FUNCTIONAL ANALYSES OF THE *DROSOPHILA* INSULIN- AND NEUROPEPTIDE Y-LIKE SIGNALING SYSTEMS: REGULATION OF FEEDING, RISK-AVERSE AND SOCIAL BEHAVIOR

by

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(Under the Direction of Ping Shen)

ABSTRACT

Neuropeptides form the most diverse group of neuronal and hormonal messengers that regulate a wide spectrum of physiological processes and behaviors. The conserved *Drosophila* neuropeptide F (NPF) signaling system is demonstrated to developmentally regulate two opposing behaviors related to food in larvae: foraging and food aversion. The expression of *npf* is strong in young larvae attracted to food, and loss of NPF signaling results in the onset of behaviors associated with older larvae, including food aversion, hypermobility, and cooperative burrowing. Conversely, the brain expression of NPF of older nonfeeding larvae is developmentally downregulated, and ectopic expression of *npf* leads to prolonged feeding, and the suppression of social burrowing behaviors. Furthermore, through a broad array of behavioral characterization of genetically altered animals, a neuronal circuit that includes the NPF and the *Drosophila* insulin-like peptides (DILP) signaling systems has been identified to adaptively mediate diverse hunger-motivated foraging behaviors under different nutritional states. In fasted animals, hunger stimuli downregulate dS6K activity in DILP2/4 neurons, which in turn leads to attenuated DILP signaling and subsequently disinhibits two downstream signaling systems: an NPF/NPF receptor (NPFR1)-dependent and an NPF/NPFR1-independent pathway. The former

selectively mediates hunger-motivated feeding of nonpreferred foods (food preference) by lowering the threshold set by a default pathway. The latter promotes a general increase in ingestion rate, thereby enabling more effective food consumption. For example, upregulation of the DILP signaling through either overexpression of DILP2 in a pan-neural pattern or targeted expression of a constitutively active form of the *Drosophila* insulin-like receptor (dInR) in the NPFR1 neurons leads to significant attenuation of hunger-motivated response to less-accessible solid media or a bitter-tasting food containing quinine. In contrast, nondeprived animals deficient in NPFR1 or dInR signaling display hyperactive feeding of solid media and the quinine-adulterated noxious food both of which are normally rejected by the controls fed ad libitum. Taken together, the *Drosophila* NPF/DILP neural network provides an important entry point into understanding how genes, molecular pathways, and neural circuits are integrated in the dynamic regulation of feeding behaviors and energy homeostasis.

INDEX WORDS: *Drosophila melanogaster*, Insulin-like peptides, DILP, Insulin receptor, Neuropeptide Y, NPY, Neuropeptide F, NPF, NPF receptor, Ribosomal S6 kinase, S6K, Feeding behavior, Food intake, Risk-sensitive foraging, Social behavior

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TABLE OF CONTENTS

Page

ACKNOWLEDGEMENTS iv

CHAPTER

1 INTRODUCTION AND LITERATURE REVIEW 1

OVERVIEW OF ENERGY HOMEOSTASIS 1

THE NEUROPEPTIDE Y-LIKE SIGNALING SYSTEM 3

THE INSULIN-LIKE SIGNALING SYSTEM 15

OBJECTIVES 29

2 DEVELOPMENTAL CONTROL OF FORAGING AND SOCIAL BEHAVIOR BY THE *DROSOPHILA* NEUROPEPTIDE Y-LIKE SYSTEM 39

3 REGULATION OF HUNGER-DRIVEN BEHAVIORS BY NEURAL RIBOSOMAL S6 KINASE IN *DROSOPHILA* 94

4 REGULATION OF AVERSION TO NOXIOUS FOOD BY *DROSOPHILA* NEUROPEPTIDE Y- AND INSULIN-LIKE SYSTEMS. 128

5 CONCLUSIONS AND DISCUSSION 157

REFERENCES 166

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

This dissertation is organized into the following five chapters:

1) Chapter I, Introduction and Literature Review, provides substantial background information and defines the objectives of the research in this dissertation. A section of Chapter I (pg. 15 – 27) was adapted from a manuscript prepared for *Annual Reviews of Entomology* by Qi Wu and Dr. Mark R. Brown. Reprinted with permission, from the *Annual Reviews of Entomology*, Volume 51 ©2006 by Annual Reviews.

2) The results of the research are presented in Chapter II, III, and IV, which have been arranged in a manuscript format and have been published in *Neuron* (2003), *Proceedings of National Academy of Sciences* (2005), and *Nature Neuroscience* (2005), respectively.

3) Chapter V, Conclusions and Discussions, aims to tie the results together and point out the overall significance of the research.

OVERVIEW OF ENERGY HOMEOSTASIS

Over the past two decades, scientists have reached a consensus that a physiological system exists in mammals, the major function of which is to maintain energy homeostasis in response to fluctuations in nutrient access and demands for energy expenditure. The system is viewed to be composed of both afferent and efferent components encompassing a large number of signaling molecules that originate from the brain and various peripheral organs. The short-

term, meal-related afferent signals include inhibitors of feeding, such as gut-derived peptide YY (PYY), glucagon-like peptide-1 (GLP-1), and cholecystokinin (CCK) as well as stimulators of feeding, such as ghrelin from the stomach. The long-term afferent signals that manifest the status of energy storage include those from fat (leptin) and pancreatic β cells (insulin). Both of these short- and long-term signals converge and are integrated in the hypothalamus region of the brain where the direction and magnitude of the efferent responses are generated. The efferent factors of this system include those regulating hunger perception and subsequent food seeking (foraging) behavior, the level of energy expenditure, as well as energy partitioning, such as the relative sizes of lean and fat mass in the body. Moreover, some of the efferent elements mediate the physiological processes of growth and reproduction associated with animal's nutritional states.

