding the nutritional status of an animal and to translate them into appropriate mechanisms for maintaining homeostasis (19, 107). Thus NE
plays a role in integrating sensory input and energy balance signals with digestive functions through its regulation of the NTS and DMV.

In the hypothalamus, $\alpha_1$- and $\alpha_2$-noradrenergic receptors are found in the PVN and VMH and act to excite or inhibit descending pathways that regulate feeding. In the PVN, $\alpha_2$ receptors have been demonstrated to promote feeding, presumably by inhibiting “satiety” neurons that project from the PVN to the brainstem (108), whereas $\alpha_1$ receptors in the PVN and VMH have an opposite effect (109, 110). $\alpha_2$ receptors are considered to be the primary effectors of NE’s enhancement of feeding in the PVN as their expression levels peak immediately prior to feeding onset and fall after meal consumption, and NE stimulation of feeding is mimicked by $\alpha_2$ agonists and not $\alpha_1$ agonists (19, 109). Disregulation of the binding affinity of $\alpha_2$ receptors is seen in the PVN of ob/ob mice, indicating that this pathway may be important in obesity (23). However, $\alpha_1$ receptors seem to play a more dominant role in regulation of the VMH. Lesions of the VMH or chronic infusion of NE induce hyperphagia, hyperinsulinemia, and obesity, and this has been found to be dependent on glucose and insulin sensitive neurons in this region (111, 112). These glucose responsive neurons respond to NE, and the direction of the response is dependent on the relative levels of $\alpha_1$ and $\alpha_2$ receptors. Studies in rats that developed dietary induced obesity (DIO) as a result of a sweet condensed milk diet display decreased binding of $\alpha_1$ receptors in the VMH, which was inversely correlated to body weight gain over several months, regardless of diet (20). Thus $\alpha_1$ receptors appear to be linked to predisposing individual rats to develop DIO. Decreases were also seen in $\alpha_2$ receptor binding in the DMV and hypothalamus, indicating that both receptors contribute to this effect but have different roles (22). Taken together, these observation indicate that NE pathways are important in the regulation of homeostatic feeding as they act to promote energy intake during times of energy depletion, their activity is linked to
circulating glucose levels, and they stimulate behaviors which promote reestablishment of energy balance.

The Role of Octopamine in Drosophila Food Intake Regulation

Among invertebrate species, octopamine (OA), a molecule chemically similar to dopamine and norepinephrine (Fig. X). OA is produced from stepwise from tyramine via tyramine decarboxylase (tdc) and tyramine beta hydroxylase (TβH) (113), and signals through four closely related G protein coupled receptors with homology to mammalian α and β noradrenergic receptors (114, 115). NE and OA exhibit a high degree of similarity, differing only in that NE contains one additional hydroxyl group in the 3-position of its phenolic ring (cite). OA receptors also have a high degree of homology to those of NE in terms of sequence, pharmacological properties, and activation of downstream pathways (116, 117). The similarities between OA receptor subtypes in invertebrates and adrenergic receptors in vertebrates suggest these two systems may have diverged from a common evolutionary origin (74).

Immunohistochemical analyses of the expression of tdc2-Gal4 construct and TβH have revealed that OA cell bodies cell bodies are located in the ventral nerve cord, which is critical in locomotion, and the subesophageal ganglion, an area that is important in integrating gustatory input and feeding regulation (118). Flp-out analysis of the OA circuit has characterized the projection pattern of each of the 137 OA neurons, and identified that they are organized in various clusters, the majority of which receive synaptic input in the area surrounding the esophageal foramen, and each cluster projects to widely varying areas including the neuromuscular junction, segmental nerves, antennal lobe, and to the mushroom body (119). They are divided into ventral medial (1-3), dorsal medial (1-2), and paramedial groups based on their
location (118). In regards to feeding, their postsynaptic input regions are ideally positioned to receive input from the esophagus as well as from gustatory receptor neurons (GRNs), and send projections to regions involved in the regulation of appetitive memory and control of muscle contraction (65, 120). This positioning strongly suggests that OA neurons may act as a weigh station centered between detection of gustatory signals and behavioral output. However, the upstream and downstream connectivity of OA neurons has only been inferred from their anatomical location and receptor distribution and from phenotypic analysis of OA receptor subtypes. Further, how separate subsets are independently regulated or coupled to physiological signals is an enticing underexplored area, which may illuminate how similar but unique OA neurons mediate their many effects on physiological processes.

*Invertebrate and Vertebrate VEGF/VEGFR Pathways*

Vesicular endothelial growth factor (VEGF) has angiogenic (121), mitogenenic (122), and vascular permeability enhancing properties (123) and has become a target of study due to its angiogenic role in tumor formation (124, 125). VEGF exerts its effects via binding with receptor tyrosine kinases (RTK) which dimerize and autophosphorylate intracellular tyrosine residues upon ligand binding, ultimately activating PI3-kinase, PKC, and Ras/MAPK pathways (126). Five types of VEGF (A-D, PLGF) have been identified which are produced as monomers that combine to form homo- or hetero-dimeric molecules (126-128). The receptors (VEGFRs) likewise come in the monomeric isoforms, which dimerize to form homo- or hetero-complexes upon activation (126, 127). Different combinations of ligands and receptors are expressed in different cell types and exhibit distinctive roles. For example, VEGFR1 is required in macrophage migration (129), and VEGFR3 regulates lymphatic system (130), while VEGFR2 is
the primary regulator of vascular permeability (123). The Drosophila PDGF- and VEGF-related Receptor (Pvr) has been demonstrated to exhibit similar roles in its regulation of border cell migration in ovaries (131), hemocyte proliferation (132), and axonal growth (133), and also displays conserved signaling properties (133). Three Drosophila proteins have been identified as containing PDGF/VEGF homology domains (Pvf1, Pvf2, Pvf3) (131, 134, 135). Only one receptor (Pvr) has been identified, though it can be alternatively spliced to produce four different isoforms (136).

The invertebrate Pvr/Pvf2 display homology to VEGF/VEGFR pathways, which have been implicated in regulating neuronal processes, such as axonal outgrowth (137, 138) and neuroprotection (139), and behavior (140). However, though several families of growth factors have been implicated in the regulation of food intake, including PDGF (141), EGF (141), FGF (142, 143), and BDNF (144), the role of VEGF and VEGFR in feeding behavior has not been explored. Some evidence exists for interaction between VEGF/VEGFR pathways and major feeding regulatory circuits. For example, NPY also acts as a mediator of neurogenic angiogenesis, in particular by modulating sympathetic neurotransmission (145). The increased levels of NPY following hypoxia, exercise, and cold allow the body to respond to ischemic signals via the sympathetic nervous system to stimulate angiogenesis, which is dependent on VEGF signaling (145, 146). Also, NE signaling is also known to have neuroprotective effects during hypoglycemia, and this effect has been linked to the upregulation of VEGF which promotes the translocation of GLUT1 transporters in neural epithelial fenestrations (147) and provides neuroprotective effects (148). Obesity is also accompanied by alterations in VEGF levels. Obese patients often display increased VEGF levels (149). Ghrelin also promotes angiogenesis in persistent ischemia by upregulating VEGF expression (150). High VEGF levels
in diabetic patients is correlated with decreased intake of carbohydrates, presumably by enhancement of GLUT1 and glucose uptake in vascular endothelia tissue surround the brain (151). Furthermore, VEGF/VEGFR2 signaling has been linked to low carbohydrate intake (6), the suppression of sucrose preference (7), and modulation of glucose transport in neuronal tissue (8, 9). However, the direct effects of VEGFR signaling on behavioral regulatory circuits is not well understood.
2.1 INTRODUCTION

The easy accessibility of energy-rich palatable food makes it difficult to resist food temptation. Drosophila larvae are surrounded by sugar-rich food most of their lives, raising the question of how these animals modulate food-seeking behaviors in tune with physiological needs. Here we describe a circuit mechanism defined by neurons expressing tdc2-Gal4 (a tyrosine decarboxylase 2 promoter-directed driver) that selectively drives a distinct foraging strategy in food-deprived larvae. Stimulation of this otherwise functionally latent circuit in tdc2-Gal4 neurons was sufficient to induce exuberant feeding of liquid food in fed animals, whereas targeted lesions in a small subset of tdc2-Gal4 neurons in the subesophageal ganglion blocked hunger-driven increases in the feeding response. Furthermore, regulation of feeding rate enhancement by tdc2-Gal4 neurons requires a novel signaling mechanism involving the VEGF2-like receptor, octopamine, and its receptor. Our findings provide fresh insight for the neurobiology and evolution of appetitive motivation.

The adaptive control of foraging decisions is crucial to survival and reproduction and is mediated by complex brain mechanisms. For example, in hungry animals, feeding behaviors can be modulated by diverse neural systems including those responsible for receiving and processing sensory properties and assigning reward and motivational significance of food stimuli (152-154).
At present, elucidation of molecular and circuit mechanisms underlying the adaptive control of feeding behavior remains highly challenging.

Our previous studies have shown that Drosophila larvae, like mammals, display diverse adaptive foraging strategies in response to appetizing odors or satiety state and food quality (66, 69, 155). For example, larvae fed for ad libitum intake tend to prefer soft, liquid sugar media that contain readily ingestible sugar solution but decline solid media in which sugar solution is embedded in gelled agar and is less accessible (66). However, as food deprivation is prolonged, larvae will become increasingly persistent in extracting the sugar solution from solid media (16). We have also shown that an evolutionarily conserved signaling cascade, involving neuropeptide F (NPF, the fly homolog of neuropeptide Y, or NPY) and insulin-like peptides (dILPs), selectively integrates motivational state (hunger) with persistence to pulverize solid food (16, 66).

The observation that the conserved NPY-like system selectively promotes food acquisition behaviors that require high energetic cost has led us to postulate that fly larvae may use other conserved neural mechanisms to regulate acquisition of readily accessible palatable food. In this work, we provide evidence which supports this hypothesis. We show that an octopamine (OA)/β-adrenergic-like receptor (Octß3R)-dependent circuit mechanism selectively regulates appetite for soft sugar media. This circuit mechanism seems to involve two subsets of tdc2-Gal4 neurons in the subesophageal ganglia (SOG). One of them mediates the hunger-driven increase of feeding and is modulated by a novel activity of the VEGF2-like receptor pathway (134). The other is required for preventing excessive appetite in fed larvae. This and our previous findings provide fresh mechanistic insights into how brain mechanisms differentially organize appetitive motivations in responses to high- and low quality food sources under different energy states.
2.2 RESULTS

2.2.1 Hunger-Driven Appetite for Liquid Sugar Media in Fly Larvae

Larvae fed for ad libitum consumption display a baseline level of feeding responses to readily accessible palatable food (e.g., 10% glucose agar paste), which can be quantified by counting the number of larval mouth hook contractions (MHCs) during a 30-s test period (Fig. 1). This baseline level of MHC rate increases in a dose dependent manner when larvae are deprived of food (Fig. 1A) and is accompanied by an increase in ingestion rate (Fig. 1B). Hungry larvae display a slightly higher peak speed of MHC (11%) relative to fed larvae (maximum number of contractions per 3 s) (Fig. S1A), as well as a large increase in persistence of feeding, as evidenced by the shorter time intervals between bites (Fig. 1C and Fig. S1B). Together, these results indicate that fasted larvae, like hungry mammals, execute a behavioral program to effectively restore energy balance following food deprivation.

2.2.2 Role of tdc2-Gal4 Neurons in Appetitive Motivation

Our previous study of the NPF system suggests that feeding incentives of hungry larvae might be regulated by distinct neural mechanisms (16, 155). The insect OA system has been implicated in behaviors associated with seeking food rewards (156). The tdc2-Gal4 driver directs reporter expression in central neurons producing OA and/or tyramine (157). We found that blocking tdc2-Gal4 neuronal activity by expressing an inwardly rectifying potassium channel protein (UAS-Kir2.1) completely abolished hunger-induced MHC rate increases in liquid food (Fig. 1D) (158). Importantly, fasted tdc2-Gal4/UAS-Kir2.1 larvae showed normal hunger-driven feeding responses to less-accessible solid sugar food, opposite to the behavioral phenotypes of fasted larvae expressing UAS-Kir2.1 in NPF neurons (Fig. 1E and Fig. S2A). We also transiently
inhibited tdc2-Gal4 neurons by expressing UAS- shi<sup>ts1</sup>, a temperature-sensitive, semi-dominant-negative form of dynamin (159). At the restrictive temperature of 30 °C, tdc2-Gal4/UAS-shi<sup>ts1</sup> larvae failed to display hunger-driven feeding response to liquid food, but their feeding responses to solid food were normal (Fig. 1F and Fig. S2B). These findings suggest that tdc2-Gal4 neurons define an uncharacterized circuit mechanism that is acutely required for hunger-motivated feeding of readily accessible sugar media. In addition, we genetically activated tdc2-Gal4 neurons by expressing a UAS-NaChBac construct that encodes a voltage-gated bacterial sodium channel (160). Fed tdc2-Gal4/UAS-NaChBac larvae displayed enhanced MHC rates and decreased intervals between bites on liquid sugar food (Fig. 1G and Fig. S2C) but showed no detectable changes in feeding response to 10% glucose solid food or liquid media without sugar (Fig. S2D).

2.2.3 Role of OA in Appetitive Motivation

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 responses of tßh<sup>abM18</sup> larvae carrying a null mutation in the tyramine β-hydroxylase (TβH) gene essential for OA synthesis (113). We found that blocking of OA synthesis completely abolished hunger-driven MHC rate increases in liquid food, phenocopying the tdc2-Gal4/UAS-Kir2.1 larvae (Fig. 2A). Moreover, pre-feeding normal fed larvae with food containing OA also led to a detectable increase in the rate of MHC and ingestion of dyed liquid sugar media (Fig. 2 B and C). The same OA treatment of fasted tdc2-Gal4/UAS-Kir2.1 larvae largely restored hunger-driven feeding response to liquid food (Fig. 2D). These findings indicate that OA signaling underlies the activity of tdc2-Gal4 neurons in selective regulation of food acquisition. Given our previous findings that
tdc2-Gal4 neuron activity stimulates a behavioral program distinct from that of npf-gal4 neurons, we investigated how simultaneous increases of OA and NPF signaling levels might affect food motivation in fed larvae. To this end, OA was introduced orally to pre-fed elav-Gal4/UAS-npf\textsuperscript{DNA} larvae that overexpress NPF. We found that fed elav-Gal4/UAS-npf\textsuperscript{DNA} larvae treated with OA behaved similarly to OA-treated fed control larvae in liquid food (Fig. S3A). However, in solid food, OA-treated elav-Gal4/UAS-npf\textsuperscript{DNA} larvae behaved similarly to untreated elav-Gal4/UAS-npf\textsuperscript{DNA} larvae (Fig. S3B). These results support the notion that the OA-mediated circuit mechanism for feeding of liquid sugar media functions independently from the NPF circuit mechanism.