Based on the common notion that starvation is much more life-threatening than obesity, one can imagine that this system is robustly tuned to take action in response to nutrient deficiency by efficiently promoting energy intake and stores. Such hunger-protective mechanisms increase odds of survival when food supplies become scarce; and the pressure of natural selection would be expected to favor such "thrifty genotypes" (Neel, 1999). On the other hand, the storage of excess energy as fat initiates adaptive responses that are supposed to prevent the development of obesity by suppression of appetite and increased energy expenditure (Weigle, 1994). In this scenario, the same energy balance system that is involved in hunger protection is considered to be switched to the opposite direction from starvation avoidance to obesity avoidance mode. Unfortunately, in a large and increasing fraction of the human population, this system does not function as robustly to suppress appetite and increase energy expenditure in response to obesity-promoting events as it does to prevent starvation. Nevertheless, many

individuals do resist obesity despite exposure to a common unhealthy environment, suggesting that the differential susceptibility to obesity is not only governed by many of the social and psychological factors, but may also be modulated at the gene and cellular level. Recent advances in transgenic rodent models have outlined several important neural circuits that exert a multitude of actions on various aspects of food intake and energy expenditure. In spite of these seminal yet rudimentary advances, we still lack a comprehensive understanding of how the body communicates with the brain to regulate appetite. Thus, identification and characterization of the responsible signaling molecules and associated regulatory pathways will facilitate our understanding of the underlying mechanisms by which environmental factors interact with specific genes and pave the way for the discovery of potential drug targets for developing safe and effective treatment of obesity and its complications

THE NEUROPEPTIDE Y-LIKE SIGNALING SYSTEM

Neuropeptide Y (NPY), a 36-amino-acid neurohormone discovered more than 20 years ago, is widely distributed throughout the central and peripheral nervous system (Dumont et al., 1992). Pharmacological studies suggest that NPY is a potent stimulator of vasoconstriction as well as food intake upon its central administration. Moreover, growing evidence from transgenic animal models demonstrates the contribution of NPY to the pathophysiology of a variety of diseases including feeding disorders, obesity, epileptic seizures, anxiety, hypertension, and drug addiction (Silva et al., 2002).

NPY and NPY receptors in mammals

NPY was first isolated from the porcine brain with structural similarities to the peptide YY (PYY) and pancreatic polypeptide (PP) (Tatemoto et al., 1982). These three molecules belong to a large family characterized by a polyproline helix folded onto an α-helix with a C-terminal tyrosine (Silva et al., 2002). Pro-NPY is generated after the removal of the signal peptide from the pre-pro-NPY in the endoplasmic reticulum. Then the cleavage of the pro-NPY at a dibasic site by prohormone covertases leads to NPY_{1-39}. The biological active form of NPY is derived from two further steps of modification at the C-terminal end by a carboxypeptidase (NPY_{1-37}) and the peptidylglycine α-amidating monooxygenase (NPY_{1-36}). The amide moiety of NPY is crucial for its physiological activity and prevents degradation by carboxypeptidase. Under different circumstances, NPY could be processed by the dipeptidyl peptidase IV or the aminopeptidase P, which produces NPY_{3-36} and NPY_{2-36} respectively.

NPY is one of the most abundant neuropeptides in the mammalian central nervous system (CNS) (Williams et al., 2001). NPY is mainly synthesized in the neurons localized in the hypothalamus, particularly PVN, ARC, superchiasmatic nucleus, and dorsomedial nucleus (Silva et al., 2002). Peripherally, NPY is highly expressed in the sympathetic nervous system, where it is co-stored and co-secreted with norepinephrine. NPY can also be detected in a subpopulation of parasympathetic neurons. In addition to its central localization, NPY is expressed in many peripheral tissues, including adrenal medulla, liver, heart, and endothelial cells of blood vessels (Cavadas et al., 2001).

The NPY receptor family includes at least six subtypes of receptor (Y1 – Y6), all of which belong to the G-protein coupled receptor superfamily and share the common character of seven transmembrane domains. Their ligand affinity profiles, distribution and physiological functions,

however, are remarkably diversified based on anatomical, pharmacological and behavioral studies (Thorsell and Heilig, 2002). Except for Y3, all these receptors have been cloned and are encoded by individual genes at different loci (Inui, 1999). In addition, mice with the null mutation of NPY, the Y1, Y2, or Y5 receptor have been generated.

The Y1 receptor requires both the N-terminal and C-terminal regions of NPY for normal binding affinity and biological activity. In the CNS, the Y1 receptor has been located in the anterior thalamus, hypothalamus, cerebral cortex, dentate gyrus of the hippocampus, medial geniculate, and amygdala. Moreover, the central regulation of feeding behavior through Y1 receptor has been well documented (Pedrazzini et al., 1998). In the periphery, the Y1 receptor is mainly localized postjunctionally at the vascular sympathetic neuroeffector junction and acts as a vasoconstrictor responses to NPY (Thorsell and Heilig, 2002).

In contrast to the Y1 receptor, the C-teriminal part of NPY is more important than the N-terminal part for its binding to the Y2 receptor (Silva et al., 2002). In the CNS, the Y2 receptor is a presynaptic receptor mainly located in the hippocampus and acts to decrease postsynaptic excitability by the inhibition of neurotransmitter release. It is also expressed prejunctionally in the peripheral nervous system (PNS), intestine and certain blood vessels.

Pharmacological studies indicate the existence of the Y3 receptor but it still has not been cloned. However, it has been reported that this putative receptor subtype is localized in the rat brainstem and the hippocampus (Silva et al., 2002). The Y3 receptor also has been implicated to mediate NPY-induced secretion of catecholamine in the human adrenal medulla (Cavadas et al., 2001).

The Y4 receptor has been cloned in rat and human, and displays a high affinity with PP. However, NPY does not bind to human Y4 receptor at all (Lundell et al., 1995). The Y4

receptor is widely distributed throughout the whole body including the brain, skeletal muscle, thyroid gland, heart, prostate, intestine, pancreas, and kidney. Due to its higher affinity for PP than for NPY, the Y4 receptor may be involved in the physiological action regulated by PP, such as pancreatic exocrine, gall bladder relaxation, and the secretion of leutinizing hormone (LH) (Silva et al., 2002).

The Y5 receptor was first discovered as an 'Y1-like' receptor, and has been implicated in the mediation of NPY-induced food intake. Some specific antagonists, which were developed recently, have been shown to be very useful in investigating the role of the Y5 receptor in the control of feeding behavior (Wyss et al., 1998). This receptor is expressed in a number of hypothalamic nuclei, CA3 region of the hippocampus, cingulated cortex, the supraoptic nucleus, as well as the amygdala (Silva et al., 2002). In the periphery, Y5 can be detected in a number of organs, including intestine, liver, skeletal muscle, kidney, spleen, placenta, heart, ovary, testis, and prostate. In the murine CNS, mRNA expression of the Y6 receptor has been described in the bed nucleus of stria terminalis, and in some hypothalamic nuclei (Weinberg et al., 1996).

In summary, these NPY receptor subtypes differ not only in ligand affinity profiles but also in their distribution in the CNS and the periphery, suggesting that NPY may exert its numerous physiological effects via signaling through different receptor subtypes.

Intracellular signaling pathway of NPY

NPY exerts its diverse physiological functions using a similar signal transduction pathway through its binding with one of the six subtypes of G-protein coupled receptors (Figure 1.1) (Silva et al., 2002). These G-proteins are all identified as pertussis toxin (PTX)-sensitive G-proteins (G_i and G_q). In most cases, NPY receptors transduce the signal by inhibition of adenylyl

cyclase (AC) to decrease the synthesis of 3′,5′-cyclic adenosine monophosphate (cAMP). NPY can also increase Ca^{2+} level by mobilizing intracellular stores via inositol triphosphate dependent or independent pathways (Perney and Miller, 1989). Moreover, NPY receptor modulates calcium and potassium channels by the coupled G_q-protein at the plasma membrane (Lemos and Takeda, 1995). Some studies indicated that NPY can induce dose- and time-dependent stimulation of prostacyclin production in cultured porcine arotic endothelial cells, suggesting it may inhibit the development of atherosclerosis (Kawamura et al., 1991). In addition, NPY was shown to activate a mitogen-activated protein kinase (MAPK) through the cascade of Y1 receptor, PTX-sensitive G-protein, and phosphatidylinositol-3-kinase (PI3K) (Keffel et al., 1999). Another signaling pathway in which NPY may be involved is nitric oxide (NO). It has been reported that Y1 receptor mediates vasodilation of human subcutaneous arteries through an NO-dependent pathway (Nilsson et al., 2000;You et al., 1995). Furthermore, in vitro assays demonstrated that NPY-induced vasodilation can be abolished by nitro-L-arginine, a potent NO synthase inhibitor (You et al., 1995).

NPY signaling regulates feeding behavior

Pharmacological studies

Upon administration into the CNS, NPY exerts a number of effects mediated by one or several of the NPY receptor subtypes. Pharmacological and behavioral studies have shown that NPY regulates a wide variety of physiological processes and behaviors, including feeding, anxiety, cardiovascular function, and drug addiction. In recent years, the stimulatory effect of NPY on food intake and body weight has been implicated and extensively studied.

Acute intracerebroventricular (i.c.v.) administration of NPY in mice or rats causes significantly increase of food intake while inhibiting thermogenesis and lipolysis (Clark et al., 1984). Furthermore, increased NPY and its mRNA levels have been detected in the hypothalamus region of obese Zucker *fa/fa* rats and fasting mice. Chronical NPY administration leads to obesity in rodents fed ad libitum, suggesting no satiety or body weight control signals are able to override the effect of NPY (Stanley et al., 1986). It has also been suggested that increased feeding motivation is the cause of NPY-induced food consumption, since NPY-treated animals are willing to work in a lever press apparatus and endure electric shock in order to obtain food (Flood and Morley, 1991;Jewett et al., 1992). Moreover, NPY infused mice will overcome a taste aversion for quinine-adulterated milk (Flood and Morley, 1991).

Many studies suggest that Y1 and Y5 receptors are involved in the orexigenic effect of this peptide. The i.c.v. injection of the Y1 antagonist (1229U911) inhibits both NPY- and fast-induced food intake (Silva et al., 2002). BIBO3304, another Y1 antagonist, also reduces food consumption induced by fasting or i.c.v. NPY infusion. Furthermore, a highly selective Y1 antagonist (J-104870) causes significant reduction of spontaneous food intake in Zucker obese rats and up to 74% decrease of NPY-induced feeding (Kanatani et al., 1999). Meanwhile, the stimulatory and inhibitory effects on food intake exerted by the specific Y5 receptor agonists and antagonists, respectively, strongly support the involvement of Y5 subtype in feeding regulation. A Y5 agonist [D-Trp_{32}]NPY induces food intake similar to NPY administration in satiated rats. However, this agonist does not increase food intake in mice. The repeated central administration of Y5 antisense oligodeoxynucleotides significantly inhibits spontaneous as well as NPY-induced food intake (Schaffhauser et al., 1997). The intraperitoneal (i.p.) injection of a Y5 antagonist, CGP71683A, also decreases fasting-induced and i.c.v. NPY-induced food intake in

lean and obese Zucker rats (Kask et al., 2001). However, this Y5 antagonist does not affect daily food intake, suggesting the Y5 receptor may be effective only under stressful or stimulated conditions. Taken together, these observations suggest that the physiological activities of different receptor subtypes depend on the animal's energetic status as well as the genetic background. Contrary to early beliefs, it seems difficult to establish single or a combination of receptor subtypes as targets for developing effective anti-obesity treatment.