2.2.4 OA Enhancement of Feeding Requires Octβ3R

Four different OA receptors have been identified in Drosophila (114, 116). To determine the downstream effectors of the OA feeding pathway, we used a mifepristone (RU486)-inducible pan-neural GS-elav-Gal4 driver to perform dsRNA-mediated conditional knockdown of individual OA receptor activity (Table S1). We found that only disruption of the β-adrenergic–like Octβ3R receptor (116) blocked starvation-induced MHC rate increases. 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\textsuperscript{dsRNA} larvae (Fig. 2E). Furthermore, Octβ3R\textsuperscript{M804794} larvae containing a transposable element that disrupts Octβ3R are also deficient in the hunger-driven feeding response (Fig. 2F). These results suggest that OA and Octβ3R define a circuit mechanism that enhances feeding of liquid sugar media in fasted larvae.
2.2.5 Subsets of tdc2-Gal4 Neurons Differentially Control Appetite

Tdc2-Gal4 is expressed in multiple clusters of OA neurons in the larval brain lobes and the ventral ganglia (Fig. 3A). Immunostaining of the larval central nervous system with anti-TβH antibodies further suggests that the somata of all central OA neurons are located in the ventral ganglia (Fig. 3B) (81). OA neurons in the larval SOG respond to gustatory inputs from gustatory receptor neurons, and unlike the OA neurons in the abdominal ganglia, they are not motor neurons (81, 161). Anatomical mapping of tdc2-Gal4 neurons at the single-cell resolution in adult flies revealed that the ventral unpaired median neurons (VUMs) in the anterior compartment (VUM1) and middle compartment (VUM2) of SOG seem to project to several common areas of the protocerebrum, whereas those in the posterior compartment of SOG (VUM3) project to the ventral ganglia (119, 162). The tsh-Gal80 construct blocks GAL4 activities in the thoracic and abdominal ganglia (163) (Fig. 3C). Fasted tsh-Gal80/tdc2-Gal4/UAS-shi larvae showed reduced MHC rates in response to liquid glucose media (Fig. 3D), suggesting that tdc2-Gal4 neurons in the SOG are required for hunger-driven response to liquid food. The SOG is proposed to act as a feeding control center in the central nervous system of insects (14, 164). To test which tdc2-Gal4 neurons in the SOG are important for OA-dependent feeding activity, we generated targeted lesions in the subsets of tdc2-Gal4 neurons using focused laser beams (165). Targeted lesions in five VUM1 with or without four ventral paired median (VPM1) neurons in SOG1 caused a significant increase in the feeding activity of fed larvae (Fig. 3E and Fig. S4A). However, this increased feeding activity was abolished when additional neurons from SOG2 (five VUM1 plus six VUM2) were lesioned. In fasted larvae, targeted lesions in six VUM2 neurons alone attenuated hunger-elicited increases of feeding response (Fig. 3F), suggesting that proper control of appetitive motivation under fed and fasted conditions may
require the negative and positive regulatory activities from VUM1 and VUM2 neurons, respectively. Lesions in all OA neurons (five VUM3 plus two VPM3) in SOG3 had no effects on larval feeding response (Fig. S4 A and B). In addition, conditioned excitation of tdc2-Gal4 neurons in fed larvae by expressing UAS-dTrpA1 triggered increased feeding response to liquid sugar media. However, this dTrpA1-stimulated effect was completely abolished by targeted lesions in VUM2 and VPM2 neurons (Fig. 3G). Together, our findings suggest that VUM1 neurons may restrict appetite for liquid sugar media in fed larvae by suppressing feeding enhancement by VUM2 neurons.

2.2.6 Drk Mediates Hunger-Induced Appetitive Motivation

From a previous genetic screen, we isolated a candidate gene downstream receptor kinase (drk), the fly homolog of human growth factor receptor-bound protein 2 (Grb2) (166), whose mutations affect larval feeding response to liquid, but not solid, food (Fig. 4). Under fed conditions, larvae transheterozygous for three independent loss-of-function drk alleles, drk^{AP24}/drk^{R1}, drk^{AP24}/drk^{10626} (167, 168), showed basal levels of feeding activity similar to wild-type larvae. However, after 150-min deprivation, the mutant larvae exhibited significantly attenuated feeding responses to liquid food (Fig. 4A). In addition, expression of UAS-drk$^{dsRNA}$ driven by elav-Gal4 also led to significantly reduced hunger-driven responses to liquid food. Conversely, overexpression of drk-cDNA in fed larvae (elav-Gal4/UAS-drk^{cDNA}) caused excessive feeding response to liquid food (Fig. 4B). Importantly, both fasted elav-Gal4/UAS-drk$^{dsRNA}$ and fed elav-Gal4/UAS-drk^{cDNA} showed normal responses to solid food (Fig. S5A). These findings suggest that the neural activity of drk is a positive regulator of hunger-driven feeding response to liquid sugar food.
Because loss of the neural activity of drk or OA signaling led to similar feeding behavioral defects, we investigated whether drk regulates OA neuronal signaling. Indeed, larvae expressing UAS-\textit{drk}^{dsRNA} in \textit{tdc2-Gal4} but not \textit{npf-Gal4} neurons displayed attenuated hunger-driven approaching response to liquid food, but showed normal food response under fed conditions (Fig. 4C). Moreover, oral administration of OA to \textit{tdc2-Gal4/UAS-drk}^{dsRNA} larvae largely restored their deficiency in food motivation (Fig. 4D). In addition, both fasted \textit{tdc2-Gal4/UAS-drk}^{dsRNA} and fed \textit{tdc2-Gal4/UAS-drk}^{cDNA} showed normal responses to solid food (Fig. S5B).

These findings suggest that drk regulates feeding of liquid food through its modulation of OA neuronal signaling.

2.2.7 \textit{Tdc2-Gal4 Neuronal Signaling Requires Pvr}

The fact that Drk is a SH2/SH3 adaptor protein that directly binds to activated receptor tyrosine kinase (RTK) strongly implicates the involvement of an as of yet-unknown RTK in modulation of OA neuronal activity. At least 14 RTK genes have been identified in the Drosophila genome (131, 169-174). To identify which RTK(s) are involved, we performed dsRNA-mediated knockdown of the 14 known RTK genes in \textit{tdc2-Gal4} neurons (Table S2). This initial screening has led to the identification of three candidates, \textit{Eph receptor tyrosine kinase (Eph)}, \textit{heartless (htl)}, and \textit{PDGF/VEGF-receptor-related (Pvr)}, which is a VEGF2-like receptor (134). To assess whether the effects of these three RTKs on \textit{tdc2-Gal4} neurons are physiological, we conditionally knocked down the individual activity of Pvr, Eph, or htl using the GS-\textit{elav-Gal4} driver. We found that only \textit{GS-elav-Gal4/UAS-Pvr}^{dsRNA} larvae showed significantly attenuated hunger-driven response to liquid food (Fig. 5A). Similarly, \textit{GS-elav-Gal4/UAS-drk}^{dsRNA} larvae also showed significantly attenuated hunger-driven response to liquid food. Moreover, the
phenotype of \textit{tdc2-Gal4/UAS-Pvr}^{DN} larvae expressing a dominant negative form of Pvr provides further verification of the essential role of Pvr in appetitive motivation (131) (Fig. 5B and \textbf{Fig. S6 A and D}). Furthermore, expression of a dominant-active Pvr (Pvr\textsuperscript{ACT}) in \textit{tdc2-Gal4/UAS-Pvr}^{ACT} fed larvae caused excessive feeding of liquid sugar media, as evidenced by increased rate of MHC and ingestion of dyed food (Fig. 5C and \textbf{Fig. S6C}). These findings indicate that the Pvr pathway in \textit{tdc2-Gal4} neurons has a previously uncharacterized role in the physiological regulation of hunger-driven food motivation. The Drosophila genome encodes three PDGF/VEGF homologs (Pvf1–3) that function as the ligands of Pvr (131). We tested the three different mutant larvae (\textit{Pvf1}\textsuperscript{MB01242}, \textit{Pvf2}\textsuperscript{d02444}, and \textit{Pvf3}\textsuperscript{EY09531}), each carrying a transposon that disrupts pvf1, pvf2, and pvf3, respectively (\textbf{Table S3}). We have found that \textit{pvf2}\textsuperscript{d02444} larvae showed attenuated hunger-driven feeding response (Fig. 5D).

To provide evidence for the functional interaction between drk and Pvr in \textit{tdc2-Gal4} neurons, we co-expressed \textit{UAS-Pvr}^{dsRNA} and \textit{UAS-drk}^{cDNA} under the direction of \textit{tdc2-Gal4}. We found that expression of \textit{UAS-drk}^{cDNA} in fasted \textit{tdc2-Gal4/UAS-Pvr}^{dsRNA} larvae completely restored the deficiency in approaching response to liquid food (Fig. 5E). Because drk signaling is mediated by Ras85D GTPase (175), we also co-expressed a dominant-negative form of mammalian Ras protein (\textit{UAS-Ras}^{DN} (176)) with \textit{UAS-drk}^{cDNA} in \textit{tdc2-Gal4} neurons. As expected, expression of \textit{UAS-Ras}^{DN} blocked the excessive food response in fed \textit{tdc2-Gal4/UAS-drk}^{cDNA} larvae (Fig. 5F). Together, these findings raise the possibility that Drk and Ras may function in the Pvr pathway to regulate OA neuronal signaling.
2.3 DISCUSSION

2.3.1 A Neural Network That Differentially Regulates Appetitive Motivations

Modulation of feeding responses to food sources is heavily influenced by nutritional quality, taste, and the energy costs of foraging. Our findings suggest that Drosophila larvae have evolved a complex neural network to regulate appetitive motivations (Fig. 6). In hungry fly larvae, OA neurons seem to mediate a specialized circuit that selectively promotes persistent feeding of readily ingestible sugar food. This OA circuit functions in parallel to the previously characterized mechanism co-regulated by the fly insulin and NPY-like systems that drives feeding response to non-preferred solid food (16, 66). Because 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 that OA neurons in the SOG may be conditionally activated by gustatory cues associated with rich palatable food to promote appetitive motivation.

2.3.2 Two Opposing OA Activities in Regulation of Appetite Control

We have provided evidence, at both molecular and neuronal levels, that the OA-mediated feeding circuit has two opposing effects on food motivation. When surrounded by liquid sugar media, the OA circuit is essential to prevent fed animals from excessive feeding. Because targeted lesions in VUM1 neurons caused excessive feeding response, these neurons may define an inhibitory subprogram within the OA feeding circuit (Fig. 3E). However, targeted lesions in VUM2 neurons attenuated hunger-induced increases of feeding response, suggesting that VUM2 neurons, along with the OA receptor Octβ3R, may define a subprogram that enhances feeding in
fasted larvae (Fig. 3F). Several lines of evidence suggest that the VUM2 neuron-mediated subprogram may be suppressed by the VUM1 neuron-mediated subprogram. First, fed larvae with double lesions in both VUM1 and VUM2 neurons failed to display excessive feeding, suggesting that increased feeding response of fed larvae deficient for VUM1 neuronal signaling requires VUM2 neurons. Second, targeted lesions in VUM2 neurons of fed tdc2-Gal4/UAS-dTrpA1 larvae completely blocked the increased feeding response induced by genetic activation of tdc2-Gal4 neurons. Finally, the anatomical data also show that VUM1 and VUM2 neurons project to many common regions of the larval brain implicated in the control of feeding (Fig. 3). Future work will be needed to determine whether VUM1 neurons inhibit directly or indirectly the activity of VUM2 neurons.

2.3.3 Functional Parallels Between OA and Norepinephrine Systems

We have obtained genetic and pharmacological evidence for the critical role of OA in the regulation of acquiring readily accessible sugar media. OA has been reported to mediate diverse neurobiological functions including appetitive memory formation and modulation of the dance of honey bee foragers to communicate floral or sucrose rewards (78, 79, 156). We postulate that the different OA receptors may mediate diverse OA-dependent behavioral responses to high-quality foods.

Norepinephrine (NE), the vertebrate counterpart of OA, has been shown to promote ingestion of carbohydrate-rich food at the beginning of a natural feeding cycle (177, 178). This feeding activity of NE resides in the paraventricular nucleus (PVN) of the feeding control center. In the PVN, α1 and α2 adrenergic receptors are organized in an antagonistic pattern (179). Activation of α1 receptor inhibits food intake (180), whereas activation of the α2 receptor stimulates food
intake (181). Our results suggest that the insect OA system, like the NE system in mammals, exerts both positive and negative effects on the intake of preferred food. The activity of NE in PVN has been shown to antagonize that of 5-HT, which suppresses intake of carbohydrate rich food (182). In Drosophila, 5-HT is also known to suppress feeding response (183). These findings suggest that the homeostatic control of intake of preferred food is likely mediated by a conserved neural network in flies and mammals.

2.3.4 The Role of Pvr in OA Neurons

We have identified a unique role of Pvr in physiological regulation of hunger-motivated feeding of preferred food (liquid sugar media). The feeding-related activity of the Pvr pathway involves two regulatory proteins, Drk and Ras, and oral introduction of OA restores the hunger-driven feeding response in tdc2-Gal4/ UAS-drk dsRNA larvae. Together, these results suggest that the Pvr pathway positively regulates OA release by tdc2-Gal4 neurons. Among the three identified ligands of Pvr (131, 134), Pvf2 is enriched in the larval CNS. Our finding suggests that Pvf2 regulates the feeding-related activity of the Pvr pathway. It is possible that Pvf2 may transduce a metabolic stimulus to Pvr/tdc2-Gal4 neurons that signals the energy state of larvae. In the honey bee brain, OA neurons from the SOG have been reported to respond to sugar stimulation (184, 185). Therefore, it would be interesting to test whether the Pvf2/ Pvr pathway is responsive to sugar stimuli.

Our previous studies showed that the fly insulin and NPY-like systems co-regulate hunger-elicited motivation to acquire solid sugar media (16, 66). We have now provided evidence that the fly VEGFR2- 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 different aspects of adaptive feeding 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 appetitive control as well as pathophysiology of eating-related disorders.