Some limitations inherent in pharmacological approaches, such as lacking suitable antagonists for some of the receptor subtypes, as well as issues of selectivity, toxicity and solubility of available compounds, have hindered the efforts in establishing an essential role of the NPY system in feeding control. In contrast, genetically modified animals provide an ideal solution to circumvent these problems and offer a superb controlled system to establish the selectivity of pharmacological tools.

Transgenic studies

Pharmacological experiments strongly support the notion that NPY acts as an endogenous stimulator of feeding behavior. However, first-generated NPY knockout mice on a mixed 129XC57BL/6 background are unexpectedly normal in feeding either under standard vivarium conditions, after fasting, or when challenged to become obese by genetic or chemical intervention (Erickson et al., 1996a). NPY deficient mice are only hypersensitive to leptin injection by showing a greater initial reduction of food intake and body weight loss over the first 2 days of leptin treatment. Other feeding inhibitors, such as corticotrophin releasing factor (CRF) and metallothionein-II (MTII), a MC4R agonist, exert no effect on NPY deficient mice (Hollopeter et al., 1998). The only phenotype shown by NPY deficiency is under conditions of

complete leptin deficiency in the *ob/ob* mice (Erickson et al., 1996b). In the absence of NPY, the obese phenotype of the *ob/ob* mice is attenuated due to the decreased food intake and elevated energy expenditure.

Why does the loss of NPY have no effect on feeding, body weight, or the response to fasting in normal mice, but counteract the obesity syndrome of the *ob/ob* mice? Firstly, it could be possible that NPY does not regulate base-line feeding activity, but only affects motivated feeding under extreme conditions. Leptin levels fall two- to three-fold by a 2-day fast in a wild-type background, although it is already life-threatening. In consideration of the leptin levels, the feeding conditions of the *ob/ob* mice with complete removal of the circulating leptin resemble extreme starvation. Perhaps a more prolonged period on a calorically restricted diet is necessary to deplete leptin further and thereby reveal a potentially significant role of NPY on fast-induced food intake.

Secondarily, normal food intake in the NPY-knockout mice may be explained by a mixed genetic background (129 X C57BL/6) under which the transgenic animals were generated. Recently, another NPY deficient mouse strain with pure C57BL/6 background has been generated by backcrossing subject mice at least seven generations onto a C57BL/6 background (Segal-Lieberman et al., 2003). NPY knockout in C57BL/6 mice leads to mild obesity and up to 50% reduction of post-fast food intake compared with their wild-type counterparts. In addition, a previous study reported that $NPY^{-/-}$ mice backcrossed for two generations onto a C57BL/6 background have a weak yet significant reduction (up to 25%) in food intake after fasting (Bannon et al., 2000). These experiments suggest that genetic background plays an important role in feeding regulation and may affect the diet-induced obesity, and a mixed genetic background may obscure differences in energy balance.

Another possible interpretation is that many redundant pathways are involved in feeding regulation and the elimination of any one of them has little or no effect. One of the compensatory candidates for NPY is agouti-related peptide (AgRP), which is co-synthesized in the NPY neurons of the arcuate nucleus. Orexigenic properties of this peptide have been reported. Anatomical studies have shown that NPY terminals in the PVN contain AgRP and AgRP terminals target neurons expressing the Y1 receptor. Thus, a similar functional relationship between the AgRP and NPY has been proposed. If AgRP was unregulated in NPY-null mice, the effect of NPY deficiency could be overridden. In contradiction to this hypothesis, a recent study showed that mice lacking both NPY and AgRP have no obvious feeding or body weight deficits and have a normal re-feeding activity after fasting (Segal-Lieberman et al., 2003). Some other neuromodulators that are known to regulate appetite when injected into the brain could also be considered as candidates. One approach is to look for variation of these molecules and their mRNA levels in the hypothalamus. The study of double knockouts of NPY and one of these candidates may provide further evidence to support the idea of compensation of neural circuits. The ultimate approach to circumvent this caveat is to generate inducible or conditional models, where cell/tissue-specific expression of a gene-of-interest could be switched on or off after development. Further studies are needed to confirm the existence of feeding-related phenotypes in NPY knockout mice.

Y1 knockout mice were generated in 1998 by two independent groups (Kushi et al., 1998;Pedrazzini et al., 1998). Surprisingly, moderate obesity and mild hyperinsulinemia without hyperphagia have been detected in Y1 deficient mice (Kushi et al., 1998). Y1 homozygous mutant mice have slightly diminished daily food intake and NPY-induced food intake, while fast-induced feeding is significantly decreased (Pedrazzini et al., 1998). Central administration

of a selective Y1 receptor antagonist, J-115814, had no effect on food intake in $Y1^{-/-}$ mice, whereas food intake of wild-type and Y5 knockout mice was markedly reduced by the treatment, suggesting the Y1 receptor is involved in the feeding regulation by NPY (Kanatani et al., 1999;Kanatani et al., 2001).