2.4 MATERIALS AND METHODS

2.4.1 Fly Strains, Media, and Larval Growth

The fly rearing and the egg collections were performed as previously described (186). After a 2.5-h synchronized egg collection, eggs were kept in a 12-h light/dark cycle in an incubator at 25 °C. Larvae were transferred to a fresh apple juice plate with yeast paste at the age of 48–52 h (<80 larvae per plate). The fly lines used include tdc2-Gal4 (157), npf-Gal4, UAS-npf cDNA (155), UAS-Kir2.1 (158), UAS-shi ts1 (159), tβR aM18 (113), drk R1, drk AP24 (167), tsh-Gal80 (163), UAS-drk cDNA (discussed below), UAS-drk dsRNA (187), GS-elav-Gal4 (188), UAS-Pvr DN, UAS- Pvr ACT (131), UAS-dTrpA1 (189), UAS-Octβ3R dsRNA, drk 10626, actin-Gal4, UAS-GFP.nls, UAS-NaChBac, elav-Gal4, UAS-Ras DN, UAS-dTrpA1, Pvf 1 MB01242, Pvf2 d02444, Pvf 3 EY09531, and Octβ3R MB04794 (Bloomington Drosophila Stock Center at Indiana University). The following lines were obtained from the Vienna Drosophila RNAi Center: UAS-Pvf dsRNA (105353), UAS-Eph dsRNA (4771), UAS-htl dsRNA (27180), and UAS-Oamb dsRNA (106511).

2.4.2 Transgenic Constructs

The UAS-drk cDNA was made by ligating drk cDNA construct into the pUAST vector. The drk cDNA clone was acquired from Berkeley Drosophila Genome Project. This vector was digested with
EcoRI and XhoI (New England Biolabs), and the resulting 1,558-bp sequence covered the whole 
drk coding sequence. This sequence was subcloned into the EcoRI and XhoI sites of pUAST 
vector, which is at the downstream of the UAS promoters. Purified $drk^{cDNA}$/pUAST was injected 
to w^{1118} (BestGene Inc.)

2.4.3 Behavioral Assays

The rate of larval food intake was quantified by following a previously published protocol with 
slight modification (16, 190). Liquid glucose agar was prepared by mixing 45 mL ddH$_2$O, 5 g D-
glucose (Fisher Chemical), and 6 g agar powder (US Biological). Solid glucose agar was 
prepared by melting a mixture of 90 mL ddH$_2$O, 10 g D-glucose (Fisher Chemical), and 2.3 g 
agar powder (US Biological). The feeding assay was performed in a 35-mm Petri dish containing 
1 g of glucose–agar paste or 2 mL of solid glucose agar. Solidified glucose agar was further cut 
into eight blocks as described (190) and allowed to dry in a 70% humidity chamber overnight. 
For assays, early third-instar larvae (10–20) were transferred to the center of the assay plate, and 
then each plate was videotaped for 2 min. The number of MHCs per 30 s was scored and 
analyzed. The dynamic patterns of larval MHC were generated with a computer program using 
the MatLab software (MatLab Inc.). All assays were analyzed under blind conditions. At least 
three separate trials were conducted for each test. Statistical analyses were performed using one-
way ANOVA followed by the Student–Newman–Keuls or Dunn test. The food ingestion assay 
was carried out by feeding a group of 30 larvae 10% (wt/vol) glucose liquid media containing 
1% food dye FD&C No. 1 (Sigma-Aldrich) for 3 min. After rinsing with a copious amount of 
water, larvae were quickly frozen in liquid nitrogen and homogenized in 100 μL 0.1 M 
phosphate buffer (pH 7.2). The homogenates were centrifuged at 30,000 × g for 10 min, and the
supernatants were analyzed with a spectrophotometer for absorbance at 625 nm. When the fly line UAS-\textit{shi}\textsuperscript{ts1} was used in the liquid food assay, the permissive temperature was room temperature, 23 °C, and the restrictive temperature was 30 °C. Twenty minutes before video recording, the experimental larva groups were pre-warmed on a 30 °C heat plate. The liquid food plate was pre-warmed on the heat plate for 3 min before use, and the videos were recorded on the heat plates. The control group assays were performed at 23 °C. To activate GS-elav-Gal4, larvae were fed with deactivated yeast paste containing 1 mM RU486 (Cayman Chemical) and 4% (vol/vol) ethanol as solvent, and the mock group used deactivated yeast paste containing only 4% ethanol. Ten to 20 second-instar larvae were transferred to RU486 or ethanol yeast plates 20 h before assay.

2.4.4 Pharmacological Treatment

OA stock solution was 500 mM OA in double distilled H\textsubscript{2}O. When used, the solution was diluted to desired concentration with deactivated yeast or with double-distilled H\textsubscript{2}O, dependent on the larval food deprivation conditions. Larvae treated with OA were fed 5 mM or 10 mM OA, in yeast or water, 30 min before video recording. For assays using \textit{GS-elav-Gal4}, the stock RU486 solution was 20 mM RU486 in 80% ethanol. Yeast was deactivated by mixing 5 g yeast powder with 10 mL boiling water and was then mixed with RU486 stock solution or 80% ethanol at the ratio of 1:20 after it cooled. RU486 and ethanol yeast were spread on apple juice plates in Petri dishes and allowed to dry for 24 h before use to evaporate extra water and ethanol.
2.4.5 **Immunohistochemistry**

Brains from larvae 76 h after egg lay were dissected out and the immunostaining were performed as previously described (24) by using chicken anti-GFP (1:1,000; Invitrogen), rabbit anti-ßgal (1:1,000; Promega), rabbit anti-tyromine-ß-hydroxylase (1:500; gift from Vivian Budnik, University of Massachusetts Medical School, Worcester, MA), Alexa 488-goat anti-chicken (1:2,000; Invitrogen), Alexa Fluor-568 goat anti-rabbit (1:2,000; Invitrogen), and rhodamine conjugated phalloidin (1:1,000; Cytoskeleton). Images were collected using a Zeiss LSM510 META confocal microscope.

2.4.6 **Targeted Laser Lesion**

The 337-nm nitrogen laser unit (337-USAS; Micro Point) was calibrated and adjusted as previously described (165). The laser lesion was performed using a previously published protocol with slight modification (69, 191). Selected *tdc2-Gal4* neurons in the subesophageal ganglion (SOG) were lesioned, using UAS-*GFP.nls* as an indicator. In early second-instar larvae (52 h after egg lay (AEL)), the *tdc2- GFP.nls* in the SOG was visible and distinguishable in vivo under 40× magnification. Six to nine second-instar larvae were rinsed briefly and transferred to 150 μL double-distilled H₂O (ddH₂O) on a microscope slide. Ether (250 μL) was added to the anesthetization chamber (90-mm Petri dish with two strips folded four times to hold microscope slide above the ether), the slide was placed inside the chamber, and the chamber was covered immediately. After 3 min, slides were removed and larvae were aligned with a brush, covered with a coverslip, and would remain anesthetized/immobile for roughly 10 min. The laser beam was focused on individual nuclei and was applied as two bursts of 15 shots at a rate of three shots per second. Roughly 50% of larvae on each slide were at the proper angle and displayed
clearly distinguishable SOG tdc2-GFP.nls neurons, which were lesioned with laser and showed the loss of GFP signal. The rest served as the mock group. After the laser treatment, the larvae were allowed to recover on fresh apple juice plates with yeast paste for 24 h before the assay. Each experimental or mock group contained at least 12 individual animals pooled from three separate trials.

2.4.7 Quantitative Real-Time PCR Analysis

For estimation of the UAS-ΨVRdsRNA efficiency, quantitative real-time PCR was conducted on the whole body of early third-instar actin-γ larvae. Total RNA of five early third-instar larvae was extracted with TRIzol (Invitrogen). Five micrograms total RNA was reverse transcribed to cDNA using the kit of SuperScript III FirstStrand Synthesis System for RT-PCR (Invitrogen). One primer was designed to bind cDNA specifically rather than genomic DNA, which overspaned a Pvr intron. RpS17 was used as the endogenous control. Quantitative real-time PCR was performed with Maxima/ROX SYBR Green qPCR Master Mix (Fermentas) in the 7500 Real Time PCR System (Applied Biosystems). Data were calculated with the 7500 System Software and analyzed with unpaired t test using Microsoft Excel.
Figure 2.1. *Quantification of hunger-driven feeding responses of Drosophila larvae.* (A) The rate of larval MHC linearly increases as food deprivation prolongs up to 150 min; the correlation coefficient R = 0.99. (B) The amount of ingested food increased after food deprivation. N = 10 trials. *P < 0.05; **P < 0.01. (C) Each dot represents one bite. The typical feeding patterns sampled from eight fed or 150-min-deprived larvae are shown. (D) Inhibition of *tdc2-Gal4*, but not *npf-Gal4*, neuronal activity using UAS-*Kir2.1* blocked hunger-induced feeding rate increases in liquid food (*P* < 0.01). Columns with identical letters indicate differences are statistically insignificant. (E) In contrast, inhibition of *npf-Gal4* not *tdc2-Gal4* neurons attenuated hunger-induced feeding rate increases in solid food; *P* < 0.01. (F) Transient inhibition of neurotransmission by *tdc2-Gal4* neurons by expressing UAS-*shits1* at 30 °C abolished the hunger-elicited approaching response to liquid food; *P* < 0.01. (G) Stimulation of *tdc2-Gal4* neurons by expressing UAS-*NaChBac* increased feeding response to liquid sugar but not sugar-free media; *P* < 0.01. All of the behavioral assays in this and following figures were analyzed under blind conditions. Unless stated otherwise, at least 12 larvae were tested for each group (*n* ≥ 12), and statistical analyses were performed using one-way ANOVA followed by Student–Newman–Keuls test in all figures. Error bars represent the SE (SEM) in this and all other figures.
Figure 2.2. Control of appetite for liquid sugar food by the OA system. (A) The null mutant $t\beta h^{nM18}$ is deficient in hunger-driven feeding response; columns with identical letters indicate differences are statistically insignificant analyzed with one-way ANOVA followed by Dunn’s test. (B) Prefeeding of fed wild-type larvae with media containing 10 mM OA for 30 min increased larval MHC rate ($P < 0.01$) and (C) ingestion rate on liquid glucose media ($P < 0.05$). (D) Oral OA treatment significantly restored the hunger-driven feeding response of fasted $tdc2$-Gal4/UAS-Kir2.1 larvae; $P < 0.05$. (E) Conditional knockdown of Octβ3R, but not Oamb, in the nervous system attenuated hunger-driven approaching of liquid food. OA treatment failed to rescue the behavioral phenotype; columns with identical letters indicate differences are statistically insignificant ($P > 0.05$). (F) $Oct\beta3R^{MB04794}$ larvae showed attenuated hunger-driven approaching of liquid food ($P < 0.05$).
**Figure 2.3.** Functional mapping of tdc2-Gal4 neurons by targeted laser lesion. (A) Expression of nuclear LacZ in the tdc2-Gal4 neurons in the central nervous system of tdc2-Gal4/UAS-nls.LacZ larvae. The tissue is counterstained with phalloidin. (Inset) The distribution of tdc2-Gal4 neurons in three SOG compartments (SOG1–3). Five VUM1 and four VPM1 neurons (arrows) in the SOG1; six VUM2 and two VPM2 neurons (arrowheads) in the SOG2; five VUM3 and two VPM3 neurons (asterisks) in the SOG3. (Scale bar, 50 μm.) (B) Expression of TβH in the larval brain lobes and ventral nerve cord. No TβH-positive somata were detected in the brain lobes. (Scale bar, 50 μm.) (C) Expression of a nuclear GFP in the central nervous system of tdc2-Gal4/tsh-Gal80/UAS-nlsGFP larvae (lateral view). The GFP expression in the OA neurons from the thoracic and ventral ganglia is blocked by tsh-Gal80. The numbers denote SOG1 to 3, respectively. (Scale bar, 50 μm.) (D) At 30 °C, fed larvae expressing tdc2-Gal4/UAS- shi<sup>ts1</sup>/tsh-Gal80 remained deficient in hunger-driven feeding response; **P < 0.01. (E and F) 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). (G) tdc2Gal4/UAS-dTrpA1 larvae showed abnormally high feeding response, which was blocked by targeted lesions in VUM2 and VPM2 neurons; P < 0.01. At least 12 larvae were tested for each group (n ≥ 12) in three separate trials.
Figure 2.4. *The neural activity of drk in appetitive motivation.* (A) The drk transheterozygous mutant larvae are deficient in hunger-driven food response; *P < 0.05 and **P < 0.01, compared with */+. (B) Knockdown of drk in the nervous system reduced hunger-driven food response (P < 0.05), whereas its overexpression increased feeding response in fed larvae (one-way ANOVA followed by Dunn’s test; P < 0.05). (C) Knockdown of drk activity in *tdc2-Gal4* neurons, but not *npf-Gal4* neurons, attenuated hunger-driven food response. One-way ANOVA followed by Dunn’s test; P < 0.05. (D) OA treatment of *tdc2-Gal4/UAS-drk* dsRNA larvae rescued the deficiency of hunger-driven response to liquid food. Columns with identical letters indicate differences are statistically insignificant.
Figure 2.5. A unique neural activity of the Pvr pathway in appetitive motivation. (A) Conditional pan-neuronal expression of drkd{dsRNA} and Pvr{dsRNA} but not Eph{dsRNA} or htl{dsRNA} reduced hunger-driven food response. One-way ANOVA followed by Dunn’s test; P < 0.05. (B) Expression of UAS-Pvr{dsRNA} and UAS-Pvr{DN} in tdc2-Gal4 neurons attenuated hunger-driven feeding response to liquid media. Columns with identical letters indicate differences are statistically insignificant. (C) Expression of UAS-PvrACT-caused increased feeding in fed larvae; P < 0.01. (D) Fasted P{vf2}d{02444} larvae showed attenuated feeding response; P < 0.01. (E) Fasted larvae expressing both drk{cDNA} and Pvr{dsRNA} in tdc2-Gal4 neurons displayed normal liquid food response; P < 0.01. (F) Fed larvae expressing both drk{cDNA} and Ras{DN} in tdc2-Gal4 neurons also displayed normal liquid feeding response; P < 0.01.
A. OA circuit for feeding regulation

Satiety state

VUM1 \( \rightarrow ? \) \( \rightarrow \) Exuberant Feeding

VUM2 \( \rightarrow \text{OCTB3R} \)