Mice with Y2 receptor deficiency have been established recently, and they developed increased body weight, food intake and fat deposition (Naveilhan et al., 1999). Y2 null mutants displayed an attenuated response to leptin administration, but a normal response to NPY-induced food intake and normal re-feeding and body weight after fasting. In addition, disruption of Y2 receptor also affects the basal control of heart rate, while blood pressure remains normal.

Y5 receptor deficient mice were also produced by Palmiter and coworkers using homologous recombination (Marsh et al., 1998). Young Y5 deficient mice feed and grow normally. Furthermore, fasting-induced re-feeding is unaltered in young $Y5^{-/-}$ mice and their sensitivity to leptin is unchanged. These results suggest that the effect of NPY on fast-induced hyperphagia is not mediated by Y5 receptor subtype. However, Y5-null mice develop certain mild obesity phenotypes characterized by increased body weight, food intake and adiposity. The feeding response to i.c.v. administration of NPY and related peptides is either reduced or disappears. Administration of an Y1 antagonist together with NPY attenuated the NPY-induced food intake in wild-type animals and completely abolished the effects in the Y5-null mutant mice. As mentioned above, NPY deficiency attenuates the obesity syndrome of *ob/ob* mice, but these effects are not mediated by NPY signaling through the Y5 subtype because Y5 deficient *ob/ob* mice are equally obese. These results demonstrate that both Y1 and Y5 are essential for proper feeding response to centrally administered NPY and its analogs, but Y5 subtype is not a critical feeding receptor in mice (Marsh et al., 1998).

The NPY-like signaling system in *Drosophila*

NPY system has been implicated in the modulation of a variety of effects in mammals, including feeding behavior, anxiety related behavior, epileptic seizure, cardiovascular function, and memory function. However, evidence from genetically modified rodent models does not always support the pharmacological data. For example, Y1 antagonists block NPY induced feeding and suppress baseline food intake, while Y1 receptor knockout mice exhibit normal food intake, moderate obesity, and mild hyperinsulinemia (Thorsell and Heilig, 2002). So far, the stimulatory effects on food intake and foraging activity of the central NPY signaling system have been difficult to establish, which may be largely due to the complicated network-based compensatory mechanisms in the mammalian CNS. In contrast, *Drosophila melanogaster* has emerged as a simple yet robust model organism for behavioral studies. It provides a large repertoire of molecular, genetic, and neurobiological tools. Moreover, *Drosophila* has a relatively simple nervous system which provides a number of advantages for identification and functional characterization of neurons and neural circuits underlying complex behaviors. Furthermore, conditional and inducible transgenic fruitflies can be easily generated in order to circumvent the problems arising from the compensatory mechanisms inherent in some important biological systems.

The *Drosophila* NPY homolog was recently isolated based on a radiommunoassay for a gut-derived peptide from the corn ear worm, *Helicoverpa zea* (Brown et al., 1999). This peptide was partially sequenced and the coding sequence was subsequently cloned by inverse PCR. The PCR results suggest that the full length prepropeptide consists of 96 amino acids of which the first 26 amino acids may serve as a signal peptide. After post-translational processing at a dibasic residue site, Lys_{64} and Arg_{65}, followed by C-terminal amidation at Gly_{63}, a mature

peptide with 36 amino acids is designated *Drosophila* NPF. Based on sequence similarities, NPF is considered as the first insect neuropeptide that belongs to the NPY family which encompasses extraordinary phylogenetic distances (Figure 1.2) (Halton et al., 1994;Larhammar, 1996;Tensen et al., 1998). Whole mount in situ hybridization reveals that the NPF transcripts are localized only in four neurons in the protocerebral region of the CNS and a number of endocrine cells in midguts of larvae and adults (Brown et al., 1999;Shen and Cai, 2001). Such remarkably definitive expression pattern of *Drosophila* NPF is reminiscent of the distribution of its vertebrate homologs: NPY is enriched in the brain and PYY/PP in the gut.

A *Drosophila* G-protein coupled receptor (NepYr; CG5811) resembling the mammalian NPY receptors was cloned (Li et al., 1992). NPY related peptides can activate inward currents in the *Xenopus* oocytes injected with NepYr cRNA. However, the identity of this receptor as a genuine NPF/NPY receptor has been challenged by the identification of another novel receptor, NPFR1 (CG1147) in the *Drosophila* genome database (Garczynski et al., 2002). NPFR1 was cloned and when expressed in Chinese hamster oocytes (CHO-K1) could be activated by NPF in a peptide binding assay. CHO-K1 cells stably transfected with NPFR1 bound radio-labeled ^{125}I-NPF specifically, and this binding could be displaced in a graded manner by the addition of unlabled NPF. In contrast, cells transfected with vector alone or with NepYr exhibited no specific biding for ^{125}I-NPF. Moreover, activation of the NPFR1 with NPF led to inhibition of Forskolin-stimulated [H^3]adenine incorporation into cAMP. Thus, the NPFR1 inhibits AC activity similar to the properties shown by the *Lymnaea* NPY receptor homolog when activated by the respective endogenous ligands in vitro; such inhibition represents a hallmark of Y-receptor signal transduction (Balasubramaniam, 1997;Blomqvist and Herzog, 1997). Again, NepYr-expressing CHO-K1 cells failed to display similar features. Furthermore, NPFR1 RNA

transcripts were detected in neurons located in the brain lobes and ventral nerve cord as well as scattered isolated cells of the midgut in *Drosophila* larvae. Taken together, these results strongly argued that NPFR1 functions as the endogenous receptor for NPF.

It has been implicated that the NPF signaling may be involved in the regulation of food intake and digestion, both as a neuromodulator within the CNS and as a regulatory hormone emanating from the gut. The NPF neuronal network has recently been characterized in *Drosophila* larvae (Shen and Cai, 2001). Gustatory stimulation with sugar was shown to increase the expression and synaptic transmission of NPF in a dose-dependent manner denoted by the changes of NPF immunostaining in the larval CNS, suggesting that NPF may play a pivotal role in the regulation of feeding behavior in *Drosophila*.

THE INSULIN-LIKE SIGNALING SYSTEM

Identification of insulin and insulin-like peptides

Insulin is the most extensively studied peptide hormone, largely because its failure to regulate carbohydrate metabolism in humans results in diabetes, and type II *diabetes mellitus* is the most common metabolic disorder worldwide. In 1925, the Nobel Prize was awarded to two Canadian scientists, Sir Frederick G. Banting and John J. R. MacLeod, for their work on the "anti-diabetic principle from the pancreas". Now some eighty years later, insulin is but one member of a peptide family that includes relaxins/insulin-like peptides and insulin-like growth factors (IGFs) in vertebrates; in fact, ten such peptides are known for humans. Although insulin is a gut hormone, IGFs are found in diverse tissues and relaxins in the brain and reproductive tract. After synthesis as pre-propeptides and signal peptide cleavage, the propeptides with contiguous B-C-A peptides are packaged in secretory granules. The A and B peptides are linked

convalently by two inter- and one intra-chain disulfide bonds, and the C peptide, which aids this linkage, is clipped out by convertases at the basic residues at each end, in all but the IGFs. The linked A and B peptides, both with 20 to 30 amino acids, are the bioactive form (5000-6000 Da) of insulin and related peptides. These peptides may be stored or released from the secretory granules at the cell surface in response to cellular or molecular stimuli. Once released, the peptides can diffuse to local target cells or be transported to distant ones where they act through different receptors and signalling pathways to regulate a great variety of processes.

A series of classic endocrine studies in the late 1970s first demonstrated that brain MNCs in flies were the source of a hormone that decreased circulating glucose and trehalose levels (Chen and Friedman, 1977;Duve, 1978;Normann, 1975). Such a hormone, when extracted from *Drosophila*, even had insulin-bioactivity in mice (Meneses and Los Angeles, 1975), but it was the more comprehensive effort by Duve and Thorpe (Duve and Thorpe, 1984) that set high criteria for subsequent work on insect insulin-like peptides (ILPs). Their quest failed to isolate an ILP from the heads of the adult blowfly, *Calliphora vomitoria*, in sufficient quantity for structural characterization, but the highly purified ILP had activity in mammalian bioassays and lowered trehalose and glucose levels in flies made hyperglycemic by the extirpation of brain MNCs, known to contain insulin-like immunoreactivity.

Given the pioneering application of genetic and molecular techniques to *Drosophila*, it is surprising that ILPs were identified long after an insulin receptor was characterized in this insect (see below). It was not until the *Drosophila* genome database was established that the seven ILP genes, *dilp1-7*, were identified (Brogiolo et al., 2001;Vanden Broeck, 2001). Five of the *dilp* genes are clustered on chromosome 3, with *dilp6* and *dilp7* on the X chromosome, and all have introns. The putative *dilp1-5* display highly conserved spacing between cysteine residues which

is typical for all known members of the insulin family. The encoded peptide sequences of *dilp6* and *7* contain single amino acid insertion between the second and the third cysteine of the putative A-chain. *dilp1-7* encode putative precursor proteins of 107 to 156 amino acid residues that are structurally similar to mammalian preproinsulin (Figure 1.3). Each precursor consists of a signal peptide, a B-chain, a C-peptide, and an A-chain.

Tissue expression patterns for *dilp* genes have been revealed in larvae and adults by RNA *in situ* hybridization and immunocytochemistry. Transcripts for *dilp1*, *2*, *3* and *5* exist in the same paired clusters of brain MNCs (Brogiolo et al., 2001;Broughton et al., 2005). Transcripts also were detected in midgut - *dilp4*, *5*, and *6*, imaginal discs - *dilp2*, ventral nerve cord - *dilp7*, and salivary glands - *dilp2* - of larvae, but none were observed in fat body. In females, *dilp5* transcripts were localized in the follicle cells surrounding oocytes (Ikeya et al., 2002). Immunocytochemistry with antisera specific to DILP2 showed that brain MNCs with axons to the corpus cardiacum (CC) were the primary source of ILPs in larvae and females, and no significant immunostaining was seen in other tissues (Cao and Brown, 2001;Rulifson et al., 2002).

Signaling pathways for insulin and related peptides

Two types of membrane-bound receptors are at the top of intracellular signalling pathways activated by insulin and related peptides in vertebrates. One type is a receptor tyrosine kinase (RTK), and although specific RTKs exist for insulin and IGF, the peptides can bind to both but with different affinities. These glycosylated receptors are homodimers of a proprotein with α and β subunits that are cleaved and joined by disulfide bridges. The first insulin receptor to be characterized for invertebrates was purified from extracts of adult *Drosophila* based on its

binding of radiolabeled insulin and found to have the same physical and enzymatic properties as the human insulin receptor (Petruzzelli et al., 1985).

The other receptor type is a "leucine-rich repeat-containing G-protein coupled receptor" (LGR), and only recently have LGRs been shown to bind relaxins and activate the cAMP pathway in mammalian cell systems (Chen et al., 2005;Ivell and Einspanier, 2002). Given the conservation of ILP signalling in animals, it seems probable that an orphan LGR identified in *Drosophila* will bind an ILP with basic amino acids in signature positions in the B peptide (e.g. DILP3 or 7), as in the relaxins.