Hunger state
Figure 2.6. *Schematic model for differential regulation of two hunger-driven appetitive motivations.* The OA-mediated circuit, regulated by the Pvf2/Pvr signaling pathway, selectively controls appetite for readily accessible liquid sugar media. This circuit functions independently of the previously identified the dILPs/insulin receptor-regulated NPF circuit that selectively promotes appetite for solid sugar media.
**Figure 2.S1.** *Quantification of hunger-driven feeding responses to liquid sugar food.* (A) The rates of mouth hook contractions (MHCs) of fed and fasted larvae during the 3 s of undisrupted meal time showed only a small difference (ca. 10%). n ≥ 12; three separate trials. (B) Box plot showing variations of time intervals between bites during a 30-s period, which are larger in fed animals. Each box represents one individual larva. (Inset) The SD of intervals from eight individual larvae. Statistical analyses were performed using one-way ANOVA followed by Dunn’s test; P < 0.05.
Figure 2.S2. Role of tdc-Gal4 neurons in hunger-driven feeding responses to liquid sugar food. (A) The dynamic patterns of MHCs of tdc2-Gal4/ UAS-Kir2.1, npfGal4/UAS-Kir2.1, and wild-type larvae. Each dot represents one bite. The larvae were fasted for 150 min, and feeding assay was performed on 10% glucose solid agar food. (B) Transient inhibition of neurotransmission in tdc2-Gal4/UAS-shi^{ts1} larvae at 30 °C caused no significant difference in solid food response relative to control larvae. (C) The dynamic patterns of MHCs of fed and fasted wild-type larvae and fed tdc2-Gal4/ UAS-NaChBac in 10% liquid glucose food. (D) Stimulation of tdc2-Gal4 neurons with UAS-NaChBac failed to increase larval MHC rate on 10% glucose solid food. n ≥ 12; three separate trials.
Figure 2.S3. *NPF activity is dispensable for hunger-driven feeding of liquid sugar food.* (A) Overexpression of NPF in elav-Gal4/UAS-npf\textsuperscript{cDNA} larvae failed to further increase feeding response to liquid food by octopamine (OA)-treated fed larvae. (P < 0.01). (B) Fed elav-Gal4/UAS-npf\textsuperscript{cDNA} larvae showed elevated feeding response to solid food (P < 0.01), which was not further enhanced by OA treatment. n ≥ 12; three separate trials.
Figure 2.S4. *Functional mapping of tdc2-Gal4 neurons by targeted laser lesion.* Analysis of feeding-related activities of OA neurons in the SOG in (A) fed and (B) fasted larvae by generating targeted lesions using focused laser beams (*P < 0.05, **P < 0.01). n ≥ 15; three separate trials.
Figure 2.S5. Characterization of the neural activity of drk in larval feeding response to solid sugar food. Manipulation of drk activity in (A) pan neurons using elavGal4/UAS-drk<sup>cDNA</sup>/UAS-drk<sup>dsRNA</sup> or in (B) tdc2-Gal4 neurons using tdc2-Gal4/UAS- drk<sup>dsRNA</sup>/UAS- drk<sup>cDNA</sup> caused no significant difference in solid food response relative to control larvae. n ≥ 12; three separate trials.
A. 

![Bar graph showing Pvr RNA level with different conditions: actin-Gal4 (±), UAS-PvrΔRNA (+, -, +, -).]

B. 

![Bar graph showing mouth hook contraction per 30s with different conditions: tdc2-Gal4 (−, +, −, +), UAS-PvrΔRNA (−, −, +, +).]

C. 

![Bar graph showing absorbance (% of baseline) with different conditions: x-PvrACT (±), tdc2-PvrACT.]

D. 

![Bar graph showing absorbance (% of baseline) with different conditions: x-PvrΔ, tdc2-PvrΔ.]

B. Solid glucose agar

- 0 min food deprivation
- 150 min deprivation
Figure 2.S6. Characterization of the activity of Pvr in larval feeding responses. (A) Actin-Gal4/Pvr\textsuperscript{dsRNA} larvae showed a reduced Pvr transcripts level. n = 9; one-way ANOVA followed by Student–Newman–Keuls test; P < 0.001. Total RNAs from actin-Gal4/UAS-pvrRNAi and control third-instar larvae (74 h AEL) were extracted using TRIzol (Invitrogen), and 5 μg 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 was performed using Maxima/ROX SYBR Green qPCR Master Mix (Fermentas) in the 7500 Real Time PCR System (Applied Biosystems). (B) Down-regulation of Pvr activity in tdc2-Gal4 neurons using tdc2-Gal4/UAS-Pvr\textsuperscript{dsRNA} caused no significant change in larval feeding response to solid sugar food (n ≥ 12). (C) Enhancement of Pvr activity in tdc2-Gal4 neurons using tdc2-Gal4/UAS-Pvr\textsuperscript{ACT} increased the amount of ingested food; P < 0.01. (D) Down-regulation of Pvr activity in tdc2-Gal4 neurons using tdc2-Gal4/UAS-Pvr\textsuperscript{DN} inhibited the increase of hunger-driven food ingestion; P < 0.05.
Table S1. Functional analysis of four OA receptors via RNA interference dsRNA specific to individual OA receptors. MHCs per 30 s ± SEM, 150-min food deprivation. Feeding responses to liquid sugar food by fasted larvae expressing dsRNA specific to each of four OA receptors driven by GS-Elav-Gal4. Statistical analysis was performed using one-way ANOVA followed by Student–Newman–Keuls test. n ≥ 10, P < 0.01.
Table 2.S2. Functional analysis of the activities of 14 receptor tyrosine kinases in tdc-Gal4 neurons via RNA interference.

<table>
<thead>
<tr>
<th>dsRNA specific to RTKs</th>
<th>0-min food deprivation</th>
<th>150-min food deprivation</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Alk</em></td>
<td>32.33 ± 1.71</td>
<td>52.40 ± 1.53</td>
</tr>
<tr>
<td><em>breathless</em></td>
<td>32.56 ± 1.16</td>
<td>49.88 ± 2.04</td>
</tr>
<tr>
<td><em>Nrk</em></td>
<td>33.38 ± 1.49</td>
<td>48.43 ± 1.21</td>
</tr>
<tr>
<td><em>Dor</em></td>
<td>32.00 ± 1.32</td>
<td>51.67 ± 2.03</td>
</tr>
<tr>
<td><em>Torpedo(EGFR)</em></td>
<td>32.80 ± 2.69</td>
<td>50.00 ± 1.30</td>
</tr>
<tr>
<td><em>sevenless</em></td>
<td>32.88 ± 0.70</td>
<td>52.00 ± 1.73</td>
</tr>
<tr>
<td><em>off-track</em></td>
<td>31.45 ± 0.69</td>
<td>45.73 ± 1.08</td>
</tr>
<tr>
<td><em>Cad96Ca</em></td>
<td>30.20 ± 3.04</td>
<td>52.22 ± 1.22</td>
</tr>
<tr>
<td><em>torso</em></td>
<td>30.20 ± 1.43</td>
<td>49.40 ± 1.72</td>
</tr>
<tr>
<td><em>Insulin-like receptor</em></td>
<td>34.60 ± 1.92</td>
<td>45.94 ± 1.55</td>
</tr>
<tr>
<td><em>derailed</em></td>
<td>33.60 ± 1.19</td>
<td>45.65 ± 1.84</td>
</tr>
<tr>
<td><em>Eph receptor</em></td>
<td>32.80 ± 0.97</td>
<td>40.47 ± 1.15**</td>
</tr>
<tr>
<td><em>heartless</em></td>
<td>34.64 ± 2.58</td>
<td>42.45 ± 2.44**</td>
</tr>
<tr>
<td><em>Pvr</em></td>
<td>31.50 ± 1.71</td>
<td>41.70 ± 1.36**</td>
</tr>
<tr>
<td><em>w</em></td>
<td>31.38 ± 0.61</td>
<td>50.70 ± 0.88</td>
</tr>
<tr>
<td><em>tdc2-Gal4/w</em></td>
<td>31.07 ± 0.77</td>
<td>49.00 ± 1.43</td>
</tr>
</tbody>
</table>

Table 2.S2. *Functional analysis of the activities of 14 receptor tyrosine kinases in tdc-Gal4 neurons via RNA interference.* Feeding responses to liquid sugar food by fed or fasted larvae expressing dsRNA specific to each of 14 receptor tyrosine kinases (RTKs) driven by tdc-Gal4. Statistical analysis was performed using one-way ANOVA followed by Student–Newman–Keuls test. \( n \geq 5, \ P < 0.01 \).
Table S3. Feeding responses to liquid sugar food by fasted *Pvf1*, *Pvf2*, and *Pvf3* larvae.

Statistical analysis was performed using one-way ANOVA followed by Student–Newman–Keuls test. n ≥ 15, P < 0.01.
CHAPTER 3:
AN OAMB/NPF PATHWAY IN LARVAL SOG PREVENTS SWEET FOOD OVERCONSUMPTION

3.1 INTRODUCTION

The adaptive control of foraging decisions is crucial to survival and reproduction and requires selective activation of motor programs which best fit both nutritional need and environmental availability of nutrients. Feeding behaviors can be modulated by diverse neural systems including those responsible for receiving and processing sensory properties and assigning reward and motivational significance of food stimuli (152-154). Foraging motor programs that require high energetic costs, risk, or low nutritional yield must be reserved for situations of high energetic need. This is demonstrated by young fruit fly larvae which prefer to feed on soft, sweet foods but when deprived of food will actively forage on non-preferred food and will increase their rate of feeding.

Our recent study of neurons expressing tdc2-Gal4 (a tyrosine decarboxylase 2 promoter-directed driver) demonstrates that activity of OA neurons is capable of providing a switching mechanism between the exuberant feeding of palatable foods demonstrated by fasted larvae and the slower feeding rate of satiated larvae. In contrast, the manipulation of NPF signaling in the larval nervous system was demonstrated to selectively modulate starvation dependent alterations in motivated feeding, as evidenced by its effect on the feeding of difficult to masticate feeding media. The role of NPF in the modulation of motivated behaviors has been well characterized.
Young, actively foraging larvae display high levels of NPF expression in the CNS, which decreases as larvae age and move toward pupation. Global disruption of NPF signaling in the brains of young larvae triggers precocious movement out of food and early onset of pupation; conversely, elevated expression of NPF in older larvae suppresses pupation. In addition, larval NPF activity is required for starvation dependent motivational processes. As larvae prefer to feed on soft, sweet media, they display aversion for foods that are hard and difficult to masticate. Their willingness to feed on such non-preferred foods increases with the duration of food deprivation. Lack of NPF or its receptor in the larval CNS diminishes the ability of larvae to display such motivated behaviors, and overexpression of the same enhances motivated foraging in satiated larvae. Together, these results suggest that starvation dependent enhancement of NPF signaling is required for appetitive motivation in adverse conditions. To date, however, very little is known regarding signals that may directly enhance or suppress NPF neuron activity or NPF release, and recent studies demonstrate that NPF/NPFR1 signaling selectively alters separate behavioral processes in different regions of the brain. For example, in the mushroom bodies, an area important for learning and memory, NPF/NPFR1 signaling is required for inhibition of tonic dopamine (DA) release in order to gate the expression of starvation dependent learning enhancement (88), whereas in the lateral horn it gates the perception of olfactory inputs (69). In this work, we provide evidence for a putative pathway acting upstream of NPF neurons. We show that an octopamine (OA)/OAMB dependent mechanism operates through NPF neurons located in the subesophageal ganglia (SOG) which acts to prevent excessive appetite in fed larvae. Given our previous finding that the Oamb receptor is required to suppress faster rates of feeding in satiated larvae, we hypothesized that OA signaling may act through a selective pathway to suppress feeding rate under times when internal energy stores are sufficient. The
selective activation of this subprogram in satiated larvae may be due to selective suppression of
OA neuron subsets, or by selective activation of an OA/OAMB dependent inhibitory pathway.
To investigate this, we began with an UAS-\(Oamb^{dsRNA}\) mediated screen to identify types of
neurons required for Oamb feeding rate suppression.

3.2 RESULTS AND DISCUSSION

3.2.1 Baseline Rate of Sweet Food Intake Requires Oamb
Four different OA receptors have been identified in Drosophila (114, 116). In previous work, we
identified a specific role for the B-adrenergic-like OctB3R receptor in promotion of food intake
under conditions of food deprivation (3). In order to identify other downstream effectors of the
OA feeding pathway, we used a mifepristone (RU486)-inducible pan-neural \(GS-elav-Gal4\) driver
to perform dsRNA-mediated conditional knockdown of individual OA receptor activity. Third
instar mutant larvae were then tested for behavioral responses to sweet, palatable media under
fed and starved conditions (Fig.1, Table S1). We found that only disruption of Oamb receptor
(116) was sufficient to trigger feeding rate enhancement in satiated larvae (Fig. 1A).
Furthermore, larvae carrying a Minos transposable element insertion at the Oamb locus,
\(Mi[ET1]Oamb^{MB00297}\) (192, 193), also displayed an enhanced feeding response (Fig. 2A), as did
\(Oamb^{286}\) larvae (194, 195) (Fig. 2B).
In our previous work, we have shown that \(tdc2-Gal4/UAS-NaChBac\) larvae expressing a
bacterial sodium channel construct in OA neurons displayed increased feeding rates only when
the assay media contained glucose (3), indicating that OA dependent enhancement of larval
mouth hook contraction rate was specific to nutritive food rather than a non-specific increase in
locomotion. Similarly, \(Oamb^{286}\) larvae were also tested for responses to media containing 0%,
5%, and 10% glucose (Fig. 2B), and demonstrated a graded increase in mouth hook contraction rate as the concentration of glucose was increased in the media, indicating that lack of Oamb specifically enhances feeding responses to sweet food. These results suggest the presence of an OA/Oamb mechanism that maintains the baseline feeding rate of larvae on sweet foods by suppressing the response to sugar.

3.2.2 OAFS-Gal4 Larvae Display Enhanced Basal Feeding Rate on Sweet Food

The OA receptor Oamb is expressed as two isoforms (K3 and AS), both of which have been shown to mediate increases in intracellular calcium, similar to the mammalian a1-adrenergic receptor (196). Expression of Oamb-K3 and Oamb-AS has been demonstrated in the mushroom body where they are required for appetitive olfactory memory formation in response to sugar (196) and courtship behavior (194). In an effort to identify and target neurons expressing the Oamb receptor underlying the suppression of food intake in fed larvae, we constructed an Oamb enhancer based Gal4 line using a 1.6 kb fragment upstream of the Oamb gene locus. This oaFS-Gal4 (OA feeding suppression, (oaFS)) line drives expression in the brain lobes, in four pairs of neurons in the SOG, and in the posterior portion of the VNC (Fig. 3B). To test whether these cells underlie Oamb-dependent feeding regulation, we tested oaFS-Gal4/UAS-Oamb\textsuperscript{dsRNA} larvae for response to sugar media and observed an increase in feeding rate (Fig. 3A). This demonstrates that the oaFS-Gal4 targets expression to neurons which functionally mediate the effects of the Oamb receptor on feeding rate suppression in satiated larvae.
3.2.2 Suppression of Sweet Food Consumption Requires NPF/Oamb

A number of neurotransmitter systems have been implicated in the regulation of food intake, appetitive motivation, and appetitive learning in Drosophila. We speculated that OA may exert suppression in food intake in satiated larvae by activating the Oamb receptor on neurons which regulate food intake. To functionally map the activity of Oamb, we performed a dsRNA-based screen with a library of Gal4 lines which drive expression in known feeding regulatory neurons (Fig. 3B). Of the chosen lines used, only npf-Gal4/UAS-Oamb\textsuperscript{dsRNA} larvae phenocopied the increase of feeding rate on sweet media seen in Oamb mutants. This suggests that NPF neurons mediate the effects of the Oamb receptor.

3.2.3 NPF SOG neurons are required to suppress food intake

Drosophila npf-Gal4 is expressed in six neurons in the protocerebrum and subesophageal ganglion of larval brain (Fig. 4B). OA neurons in larval SOG respond to gustatory inputs from gustatory receptor neurons (81, 161), and the SOG is proposed to act as a feeding control center in the central nervous system of insects (14, 164). When oaFS-Gal4/UAS-mCD8GFP larvae were co-stained with anti-NPF, co-localization was seen only in NPF neurons located in the SOG region. To determine whether npf-Gal4 neurons in the SOG are important for the Oamb dependent suppression of feeding rate, we generated targeted lesions in the DM, DL, and SOG subsets of npf-Gal4 neurons using focused laser beams (165). Following lesioning of the DM or DL pairs, npf-Gal4/UAS-GFP.nls larvae behaved similarly to mock treated controls, however, lesions of the SOG subset caused a significant increase in the feeding activity (Fig. 4A). We have previously shown that alterations in NPF or NPFR1 activity in the larval CNS is sufficient to disrupt starvation dependent motivated feeding behaviors, such the willingness to work to
procure food (15) or to consume foods with aversive tastes (66) or temperature. These findings present the first evidence of a role for NPF signaling in the regulation of intake of preferred foods. The reduction of Oamb levels in the CNS or specifically in OaFS-Gal4 and npf-Gal4 neurons results in starvation-like feeding behavior in larvae. In conjunction with our finding that VUM1 OA neurons mediate a feeding suppression mechanism, these results suggest that OA signaling in satiated larvae acts to suppress overconsumption of sweet foods via a local circuit involving OA and NPF neurons located in the SOG.

3.4 MATERIALS AND METHODS

3.4.1 Fly Strains, Media, and Larval Growth

The fly rearing and the egg collections were performed as previously described (186), with a slight modification for glucose feeding assays using 0%, 5%, and 10% glucose. After a 2.5-h synchronized egg collection, eggs were kept in a 12-h light/dark cycle in an incubator at 25 °C. Larvae were transferred to a fresh apple juice plate with yeast paste at the age of 48–52 h (<80 larvae per plate). The fly lines used included tdc2-Gal4 (157), npf-Gal4 (15), Oamb^{286} (194, 195), npfr1-Gal4 (69), UAS-dilp2-Gal4 (16). UAS-GFP.nls, Mi[ET1]Oamb^{MB00297} (192, 193), UAS-mCD8-GFP, GS-elav-Gal4, UAS-OctB2R^{dsRNA}, UAS-OctB3R^{dsRNA}, TH-Gal4, vglut-Gal4, TRH-Gal4, were obtained from Bloomington Drosophila Stock Center at Indiana University. UAS-Oamb^{dsRNA} (#2861) (197), UAS-qa2^{dsRNA} (#47896) (197) were obtained from the Vienna Drosophila RNAi Center.
3.4.2 Transgenic Constructs

To construct the `oaFS-Gal4` driver line, a 1.6 kb DNA fragment containing the 5’ regulatory sequence for the Oamb gene was amplified by 5’-

ATACATACTAGAATTCTCTGAAAGCTGCGGGATA-3’ and 5’-

GGGCGAGCTCGAATTCCGGCAAGAACCGTTAGTTC-3’ and cloned into the pCaSpeR-Gal4 vector at the EcoR1 site. The purified construct was injected to `w^1118` background (BestGene Inc.).

3.4.3 Behavioral Assays

The rate of larval food intake was quantified by following a previously published protocol with slight modification (16, 190). Liquid glucose agar was prepared by mixing 45 mL ddH2O, 5g D-glucose (Fisher Chemical), and 6 g agar powder (US Biological). Solid glucose agar was prepared by melting a mixture of 90 mL ddH2O, 10 g D-glucose (Fisher Chemical), and 2.3 g agar powder (US Biological). The feeding assay was performed in a 35-mm 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 (190) and allowed to dry in a 70% humidity chamber overnight. For assays, early third-instar larvae (10–20) were transferred to the center of the assay plate, and then each plate was videotaped for 2 min. The number of MHCs per 30 s was scored and analyzed. All assays were analyzed under blind conditions. At least three separate trials were conducted for each test. Statistical analyses were performed using one-way ANOVA followed by the Student–Newman–Keuls or Dunn test.
3.4.5 *Immunohistochemistry*

Brains from larvae 76 h after egg lay were dissected out and the immunostaining were performed as previously described (24) by using chicken anti-GFP (1:1,000; Invitrogen), rabbit anti-NPF (cite), Alexa 488-goat anti-chicken (1:2,000; Invitrogen), Alexa Fluor-568 goat anti-rabbit (1:2,000; Invitrogen), Images were collected using a Zeiss LSM510 META confocal microscope.

3.4.6 *Targeted Laser Lesion*

The 337-nm nitrogen laser unit (337-USAS; Micro Point) was calibrated and adjusted as previously described (165). The laser lesion was performed using a previously published protocol with slight modification (69, 191). Selected npf-Gal4 neurons in the subesophageal ganglion (SOG) were lesioned, using UAS-GFP.nls as an indicator. In early second-instar larvae (52 h after egg lay (AEL)), the npf- GFP.nls in the SOG was visible and distinguishable in vivo under 40× magnification. Six to nine second-instar larvae were rinsed briefly and transferred to 150 μL double-distilled H₂O (ddH₂O) on a microscope slide. Ether (250 μL) was added to the anesthetization chamber (90-mm Petri dish with two strips folded four times to hold microscope slide above the ether), the slide was placed inside the chamber, and the chamber was covered immediately. After 3 min, slides were removed and larvae were aligned with a brush, covered with a coverslip, and would remain anesthetized/immobile for roughly 10 min. The laser beam was focused on individual nuclei and was applied as two bursts of 15 shots at a rate of three shots per second. Roughly 50% of larvae on each slide were at the proper angle and displayed clearly distinguishable SOG npf-GFP.nls neurons, which were lesioned with laser and showed the loss of GFP signal. The rest served as the mock group. After the laser treatment, the larvae were allowed to recover on fresh apple juice plates with yeast paste for 24 h before the assay.
Each experimental or mock group contained at least 12 individual animals pooled from three separate trials.
Figure 3.1. *Oamb* is required for suppression of food intake in fed larvae. A) Conditional knockdown of *Oamb*, but not other octopaminergic receptors, with the *GS-elav-Gal4* inducible pan-neural driver line resulted in enhancement of feeding rate in satiated larvae (**p<0.01). B) Expression of two different *Oamb*dsRNA constructs similarly triggered increased feeding response. Columns with identical letters indicate differences are statistically insignificant analyzed with one-way ANOVA followed by Dunn’s test (**p<0.01).
**Figure 3.2.** *Oamb* mutant larvae display enhanced basal rates of feeding.

A) *Mi[ET1]*Oamb<sup>M11478</sup> larvae carrying a Minos transposable element insertion in the Oamb locus display an enhanced feeding rate (**p<0.001**). B) *Oamb<sup>286</sup>* null larvae with a deletion of the Oamb gene display enhanced feeding rate only when feeding media contains glucose (**p<0.001**).
A

**MOU**

TH HOOK C

TRACTIONS 30 se

wt OaFS

OaFS

OaFS x Oamb

B

C

**MOU**

TH HOOK C

TRACTIONS 30 se

wt Oamb

npf

yglu

dilp

ten

trh
Figure 3.3. *Oamb* is required in NPF and *oaFS-Gal4* neurons for feeding suppression. A) *OaFS-Gal4/UAS-Oamb*dsRNA larvae display increased feeding rate on sweet food, similar to *Oamb* mutants (**p<0.001**). B) Expression pattern of *OaFS-Gal4/UAS-mCD8GFP*. C) Functional mapping of *Oamb* regulation of feeding via Gal4 mediated screen in cells expressing feeding regulatory neuropeptides. Only larvae expressing *npf-Gal4/UAS-Oamb*dsRNA displayed increased ingestion rate (**p<0.01**).
A

npf-Gal4<UAS-nls,GFP

MOUTH HOOK CONTRACTIONS/30 sec

<table>
<thead>
<tr>
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* p < 0.05

B

DM
DL
SOG

C

α-NPF

D

OaFS-Gal4>
UAS-CD8GFP

E

α-NPF

OaFS-Gal4>
UAS-CD8GFP
Figure 3.4. NPF SOG neurons are required for suppression of sweet food intake and co-express Oamb. A) Targeted laser lesion of dorsal medial (DM), dorsal lateral (DL), and subesophageal ganglion (SOG) subsets of npf-Gal4/UAS-nls.GFP neurons in fed larvae. Only lesion of the SOG subset resulted in an enhancement of sweet food intake (*p<0.05). B) Expression pattern of npf-Gal4/UAS-mCD8GFP shows DM, DL, and SOG NPF neuron subsets. C) Anti-NPF immunoreactivity, D) expression of OaFS-Gal4/UAS-mCD8GFP, E) merged images. Co-localization is observed only in the SOG subset of NPF neurons.
CHAPTER 4:

PREVENTION OF PALATABLE DIET-INDUCED HYPERPHAGIA IN RATS BY CENTRAL INJECTION OF A VEGFR KINASE INHIBITOR

4.1 INTRODUCTION

Humans and animals alike are vulnerable to the temptation of overeating appetizing foods. Many studies have implicated the “rewarding” nature of tasty foods in the drive to overeat (77, 198). Palatable foods are more likely to be eaten in excess relative to bland foods, and the pleasurable effects of consuming them may override homeostatic signals (77, 198-200). It is speculated that obese individuals may be more susceptible to the temptations of food. In support of this, obese humans display heightened activity in neural reward circuits in response to palatable food or food-associated cues (201, 202). The taste of palatable food may also contribute to overeating by suppressing satiety. For example, in the presence of palatable food, endocannabinoids increase appetite by stimulating the desire to eat while also blocking signals for meal termination (203).

Our recent study using the *Drosophila* larva model points to the essential roles of a vesicular endothelial growth factor (VEGF)-like ligand (Pvf2) and its receptor (dVegfr, also known as Pvr) in promotion of hunger-driven intake of palatable food (3). Furthermore, the enhancement of feeding by Pvf2/dVegfr signaling positively correlated with the concentration of sugar in the media. These findings have led us to postulate that a homologous VEGF signaling pathway in mammals may have a parallel function in regulation of consumption of palatable food. An N-cyclopropynaphthamide compound, named as VEGFR Tyrosine Kinase Inhibitor V (VTKI-V),
has been shown to selectively inhibit VEGF2 receptor kinase at subnonamolar concentrations (204). Oral and intraperitoneal administration of VTKI-V in mice has been demonstrated to inhibit angiogenesis and to promote regression of established HT29 tumors (205). In this work, we examined its potential effect on the feeding behavior of male Sprague Dawley rats. We find that i.c.v. injection of VTKI-V at a lower single dose attenuates overeating induced by sweet and fatty food. The same drug treatment also reduced body weight gain of animals fed with CHOW or sweet and fatty food. Our results suggest that i.c.v. VTKI-V may interfere two separate VEGFR-mediated mechanisms that promote overconsumption of palatable food and body weight gain, respectively. Therefore, components of the VEGFR pathway may have potential diagnostic and therapeutic applications for prevention of overeating and obesity.

4.2 RESULTS

4.2.1 Effects of i.c.v. Injection of VTKI-V on CHOW-Fed Rats

Our previous finding of the role of neural VEGFR-like signaling in promoting hunger-driven feeding of Drosophila larvae led us to speculate that a mammalian VEGFR system(s) might perform a similar neural function. To test this hypothesis, we selected a small molecule inhibitor of VEGFR tyrosine kinase, VTKI-V, which has been characterized pharmacologically for its selectivity profile against tyrosine kinase receptors and its efficacy in inhibition of VEGF dependent VEGFR2 phosphorylation and tumor cell growth (204). In order to target the neural activity of the VEGFR system, the compound was injected i.c.v. In a pilot dose response study, a single dosage of 2 µg dissolved in 2 µl of DMSO was determined to be sufficient to elicit a reduction in weight gain (data not shown). On a per animal basis, this dose is >500 times lower
than the minimal effective oral dose (3 mg/kg) demonstrated to block VEGF mediated processes (205). This dosing regimen was subsequently adopted throughout the following experiments.

Following recovery from surgery, rats of roughly equal starting weight (control group: 288 ± 22.1; drug group: 287 ± 25.8) were habituated to home cages for one week. Rats were then injected with one dose of VTKI-V, followed by monitoring for body weight, food intake, and water intake for five days post injection (Fig. 1). The treatment resulted in a rapid reduction in the rate of body weight gain detectable within 24 hours, and it persisted during the test period (Fig. 1A). This was accompanied by a slight but significant reduction in cumulative daily intake (p<0.05 for day 3 and 5; Fig. 1B). By five days post injection, animals treated with VTKI-V gained approximately 35% less weight than vehicle treated controls and reduced their caloric intake by roughly 10%. However, no significant alteration of water intake was observed (Fig. 1C). These results suggest that a single dose of i.c.v. VTKI-V is sufficient to inhibit the weight gain in the male rats over at least 5 days. It also appears to cause a slight but significant reduction in cumulative consumption of CHOW.

4.2.2 Effects of i.c.v. Injection of VTKI-V on Rats Fed a Palatable Diet

We previously demonstrated that reduced VEGFR-like signaling in *Drosophila* larvae selectively attenuated feeding rate when hungry larvae were placed on media with higher sugar concentrations. Therefore, we postulate that a mammalian VEGFR system might have a similar function. To test this, we acclimated animals to a palatable diet rich in fat and sugar (Cat No. RD12506; Research Diets). This high-calorie diet (HC) has been widely used in studies of diet induced obesity (206) and hedonic feeding (144). Animals with roughly equal body weight (DMSO group: 346.9 ± 5.7; VTKI-V group: 366.6 ± 3.4) were injected with either VTKI-V or
vehicle as described before. Treatment again resulted in a rapid as well as persistent reduction in the daily rate of body weight gain (Fig. 1D). By five days post injection, animals treated with VTKI-V gained approximately 60% less weight than vehicle treated controls (p < 0.0001). Surprisingly, however, a more pronounced effect on cumulative daily intake was observed under this condition (Fig. 1E). The drug-treated animals consumed 30% less calories than the controls (p < 0.0001). Again, the VTKI-V treatment had no significant effect on water intake (Fig. 1F). Together, these results indicate that the VTKI-V treatment results in more drastic attenuation of food intake when the fat and sugar content of the diet is increased. However, the effect of the drug on body weight gain remains substantial under both feeding conditions.