In mammalian cell systems, insulin and IGF receptors potentially can activate two signalling pathways that affect cell metabolism and growth. Once ligand binding occurs at the extracellular α subunits, the kinase regions of membrane spanning β subunits undergo extensive tyrosine phosphorylation. In this state, the receptors interact at the intracellular membrane with the adaptor proteins, insulin receptor substrate 1-4 (IRS1-4), which are phosphorylated by the receptor's kinase region. These proteins then bind and activate either a growth factor receptor bound protein (GRB2) or a PI3K, each of which initiates a distinct pathway. The activated GRB2 activates the MAPK pathway, resulting in cell proliferation. In the other pathway, the activated PI3K, p110 – the catalytic subunit, generates a membrane lipid messenger, phosphatidyl-inositol-3, 4, 5-triphosphate (PIP3), that activate kinases, including phosphoinositide-dependent protein kinase 1 (PDK1) or Akt/protein kinase B. These activated proteins in turn alter other proteins associated with insulin action, such as glucose uptake and lipid synthesis, or gene expression. An increasing number of proteins are known to block or aid insulin action at specific steps in this pathway. This whole array of protein interactions forms a conserved pathway (Figure 1.4) that has been extensively investigated in *Drosophila*, for its

effect on growth, longevity, and an increasing number of physiological processes (Garofalo, 2002).

Ten years after the identification of an insulin receptor in *Drosophila*, its gene structure was reported (Ruan et al., 1995), and its expression in embryos and cell lines was described, along with the effects of mutagenesis (Fernandez et al., 1995;Ruan et al., 1995). The *Drosophila* insulin receptor (dInR) is an RTK of ~400kDa, and the degree of amino acid sequence conservation between dInR and the human insulin receptor is remarkable, as exemplified by the high affinity (K_d = 15 nM) that dInR binds human insulin (Fernandez et al., 1995;Yamaguchi et al., 1995). Though to date, there is no report that the dInR binds or is activated by a DILP or any other insect ILP. Another distinguishing characteristic is the 368 amino acid extension of the β subunit, which contains additional tyrosine phosphorylation sites (Fernandez et al., 1995), and its function has been examined (Marin-Hincapie and Garofalo, 1999). In some cells, this extension is cleaved from the receptor to give one isoform, which exists along with the extended isoform. In addition, partial nucleotide sequences for putative IGF receptors have been identified in *Drosophila* (Pietrokovski and Shilo, 2001). The tissue distribution of dInR is poorly defined for all life stages of *Drosophila*, although dInR transcripts are reportedly ubiquitous up to mid-embryogenesis and then more localized in the nervous system and imaginal discs of larvae and in the nurse cells of ovaries and nervous system of females (Chen et al., 1996;Fernandez et al., 1995;Garofalo and Rosen, 1988).

Functional significance of the conserved insulin-like signaling pathway

Metabolism

Insulin in mammals induces cells to take up glucose and convert it to glycogen, to inhibit glycogen breakdown and gluconeogenesis, and generally to shift from catabolic to anabolic lipid and protein metabolism. Genetic manipulation of *Drosophila* offers another approach to define the regulation of carbohydrate and lipid metabolism through this pathway. Targeted expression of a cell death-promoting factor ablated brain MNCs in larvae that contain DILPs and elevated trehalose and glucose levels in hemolymph (Broughton et al., 2005;Rulifson et al., 2002). Although there is no significant difference in levels of proteins and glycogen, dwarf flies with mutations in *dInR* or *chico* (the IRS orthologue in *Drosophila*) exhibit up to a five-fold increase in lipids (Bohni et al., 1999). Furthermore, insulin had no effect on glucose uptake or lipid synthesis and reduced glycogen in *Drosophila* Kc cells, but it did increase glucose oxidation and lactate production, thus providing biosynthetic precursors needed for growth (Ceddia et al., 2003). Relevant studies, described below, have focused on the regulation that this pathway exerts on cell size and growth in *Drosophila*, as mediated by nutrients.

Cell Size and Growth

Altered expression of genes for ILPs and proteins in the conserved pathway produced phenotypic effects on the growth and organs of *Drosophila*. Overexpression of *dilp* genes ubiquitously results in bigger flies by increasing cell size and cell number of individual organs in a *dInR*-dependent manner (Brogiolo et al., 2001). Genetic ablation of DILP MNCs caused developmental delay and growth retardation (Rulifson et al., 2002), and cells in the wings of these flies showed reductions in both size and number. These phenotypes are similar to those

observed in flies homozygous for a partial loss-of-function mutation in *dInR* (Brogiolo et al., 2001), but strong *dInR*-deficient mutants are recessive embryonic lethal, indicating a key developmental role for dInR (Chen et al., 1996;Fernandez et al., 1995). Flies with a mutation in the IRS gene, *chico*, display reduced body size due to an autonomous reduction in cell number and cell size (Bohni et al., 1999). The growth deficiency phenotype of *chico* mutants resembles that of *dInR* mutations, suggesting that dInR requires IRS to affect growth. Targeted expression of the PI3K subunit gene, *p110*, as a dominant-negative, in the developing wing imaginal discs causes a reduction in both cell size and cell number in the wing (Weinkove et al., 1999). Conversely, overexpression of an active, membrane-targeted variant of *p110* increased cell size and cell number. Furthermore, *p110* mutant larvae are incapable of growth beyond the size reached in the early third instar, indicating that the increase in larval cell size depends on PI3K (Weinkove et al., 1999).