4.2.3 Effect of Drug Treatment on Conditioned Taste Aversion

The experience of DMSO or VTKI-V injection (unconditioned stimulus) was paired with a novel neutral taste (conditioned stimulus) with the experimental paradigm outlined in Figure 5A. Briefly, prior to drug administration, animals were trained to drink sweetened condensed milk from 25-mL graduated drinking tubes presented for one hour periods in a pattern of two days on/one day off, and 30 minute ingestion amount was recorded. Once animals reached a stable baseline of ingestion of the solution, the conditioning trial was performed in which novel orange flavoring was added (0.5 ml/liter). Immediately following the removal of the orange flavored milk, animals received and injection of DMSO (n=7) or VTKI-V (n=8). Three days later, animals were again presented with orange flavored milk solution and ingestion was recorded. Animals displayed no aversion to the orange flavored milk after treatment with either DMSO or VTKI-V (Fig. 5). This demonstrates that the effects of VTKI-V on body weight gain and food intake are not due to nausea or malaise.
4.2.4 Effects of i.c.v. VTKI-V on Meal Pattern

To better understand the behavioral effects of VTKI-V, we assessed potential changes in meal pattern that contributed to altered food intake. CHOW fed control animals treated with DMSO or VTKI-V ate similar amounts of food in diurnal and nocturnal phases of the five-day period in terms of calories or mass (Fig. 2A, B). This observation is largely consistent with the minor effect of VTKI-V on cumulative food intake shown in Fig. 1B. In addition, the meal patterns of both groups showed no significant changes, as evidenced by the meal number, size and duration as well as bouts per meal (Fig. 2C-F).

However, control animals given access to the palatable diet exhibited hyperphagic behavior. Their diurnal and nocturnal caloric intake increased by roughly 50%, although the mass of food consumed remained the same (Fig. 2A, B). We found that this increased calorie intake is due to an increase in average meal size rather than meal number (Fig. 2C, D). Contrasting, VTKI-V-treated animals showed reduction in the mass of palatable food ingested, bringing their caloric intake down to a level comparable to that of CHOW fed animals (Fig. 2A, B). Furthermore, we observed that the drug treatment reduced meal size and duration as well as the number of bouts per meal (Fig. 2C, F). However, the meal number remained unaltered (Fig. 2D). Together, these results suggest that i.c.v. VTKI-V may affect meal consumption but not initiation in both dark and light phases.

To evaluate the time course of VTKI-V effects, we further analyzed the meal patterns of the four animal groups by individual 12-hour periods over five consecutive days. We found that VTKI-V injection caused no significant changes in all parameters examined under CHOW fed conditions (Fig. 3A-E). In contrast, the drug treatment resulted in significant reductions in food intake, meal size, bouts per meal, and meal duration. These effects were evident within 12 hours and persisted
for at least five days (Fig. 3F-I). Again, the average number of meals for the light or dark period remained constant throughout the 5-day test period (Fig. 3J). These findings suggest that a single dose of VTKI-V is sufficient to inhibit feeding of palatable food over a significant length of time without detectable compensatory consumption as normally seen in calorie restricted animals (207). These results also reveal that the magnitudes of the drug effects on meal consumption and duration were comparable for five consecutive days.

4.2.5 Effects of Reduced Neural dVEGFR Activity on Body Weight Gain in Drosophila

Given that i.c.v. injection of VTKI-V resulted in a significant reduction of body weight gain in rodents, we sought to investigate whether reduction in neural activity of the fly VEGF-like pathway had a similar effect. Activity of dVegfr was knocked down by expressing its double-stranded RNA (UAS-dVegfr dsRNA) using a pan-neural elav-Gal4 driver. The body mass and protein content of elav-Gal4/UAS-dVegfr dsRNA and male flies were significantly lower relative to the controls in 4 day-old adults (Fig. 4A, B). These results suggest that the neural VEGFR systems in insects and mammals may have parallel regulatory functions in the central control of body weight as well as intake of palatable food.

4.3 DISCUSSION

We have shown that i.c.v. injection of a single dose of a VEGFR tyrosine kinase inhibitor causes reduced body weight gain and attenuated consumption of palatable food, and that these effects are both rapid and persistent. Therefore, these results raise the possibility that VTKI-V may interfere with two separate functions of VEGF system(s) that acutely regulate body weight gain and consumption of palatable food, respectively. In our assay, VTKI-V is administered into the
lateral ventricles at a single dose of 2 µg. This is >500 fold lower than the minimal effective intraperitoneal dose shown to reduce VEGF-mediated angiogenesis and tumor growth (205). This result argues for a neural target(s) that mediates the behavioral effects of i.c.v. VTKI-V. Very little is known regarding central regulation of feeding behavior by VEGFR. Inhibition of VEGFR2 has been demonstrated to block the effects of fluoxetine on sucrose preference (140). VEGFRs are essential for a number of neuronal activities such as axonal outgrowth and membrane excitability (208). VTKI-V may exert its effects by altering activity of a neural system (e.g., sympathetic nervous system) to regulate peripheral metabolism of stored fat and/or regulation of energy expenditure (209). It is also possible that VTKI-V might interfere with a central feeding circuit (e.g., the norepinephrine circuit implicated in intake of carbohydrates) that promotes appetitive response to food rich in sugar and fat (210, 211). Future determination of the target(s) of i.c.v. VTKI-V will likely yield fresh insights into novel strategies for prevention of overeating and the development of obesity.

This and other studies have shown that animals fed with palatable diets exhibit rapid alterations in meal pattern, including increased meal size and duration (206, 212). These changes persist over time and lead to diet induced obesity (206, 213). In our studies, the control animals acclimated to the palatable diet displayed a similar trend. VTKI-V injection led to a roughly 35% reduction of the average mass of food ingested, bringing their caloric meal size down to a similar level as CHOW fed animals. Intriguingly, the drug treatment caused at most a minor reduction in CHOW consumption, which is only detectable by measuring cumulative intake over more than three days. These findings suggest that i.c.v. VTKI-V may selectively affect a VEGFR-mediated mechanism that regulates palatable-diet induced hyperphagia.
An in-depth analysis of meal pattern reveals that the reduced consumption of palatable food is due to smaller, shorter meals with fewer bites. We find that control animals fed on the rich diet behave similarly to the CHOW fed animals in terms of meal number, duration and mass. This translates to a 55% increase in daily caloric intake over the testing period. Following VTKI-V treatment, animals consumed a smaller mass of food, and therefore fewer calories. This appears to be the result of eating shorter and smaller meals (Fig. 2, 3). Together, these findings suggest that VTKI-V treatment selectively alters the mass of food ingested, but only when the energy density of that food exceeds a baseline need of calories.

We also observed that VTKI-V treatment caused a reduction in the rate of weight gain. This effect was rapid and persistent, with a five day reduction of 35% and 60% for rats fed on CHOW or the rich diet, respectively. Given that drug-treated rats fed with CHOW diet display a modest reduction of cumulative food intake, this suggests that the comparatively large effect of VTKI-V on body weight is largely due to its interference with a physiological process that controls body weight gain. In contrast, the more pronounced reduction in body weight gain for animals fed with the rich diet could be due to the compounding effects of the drug on mechanisms underlying both feeding and metabolism. These results are reminiscent of diet-induced obesity resistant rats that display a reduction of meal size and caloric intake when fed with palatable diets relative to normal controls (206).

In Drosophila larvae, attenuation of neural signaling by the VEGFR-like system blocks hunger-driven feeding activity in sugar-rich media, and the magnitude of this effect becomes smaller as the concentration of sugar is reduced (3). Interestingly, we find that i.c.v. injection of VTKI-V caused pronounced reduction in consumption of the food rich in sugar and fat, but showed much less effect on CHOW intake. These parallel observations across phylogenetically divergent
species raise the possibility that the VEGFR system may play a conserved role in neural circuitry that underlying palatable food-induced overeating. In addition, the regulatory activity of the fly VEGFR system for feeding of sugar media has been mapped to a subset of neurons producing octopamine. Future work will test whether the effect of i.c.v. VTKI-V on overeating of palatable food is mediated by norepinephrine neurons.

4.4 MATERIALS AND METHODS

4.4.1 Animals and Diet

Sixty male Sprague-Dawley rats approximately 7-8 weeks old (250 – 270g of initial body weight) purchased in batches of twelve from Harlan Inc. (Indianapolis, IN, USA) were individually housed in plastic shoebox cages equipped with BioDAQ® Food Intake Monitors (Research Diets, Inc., New Brunswick, NJ). Rats were maintained on a 12/12-hr light-dark cycle (lights on at 06.00h) in a temperature controlled environment (23 ± 1°C) with ad libitum access to food and water throughout the study. CHOW fed animals received 5053-Rodent Diet (LabDiet, St. Louis, MO) (3.07 kcal/g, 13% fat, 5% sugar) and HC groups received 12451-Research Diets (4.73 kcal/g, 45% fat, 17% sucrose). Rats were removed from and returned to their homecages daily (between 09.00h and 10.00h) for food hopper maintenance and monitoring of body weights and water bottle weights. Food intake was monitored as described below. All experimental and surgical procedures were approved prior to initiation of the study by the Institutional Animal Care and Use Committee of the University of Georgia.

Fly rearing and egg collections were performed as previously described (3). Following a 2.5-h synchronized egg collection, petri dishes containing egg plates were maintained in a 12-h light/dark cycle incubator at 25 C. Larvae were transferred to fresh apple juice plates at 48-52h
(>80 per plate), and returned to incubator until adults began to emerge (9-10 days). Male adults were collected and transferred to standard yeast, cornmeal, and molasses fly media for 4-5 days. Samples were prepared with five flies per Eppendorf tube, which were flash frozen in liquid nitrogen. Following freezing, flies were thawed and weighed to determine average body mass. Flies were then homogenized and average protein content per fly was determined using a BCA protein assay (Peirce, Cat No. 23225). Fly lines included Canton S (BL1) and elav-Gal4 (BL8760) (Bloomington Stock Center at Indiana University (BL)). UAS-dVegfr\textsuperscript{TKI} (105353) was obtained from the Vienna Drosophila RNAi Center.

4.4.2 Drug Treatment

VEGFR2 Tyrosine Kinase Inhibitor V or VTKI-V (Cat No. 676501) was purchased from EMD Millipore, Darmstadt, Germany. It is a cell-permeable, ATP-binding pocket-targeting N-cyclopropynaphthamide compound with high selectivity against VEGFR2 (IC\textsubscript{50} = 0.6 nM) (204). The compound was dissolved in DMSO (Sigma) at a concentration of 1 µg/µl, and aliquots were stored at -80 °C. For all injections, 2 µL of VTKI-V solution or vehicle (DMSO) were delivered via i.c.v. cannula at a rate of 1 µL/min.

4.4.3 Surgical Procedures

Following a one-week acclimation period, anesthetized animals were implanted with a chronic 22 gauge i.c.v. cannula (Plastics One, Roanoke, VA) stereotaxically positioned in the lateral ventricle with the coordinates 1.0 mm posterior, 1.5 mm lateral, and 3.2 mm ventral to bregma. The cannula was affixed to the skull by three stainless steel screws and cranioplastic cement.
Following surgery, animals were transferred back to their home cages and allowed to recover for three days prior to treatment.

### 4.4.4 Cannula placement verification

Angiotensin II drinking tests were performed one week after surgery. Animals were given one intracerebroventricular (i.c.v.) injection of 100 ng human angiotensin II (Sigma, St. Louis, MO) dissolved in 5µL volume of saline. Rats that drank >5 mL of water in 30 minutes were considered to have the cannula correctly placed.

### 4.4.5 Conditioned taste aversion test

Behavioral testing was performed during the light cycle between 10.00 and 12.00 h. On days 1, 2, and 4 of behavioral testing, food and water were removed for one hour and animals presented with sweetened condensed milk (diluted, 1 part milk: 2 parts distilled water) in 25 mL calibrated drinking tubes secured to the homecage. Consumption of solution was measured after 30 minutes. On day 5 of the experiment a conditioning trial was performed in which animals were offered the sweetened condensed milk solution flavored with orange extract (0.5 mL/liter). Consumption was recorded at the time points of 15 and 30 minutes. Immediately following removal of the drinking tubes, animals received an injection (2 µl of 1 µg/µl VTKIIV in DMSO). On day 8, animals were again offered the orange flavored solution, and ingestion was recorded after 30 minutes. Conditioned taste aversion was calculated as the percentage of orange flavored SCM ingested on the test day relative to baseline ingestion of non-flavored SCM during the training period (214).
4.4.6 *Food Intake Monitoring and Analysis*

All animals were individually housed in homecages equipped with the BioDAQ® Food Intake Monitoring System (215). It provides accurate and continuous collection of food intake with minimal experimenter intervention. The system consists of individual low spill food hoppers attached to the outside of each homecage which rests on a strain gauge-based load cell (peripheral sensor controller, PSC) that measures the weight of the hopper 50 times/second. Each PSC is linked to a central controller, which transmits raw data to a peripheral computer where the mean mass and standard deviation of the hopper weight is calculated each second. When the computer detects a difference in hopper weight of ≥0.01g for a minimum of five seconds, it is recorded as the beginning of feeding event (bout). Termination of a feeding event is reached when the hopper is undisturbed minimum of five seconds. Upon termination of a bout, the computer records the difference in beginning and ending weight (bout grams), and the duration of the event. This information is stored using BioDAQ® Monitoring Software 2.1.00 which allows viewing, filtering, and export of the raw data for further analysis.

4.4.7 *Data Processing and Statistics*

Continuous raw data for each animal exported in 5 day blocks from BioDAQ® Monitoring Software 2.1.00. Data was imported into MATLAB, in which all filtering, bout, and meal calculations were performed. Minimal filtering was applied to remove data points indicative of mechanical error or human intervention (values between 0.01/-0.01, negative values, and bouts ≥3g). Data occurring within the hopper and animal maintenance period (9.00 – 10.00h) was also filtered.
Bouts were defined as a difference in hopper weight of >0.03g, and bouts were binned to define a meal when a net food intake of ≥0.23 g occurred within 15 min (206, 215). A meal was considered terminated when no bouts occurred for a period of 15 minutes. Bouts not occurring within a meal were included in calculation of total intake, but excluded from meal pattern analyses. Duration of a meal includes bouts that are not separated for more than 15 minutes. Meal size is calculated based on calorie intake (food mass multiplied by its caloric content) per meal.

Feeding data for each animal was binned by 24 h (daily) or 12 h (light-dark phases) to calculate total, diurnal, and nocturnal meal patterns. Data are expressed as means ± S.E.M., with consideration of significance at $p \leq 0.05$ as measured by one way ANOVA computed between treatment groups for each diet, followed by pair-wise ANOVA on ranks where appropriate. Conditioned taste aversion data was analyzed using a two-by-two mixed-model ANOVA, and significance was again defined as $p \leq 0.05$. 
Figure 4.1. Cumulative daily effects on body weight and intake following treatment. CHOW (3.07 kcal/g, left) and HC (4.73 kcal/g, right) fed rats (3.07 kcal/g) evaluated by linear regression (r >0.976). Rats were given one i.c.v. dose of vehicle (2 µL DMSO, open circles) or VTKI-V (2 µg/2 µL DMSO, grey circles). Panels are daily cumulative (A, D) body weight change, (B, E) food intake, (C, F) water intake. Data are means ± S.E.M. (CHOW: n = 15 (DMSO) n = 16 (VTKI-V); HC: n = 8 DMSO), n = 9 (VTKI-V)). Statistical tests are one-way ANOVA between treatments, *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001.
Figure 4.2. Five day average light and dark phase differences in meal variables. Bars are CHOW fed animals treated with DMSO (white) or VTKI-V (grey); HC fed animals treated with DMSO (white, stripes) or VTKI-V (grey, stripes). Panels are (A) caloric intake, (B) mass of intake, (C) average caloric intake per meal, (D) number of meals, (E) average number of bouts per meal, (F) average meal duration. Data are 5-day means by light period ± S.E.M. (CHOW (n = 14 (DMSO), n = 15 (VTKI-V)); HC (n = 8 (DMSO), n = 9 (VTKI-V)). Statistical tests are one-way ANOVA between treatments within diet groups, *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001.
Figure 4.3. Meal pattern for ten consecutive light periods of rats fed CHOW or HC diet. White symbols (DMSO); grey symbols (VTKI-V). Left panels (circles) are CHOW fed animals (n = 14 (vehicle) n = 15 (drug)); right panels (squares) are HC fed animals (n = 8 (vehicle) n = 9 (drug)). (A, F) Total kilocalorie intake, (B, G) average calories per meal, (C, H) average number of bouts per meal, (D, I) average meal duration, and (E, J) number of meals. Data are per period means ± S.E.M. Statistical tests are one-way ANOVA between treatments within diet groups, *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001.
Figure 4.4. Effects of Drosophila UAS/Gal4 mediated dVegfr knockdown on adult body size.

Canton S flies were used as wild type controls (wt). *elav*: a pan-neural Gal4 driver; *dVegfr^{dsRNA}*: an UAS-*dVegfr^{dsRNA}* effector line for cell-autonomous RNA interference. A) Average body mass of adult flies; B) average protein content of adult flies. Data represent averaged readings from at least 3 separate assays, each involving 5 flies per sample. Data are means ± S.E.M. Statistical tests are one-way ANOVA between control crosses and mutants, *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001.
Figure 4.5. *Conditioned taste aversion test.* (A) Training paradigm: dots indicate days with one hour training with sweetened condensed milk (SCM) alone, grey boxes indicate days with SCM + orange flavor, the day of the conditioning trial is indicated with an arrow. Animals did not exhibit aversion to unconditioned stimulus (orange flavor) following conditioning trial with one dose of DMSO or VTKI-V. Data are change from baseline means ± S.E.M. (n = 5 (vehicle) n = 7 (drug)). *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001.
CHAPTER 5:
DISCUSSION AND GENERAL CONCLUSIONS

Integration of Nutrient Detection and Satiety State

Feeding behaviors are dynamically tuned according to sensory determinants of palatability and physiological need. A state of internal energy depletion dictates that an animal must activate foraging relevant behavioral programs to maximize food intake while suppressing all other extraneous activities such as reproduction or avoidance of predators. While in this state, metabolic processes minimized, foraging relevant behaviors are activated or promoted, sensory perceptions of food related stimuli are heightened, and mechanisms of memory formation are potentiated. Once an energetically appropriate amount of food has been obtained and ingested, internal energy sensing mechanisms activate negative feedback signals which act to suppress ingestion and foraging behaviors, allowing the fully satiated animal to return to other behaviors. While the states of “starvation” and “satiation” are the maximal endpoints of the energetic scale, “hunger” and “satiety” are non-absolute states positioned somewhere between these opposite extremes. Where an animal falls within the satiety spectrum determines how likely it is to execute or terminate feeding-relevant behavioral programs. The predisposition of an individual to overconsumption of calories and weight gain can develop from disruption or maladaptation of these basic biological processes. The mechanisms by which consumption is controlled by external sensory cues and internal metabolic status therefore remain a critical topic of investigation in efforts to prevent weight gain and obesity. Satiety-state dependent mechanisms
which block overconsumption of high energy foods are of particular interest, as they offer potential targets for preventative treatments against weight gain. Studies of food intake in model organisms have demonstrated that feeding behavior plasticity is achieved in part by alterations of feeding thresholds in response to internal energy needs and external food availability (40, 68). However, the mechanisms coupling satiety state to sensory perception and coding are continuing to be elucidated. The primary driving force for feeding initiation and ingestion in Drosophila is the detection of gustatory cues signaling the availability of nutritive foods (64, 65). The character of the behavioral responses elicited by such sensory cues are dynamically modulated based on internal metabolic status via circulating hormones from neuroendocrine cells and fat body tissue such as the Drosophila insulin-like peptides (16, 61), adipokinetic hormone (58), and leptin homolog Unpaired 2 act as coding mechanisms for the availability of carbohydrate and lipid stores (57). Post-ingestive feedback from the gut following a meal may also inhibit feeding via the recurrent nerve or medial abdominal nerve (68). This behavioral plasticity is evident in the responses of larvae to nutrient detection and satiety state. The baseline activities of well-fed larvae are optimized to promote the ingestion of nutritive foods and minimize energy expenditure. If provided with sweet nutritive foods, they tend to eat at a steady baseline rate, but decline foods that are difficult to masticate or have aversive taste (15, 16). The character of baseline behaviors are modified in response to food deprivation, which enhances the rate of feeding (16) and food preferences (15) in order to restore energy balance. Similarly in the adult fly, gustatory information is obtained by gustatory receptor neurons in the periphery respond to bitter and sweet compounds and promote either acceptance or rejection behaviors (64, 65) by regulating motor neurons subprograms for proboscis extension and ingestion (14, 216-218). The thresholds for gustatory gating of acceptance or rejection is
dependent on satiety state, as food deprived flies display alterations in responses to both sweet and aversive gustatory inputs (219). However, how the detection of peripheral signals of metabolic state is translated into alteration of feeding thresholds in adult and larval stages remains a topic of investigation (220), and whether this reflects a satiety state-dependent neural mechanism or results from the failure to ingest enough sugar is unclear (88). In order to determine how the expression of different behavioral characteristics is constrained by satiety or promoted by hunger, we must first identify the neuronal circuits whose activity is necessary for the maintenance of basal feeding behavior qualities.

Evidence for satiety-state dependent mechanism for feeding rate suppression

Taken together, several aspects of the feeding behavior demonstrated larvae (16, 66) provide evidence for the existence of satiety-state dependent processes regulating their rate of ingestion. First, satiated larvae steadily feed at a rate of roughly one bite per second when provided with nutritive food that is easily masticated, and this baseline rate is maintained over long periods of time. However, they are capable of feeding at a maximum rate of 2 bites per second when deprived of food for 150 minutes or more. Given that the maximum feeding rate is only utilized when larvae are experiencing a deficit of internal energy, this maximum rate is likely to be energetically unfavorable. Furthermore, when larvae that have been deprived of food are placed on nutritive media, the initial maximum rate of feeding gradually declines over time, and returns to a steady baseline. The selective execution maximum feeding capacity indicates that it is expendable for the maintenance of internal energy stores. The observation that re-feeding results in time dependent decay of feeding rate suggests that ingestive output is modulated by post-ingestive inhibitory mechanisms, and argues against the presence of starvation dependent feeding
rate enhancement mechanisms. In addition, several studies have shown that larval and adult feeding behaviors are dependent on factors such as Drosophila insulin-like peptides (16), the leptin-like Unpaired-2 protein (221), and circulating metabolite levels (222), whose levels are dependent on food intake, and exert an inhibitory effect on feeding rate.

Recent studies in larvae and adults have identified two separate central circuits in with roles in satiety-state dependent feeding regulation. Following the characterization of pumpless and klumpfuss mutants, which fail to appropriately move food from the pharynx into the esophagus and display reduced food intake (223), it was shown that these mutants also display increased expression of a neuropeptide called hugin, a putative homologue of mammalian neuromedin U (224), whose expression is found in roughly 20 neurons located in the larval SOG. Hugin expression is suppressed during following starvation and amino acid deprivation, and its up-regulation is correlated with decreased food intake and food seeking behavior, suggesting that hugin levels act to encode satiety status. Circuit analysis revealed that hugin neurons receive input from gustatory sensory neurons and chemosensory neurons of the pharynx, and extend axonal processes to the ring glad (a major endocrine organ) and pharyngeal muscles (required for mouth hook movement and ingestion), indicating that hugin coordinates sensory inputs with growth, metabolism and food intake (223). In contrast to the hugin circuit, which is nutrient responsive and satiety dependent, Scott et. al recently identified a novel circuit which exerts a tonic inhibitory tone to suppress meal initiation and consumption (220) by selectively gating motor neuron output in adult flies. Mediated by four gabaergic neurons in the ventral region of the SOG, which receive input from gustatory receptor neurons and project to motor neurons regulating food intake, this circuit is unique in that its activity is not dependent a homeostatic pathway or taste detection, but rather it establishes a central threshold on multiple feeding motor
neurons which must be overcome in order to initiate feeding (220). Together, these findings indicate that 1) excessive food ingestion is suppressed by nutrient ingestion, and 2) both homeostatic and taste dependent pathways modulate ingestive behavior rate by overcoming feeding inhibition circuits. This suggests that the maximum capacity of larval ingestive motor circuits is likely to be directly or indirectly inhibited in satiated larvae, and the maximum feeding rate demonstrated by starved larvae is likely to be due to dis-inhibition of these circuits by nutrient and gustatory dependent signals.

The Overlapping Roles of Octopamine and Dopamine in Appetitive Learning

The vast majority of investigations into the role of OA signaling and behavior in flies and other insects have centered on its relationship to appetitive learning and reward driven behaviors. In *Drosophila*, the prevailing paradigm regarding the contributions of dopamine (DA) and OA to learning and behavior has been that DA is required for aversive learning but not reward learning, and OA is required for reward learning but not aversive learning (78-81). Studies of the appetitive behaviors of bees demonstrate that OA treatment effects behavioral responses to sucrose reward (85, 225-227) and acts as a taste driven reward signal to enhance food seeking (228), food intake (229), and appetitive learning (78, 79), as well as circadian rhythms (230, 231). In particular, studies in bees show that OA treatment effects behavioral responses to sucrose reward (85, 225-227) and OA injection into the MB or AL can substitute for sucrose presentation or pairing in conditioned learning paradigms (85). Also, flies lacking OA due to a mutation in the tyramine-beta-hydroxylase (tβh) enzyme showed no impairment in aversive tests involving the pairing of a novel odor with a shock, but did not learn to associate a sugar reward with odor (78). Conversely, the acute inhibition of dopaminergic neurons was demonstrated to
block aversive learning without affecting sugar learning (78). Furthermore, light-induced activation of dopaminergic neurons via channel rhodopsin expression was shown to substitute for shock pairing to elicit aversive memory formation, while OA neuron stimulation was shown to substitute for sugar reward (79). The requirement for dopamine signaling was mapped to three clusters of cells in the MB, each with distinctive roles in the phases of aversive memory encoding (232, 233).

More recently, however, the advent of a wide array of acutely inducible constructs for the manipulation of neural activity, coupled with tools allowing fine spatial control of their expression, has provided more insight into the relative contributions of OA and DA to learning. In particular, recent studies manipulating small sets of dopamine neurons demonstrate that the dichotomy of OA/food reward and DA/punishment is an oversimplification. For example, small subsets of DA neurons and DA receptor neurons in the mushroom bodies (MB) have shown that some dopamine signals may also modulate reward responses (82, 88, 234). In the insect brain, the MBs comprise a protocerebral higher brain center known for roles in olfactory processing and learning and memory (86), and in particular is required for associative learning (78, 235-238). The dopamine receptor DA1, which acts by increasing adenylyl cyclase activity, is highly expressed in adult MB (234). Studies using DA1 mutants demonstrated that lack of DA1 signaling in the adult MB blocked learning association of an odor stimulus with an electric shock, which is supportive of a role for DA in aversive learning (239). However, the same mutants also showed impairment of learning an odor associated with sucrose reward (239). In larvae, DA1 mutants also showed defects in aversive and appetitive learning assays. Another major finding came with the demonstration that satiety levels influence performance of flies in appetitive memory tests via overlapping DA and NPF/NPFR1 signaling mechanisms (88). It was
demonstrated that the lower appetitive memory performance observed in satiated flies was due to tonic inhibition of MB neurons medial lobe/pedunculus DA neurons (88). Furthermore, stimulation of neurons expressing NPF promoted appetitive memory in satiated flies by suppressing the inhibition of MB neurons by DA, enabling the expression of enhanced food associated conditioned responses (88). It has since been discovered that the driver lines used in earlier reward/aversion learning studies omitted a subset of dopaminergic neurons in the PAM region of the MB, which are required for reward learning (89, 240). Activation of PAM DA neurons paired with odor presentation stimulates robust appetitive memory formation, even in flies lacking OA, and blocking these neurons disrupts appetitive learning with both sweet/nutritive and sweet/non–nutritive sugars (89, 240). Also, pairing OA neuron activation with odor presentation led to only short-term memory formation, which is rendered dispensable by downstream PAM DA neuron activation (89). Moreover, it was demonstrated that part of the OA-dependent short-term memory encoding is mediated via activation of its Oamb receptor on a subset of DA neurons in the MB, as activation of DA/Oamb neurons was shown to bypass the need for sugar in appetitive memory formation (89). It therefore appears that subsets of DA neurons in the MB represent short-term and long-term reinforcing effects of nutritious sugar in learning and act downstream of OA signals (89).

From these studies, a general picture of fly pathways regulating appetitive motivation is emerging which involves parallel and overlapping contributions from DA, NPF/NPFR1, and OA. DA appears to provide a tonic inhibitory signal which can be modulated by selective NPF/NPFR1 gating mechanisms to promote execution of either appetitive or aversive behavior (88), and also by OA/Oamb gated signaling which provides gustatory signals regarding food quality (89). With this model in mind, DA neurons in the MB can be thought of as a central
behavioral subprogram selection circuit, in which subprogram selection is gated by both nutrient detection and satiety state.

*The Role of OA in Sweet Taste Encoding*

While it is clear that OA signaling mediates the short-term reinforcing properties of sugar in appetitive behavior, it remains to be determined which OA neurons are responsible for this process, how they do so, and how OA-dependent sweetness encoding mechanisms interact with other appetitive motivation pathways. One consistent observation is that both tβh mutants and OA receptor mutants display inappropriate detection of, and responses to, sugar. Given the ability of OA injection and tdc2-gal4 neuron activation to substitute for sugar in appetitive learning paradigms, it seems clear that OA signaling is at least one component coupling detection of external nutrient cues to appetitive responses. More recent work has demonstrated that sweet taste and nutrient value represent parallel appetitive reinforcement pathways in flies (90). OA-dependent sugar learning appears to provide the transient reinforcing properties of the detection of sweet taste, but the need for OA reinforcement is bypassed by the nutrient value of sugar. For example, acute inhibition of OA neurons blocked appetitive learning with arabinose, a sweet but non-nutritive compound, but had no effect when sweet and nutritious sucrose was used (89), which implicates OA in encoding the gustatory detection of “sweetness”. The presence of OA neurons in the SOG, which is known to be an important site for integration of gustatory inputs, provides anatomical support for this idea (119), and it may be that OA neurons act as second order transducers of olfactory receptor neuron signals.
Overlapping functions of VUM1 and VUM2 OA neurons in feeding regulation

OA signaling is required for the establishment of basal feeding rate, as well as for starvation dependent feeding rate enhancement. These regulatory roles can be mapped to two small clusters of OA neurons in the SOG. Targeted lesioning of only the VUM1 group resulted in a significant increase in the feeding activity of fed larvae. However, this increased activity was blocked when the VUM2 group was ablated in tandem. In fasted larvae, lesioning of the VUM2 group reduced hunger-dependent feeding rate enhancement, and this reduction was compounded by joint ablation of both the VUM1 and VUM2 groups. This suggests that the proper control of feeding rate in fed and fasted conditions requires negative regulatory input from VUM1 neurons, and positive input from the VUM2 group. These findings suggest that components of the OA circuit are selectively responsive to satiety state. As tdc2-gal4 neuron activation selectively promotes the ingestion of sweet foods, it is likely that OA signaling is a component of a system that guides larvae toward nutritive substances.

While we have shown that OA signaling is required for both starvation dependent feeding rate enhancement and the suppression of food intake during satiety, further work will be necessary to determine the relative contributions of OA neurons in the SOG in these processes, and what downstream pathways mediate their effects. Lesioning of VUM2 OA neurons was sufficient to block enhancement of food intake due to tdc2-Gal4 neuron excitation, while both VUM1 and VUM2 lesion is necessary to reduce starvation dependent feeding rate to the same level as is observed following inhibition of tdc2-Gal4 neurons. This suggests that both VUM1 and VUM2 groups contribute to starvation dependent feeding rate increases. The unique role of the VUM1 subset of OA SOG neurons in this behavioral response suggests that OA dependent feeding rate enhancement is blocked under conditions of satiety and operates in a similar pathway as
Oamb/NPF, and it is possible that a VUM1 or VUM2 dependent mechanism directly or indirectly links OA signaling to NPF.

**A Novel OA/Oamb/NPF Pathway Maintains Basal Feeding Rate**

While many studies have demonstrated that NPF/NPFR1 signaling is required for starvation dependent alterations in motivational state and appetitive behavior (14, 66, 69, 88), targeted manipulation of subsets of NPFR1 neurons reveals that NPF neurons mediate spatially and functional distinct regulatory roles (69, 89). The selective manipulation of NPF SOG neurons has yet to be investigated, and our experiments provide the first indication that NPF may also play an important role in determining the character of basal feeding activity. Targeted lesioning of NPF SOG neurons reveals an enhancement of palatable food ingestion, suggesting that these neurons may act as a satiety level dependent braking system to hold the feeding rate of satiated larvae at a baseline level. This is reminiscent of findings from mammals which demonstrate that hypothalamic NPY expressing neurons are inhibited by high levels of leptin and insulin (Figlewicz, 2009), and some are directly inhibited by glucose (Levin 2006). When animals are deprived of food, the levels of leptin, insulin, and glucose fall, which results in a loss of NPY neuron inhibition, and a subsequent enhancement of food intake. Currently, little is known regarding how the activity levels of *Drosophila* NPF neurons are regulated. NPF-expressing neurons innervate broad regions of the brain and may simultaneously modulate distinct neural circuits to promote food seeking (88). NPF neurons in the SOG have extensive dendritic arbors which are likely to receive inputs from multiple upstream pathways, and their proximity to the esophagus makes them likely targets for directly or indirect targeting by circulating satiety factors, sugars, or other nutrients.
The unique roles of subesophageal ganglion neuropeptide F (NPF) and octopamine (OA) neurons in feeding rate regulation provide support for the presence of novel circuit involved in satiety-state dependent inhibition of sugar overconsumption. Laser lesion analysis indicates that the presence of two NPF neurons in the SOG is necessary for the expression of basal feeding rate characteristics on sweet, palatable food, as lack of these neurons enhances basal feeding rate. Our findings demonstrate a phenotypic and functional overlap between NPF SOG neurons and OA SOG neurons, as loss of the VUM1 OA cell group also enhances basal feeding rate. Furthermore, manipulation of the expression of the Oamb OA receptor indicates that NPF expression of Oamb is required for the inhibitory role of OA on basal feeding rate. Global CNS knockdown of Oamb resulted in an enhancement of feeding rate, which was phenocopied by targeting of the knockdown to NPF expressing neurons, as well as in an enhancer-trap based Oamb driver line which colocalizes with NPF neurons in the SOG. In addition to the well characterized role of OA in coding for gustatory detection of sugar (78, 81, 85, 89, 228), these findings indicate that OA may act upstream of NPF to enhance their activity when sugar is ingested. This is corroborated by previous experiments which demonstrate that NPF immunoreactivity is selectively enhanced in the SOG region following prefeeding with both nutritive sugar and non-nutritive sweeteners (186), suggesting that the activity of these neurons is dependent on the detection of sweet taste, though they have not been demonstrated to directly connect with gustatory receptor neurons.

The anatomical proximity of SOG NPF and OA neurons suggest that they may exert their regulatory roles via parallel and overlapping downstream pathways in the SOG. Artificial excitation of tdc2-Gal4 neurons or loss of OA neurons in the SOG results in the expression of increased ingestive rate in satiated larvae, indicating the presence of an OA dependent
mechanism in the SOG for promotion of sweet food intake which is functionally silent at when internal energy stores are at baseline. The finding that the Oamb is required in NPF neurons for appropriate basal food ingestion suggests that OA and NPF work in a concerted fashion to suppress excessive feeding of sugar during satiety. However, inhibition of OA signaling in tdc2-Gal4>UAS-shibereTS larvae does not affect basal feeding rate, indicates that overlapping mechanisms exist to regulate food intake during satiety.

NPF/NPFR1 signaling acts to inhibit downstream neurons, therefore lesioning of SOG NPF neurons is likely to reduce an NPF/NPFR1 signal which exerts an inhibitory effect on local neurons in the SOG in satiated larvae. Loss of Oamb in these neurons may also reduce this inhibitory effect. It has been suggested that satiated flies receive maximum inhibitory feedback on behavioral output such that sensory input is behaviorally ineffective, but this inhibition may be decreased in a dose dependent fashion as food deprivation is prolonged, allowing sensory input to become increasingly effective in initiating feeding (88). With such a mechanism, the likelihood that an appetitive behavior is triggered by a conditioned odorant or gustatory cue would therefore be determined by competition between inhibitory systems in the brain. If this is the case, appetitive behaviors may be given their baseline characteristics via active tonic inhibition by circuits sensitive to circulating nutrients.

Our findings provide the first evidence for an upstream regulatory pathway that specifically targets NPF SOG neurons. Given the role of OA in gustatory encoding of sweet taste, local OA signaling in the SOG may link NPF neuron activity indirectly with the detection of sweet tasting compounds, which may provide the input to link a NPF/NPFR1 dependent braking system to upstream gustatory inputs. It will be illuminating to determine how the basal activity level of these neurons may be altered in different satiety states, and their responsiveness to known
regulators of food intake and satiety such as dILPs. It remains unclear how the activity of NPF SOG neurons exerts an effect on feeding rate, which must be determined by functional characterization of local SOG NPFR1 expressing neurons. Anatomical analysis and application of the GRASP technique for mapping synaptic connections may reveal what direct connectivity may exist between the NPF and OA cell groups in the SOG, and how OA neuron activity levels correlate with NPF neuron activity in different satiety states.

A VEGFR Pathway for Behavioral Plasticity

Animal growth is determined by environmental, genetic, hormonal, and nutritional factors, and is critically mediated by appropriate expression of growth factors. Despite important links between growth factors and nutrition, direct roles for these proteins in the regulation of feeding behaviors has been underexplored. Intriguingly, the levels of various growth factors have been found to be elevated in the CSF of rats who were fed or injected with glucose relative to starved rats, and intracerebroventricular administration of inhibitors selective for growth factor receptors have been shown to transiently decrease food intake (141). We provide evidence of a novel regulatory role for a PDGFR/VEGFR-related receptor (Pvr) required for OA-dependent homeostatic control of feeding behavior and growth in flies. Pvr has been identified as sharing structural homology with receptors for both platelet derived growth factor (PDGFR) and vesicular endothelial growth factor receptor (VEGFR), though it shares greater similarity with the latter (132). Our findings in the fly model of feeding behavior suggest that the neuronal effects of VEGFR signaling pathways have relevance to the regulation of behavioral output. Furthermore, the extension of these findings into a rodent model system of food intake and diet-induced hyperphagia indicate
that a conserved role exists for the central regulation of food intake by a mammalian VEGFR pathway.

When given free access to a palatable, energy dense diet, humans and other animals tend to consume more calories, and this results in an increase in the amount of stored fat they maintain and defend (241-243). Many studies have implicated the “rewarding” nature of tasty foods in the drive to overeat (77, 198). Palatable foods are more likely to be eaten in excess relative to bland foods, and the pleasurable effects of consuming them may override homeostatic signals (77, 198-200, 210, 244). Food that have tastes which provide cues of palatability activate reward pathways (77), and it is speculated that obese individuals may be more susceptible to the temptations of food. In support of this, obese adults display heightened activity in neural reward circuits in response to palatable food or food-associated cues (201, 202, 245). Alternately, the taste of energy dense food may contribute to overeating by suppressing satiety. For example, in the presence of palatable food, endocannabinoids increase appetite by stimulating the desire to eat while also blocking signals for meal termination (203).

However, it may be that high energy density foods bypass satiety pathways based on the mass of food ingested. Many studies find that when rodent models raised on balanced diets are given free access to diets that contain high levels of sugar and fat, animals continue to eat the same mass of food (246) and similar findings have been shown in humans (247). If maintained on an energy diet for long periods, rodents will consume excessive calories and become obese (248, 249), and will maintain an elevated body weight (241, 242). While much is known regarding the initiation and consumption phases of feeding behavior, much less is known of the complex processes regulating the termination and postconsumption phases of feeding, the dysregulation which are likely to contribute to the hyperphagia seen in diet dependent obesity (249).
In our study, young male rats were given ad libitum access to standard rodent CHOW, or were acclimated to an energy dense palatable HFHS diet prior to treatment with VTKI-V. Animals given long term access to the diet used in this study have been shown to exhibit rapid alterations in meal pattern, including increased meal size and duration, which correlate with the development of diet induced obesity over time (206, 213, 250). Our results show a similar trend. DMSO injected control animals maintained on CHOW and HFHS diet displayed similar patterns of food intake in terms of meal number, mass, duration, and number of bouts. Given the higher energy density of the diet presented to the HFHS control group, consumption of the same mass of food containing more calories resulted larger caloric intake overall, and larger average meal size. After VTKI-V administration, CHOW fed animals reduced their intake slightly in terms of both mass and calories, but the effect was only significant when overall cumulative intake was assessed, and no detectable alteration was seen in other meal pattern variables. However, drug treatment resulted an acute effect on the mass of food ingested by the HFHS diet group. Animals decreased the mass of their meals by roughly 30% due to a reduction in the number of bouts per meal and meal duration. This brought their average caloric meal size down to a similar level as CHOW fed animals.

Our findings suggest that VTKI-V treatment selectively alters the mass of food ingested, but only when the energy density of that food exceeds caloric need. Rats predisposed to diet induced obesity display larger meal sizes and decreased satiety ratios on high energy palatable diets, relative to diet induced obesity resistant rats (206). Rodent models of diet induced obesity have central resistance to leptin (251), which has been correlated with reduction in meal size, not number (56). Further work will be needed to determine if VTKI-V targets similar systems involved in the regulation of food intake.
Additionally, we find that VTKI-V treatment results in a reduction in the rate of weight gain. This effect was immediate and persisted over the five days following treatment. Interestingly, reduction in weight gain was seen in both CHOW and HFHS fed groups, despite the fact that CHOW fed animals displayed very little alteration in their pattern of food intake following drug treatment. This suggests that there is some component of the variance in the rate of weight gain between control and drug treated animals is not fully accounted for by alterations in food intake. It is possible that VTKI-V interacts with other neural systems involved in metabolism. Given that obesity is correlated with alterations in sympathetic nervous system output (252, 253) and fat metabolism (27) there may be an effect on peripheral metabolism of stored fat or regulation of energy expenditure. Rats acclimated to energy dense diets achieve and maintain a higher body weight set point (207, 241), which is correlated with alterations in energy expenditure and metabolism (212, 243). Furthermore, this is accompanied by alterations in the hypothalamic and peripheral activity of noradrenergic systems, including differences in neural sympathetic output and responsiveness (209, 252), ventromedial hypothalamus morphology (253), and neural monoamine metabolism (252). Further work will be needed to determine if VTKI-V targets VEGFR2 on norepinephrine neurons, sympathetic nervous system output, or metabolism.
REFERENCES