A tumor suppressor protein in humans, PTEN (phosphatase and tensin homolog), is a negative regulator of this pathway, because its phosphatase activity opposes that of PI3K. Overexpression of *PTEN* in *Drosophila* inhibits cell cycle progression at an early stage of mitosis and promotes cell apoptosis during eye development (Huang et al., 1999). The small-eye phenotype caused by *PTEN* overexpression can be rescued by overexpression of wild-type *p110*. Interestingly, overexpression of *dInR* also results in eye proliferation in the few survivors, but overexpression of both *dInR* and *PTEN* completely rescues this phenotype and blocks lethality, thus supporting the role of PTEN as a negative regulator.

PDK1 and Akt are mediators of the growth and proliferative responses regulated through this pathway. Biochemical studies demonstrate that Akt is activated by phosphorylation of a specific Thr by PDK1 and a particular Ser by the target of rapamycin (TOR) kinase and its

associated protein, rictor (Brazil and Hemmings, 2001;Sarbassov et al., 2005). Flies deficient in *PDK1* exhibit lethality and apoptosis during embryogenesis, and overexpression of *PDK1* increases cell and organ size in a PI3K-dependent manner and enhances *Akt* gain-of-function phenotypes during eye morphogenesis (Cho et al., 2001;Rintelen et al., 2001). Mutations in *Akt* reduce cell size, whereas its overexpression leads to increased cell size (Scanga et al., 2000;Verdu et al., 1999). Overexpression of *Akt* rescues the phenotypes caused either by dominant-negative *p110* or overexpression of *PTEN*, which indicates Akt is a downstream of both (Scanga et al., 2000). Notably, unlike mutations in the upstream genes, *Akt* mutations do not affect cell proliferation, thus suggesting that it may be part of downstream branch in this pathway (Figure 1.4).

Recent studies show a tight but complex connection between this pathway and that mediated through TOR in response to nutrient availability. Null mutation of *TOR* in *Drosophila* results in severe growth defects, reductions in larval body size and imaginal discs, and lethality during the second instar (Oldham et al., 2000;Zhang et al., 2000). Clonal analysis of imaginal discs shows that *TOR* mutant cells are smaller and have a slower proliferation, thus indicating that TOR autonomously regulates both cell growth and proliferation. Partial *TOR* mutation in larvae has the same effect as the withdrawal of amino acids, which is consistent with a role for TOR in growth control by sensing nutrient levels (Zhang et al., 2000).

Genetic interaction studies indicate that TOR signalling is coupled to the conserved pathway by a complex of two tumor suppressor proteins, TSC1 and 2 (Pan et al., 2004). Mutation of *TSC1* and *TSC2* in *Drosophila* recapitulate alterations in the activity of *PTEN*-increased cell size and number, while co-expression of *TSC1* and *TSC2* have the opposite effects (Potter et al., 2001;Tapon et al., 2001). On the other hand, over-expression of *TSC1* and *TSC2*

blocks the over-growth phenotypes caused by *dInR* over-expression, while dominant-negative *TSC1/2* can rescue the lethality associated with loss of *dInR* function. Furthermore, the large cell phenotype of *TSC1* mutants cannot be suppressed by mutations in *dInR* or *Akt* or by over-expression of *PTEN* (Potter et al., 2001). These results and the fact that Akt destabilizes the TSC1/2 complex indicate that the negative regulator role of this complex is downstream of Akt.

The connection between TSC1/2 and TOR has been genetically mapped to a small GTP-binding protein Rheb (Ras-homologue expressed in brain) in *Drosophila*. Mutation of *Rheb* results in growth defects, similar to those caused by mutations in *chico* or other components in the pathway (Saucedo et al., 2003;Stocker et al., 2003;Zhang et al., 2003). Moreover, the phenotype of larval lethality caused by loss of TSC1 function is rescued by *Rheb* mutations. Genetic and biochemical evidence suggest that Rheb is a critical upstream activator of TOR (Stocker et al., 2003), but its role is elusive.

Studies of animal models have shown that a ribosomal protein, S6 kinase (S6K), is a downstream effector of this pathway. Loss of S6K function in *Drosophila* causes a severe reduction in body size by decreased cell size but does not alter cell number (Montagne et al., 1999). Moreover, S6K activity appears normal in *chico* mutants, suggesting that S6K regulates cell size by acquiring additional signals from other pathways (Oldham et al., 2000;Radimerski et al., 2002). Disruption of PI3K affects both cell size and cell number, thus implying that S6K is downstream of PDK1 and resides in a branch that controls cell growth and size but not cell number (Kozma and Thomas, 2002)(Figure 1.4). The large cell phenotype of *TSC1* mutation is suppressed by *S6K* mutation (Potter et al., 2001), thus indicating TSC1 and TSC2 are upstream of S6K.

In addition, the transcription factor, FOXO (forkhead box-containing protein, O subfamily), in *Drosophila* has been identified as another critical target of Akt through genetic and biochemical approaches (Puig et al., 2003). Clonal analysis and genetic interaction assay show that FOXO regulates cell and organismal size by specifying cell number but not cell size (Junger et al., 2003;Puig et al., 2003). Gain-of-function analysis reveals that under normal conditions excess FOXO is phosphorylated by Akt and kept inactive in the cytoplasm, but when deprived of nutrients or signalling through the dInR-IRS-PI3K pathway, unphosphorylated FOXO is transported to the nucleus where it promotes factors that impede cell growth and proliferation (Junger et al., 2003).

Growth of insects is dependent on the metabolism of ingested nutrients or ones mobilized from reserves, and as alluded above, nutrient levels modulate signalling through this pathway. Results from a study of *Drosophila* larvae support this notion: 1) mutation of *PI3K* suppresses cell growth in the same way as starvation, 2) ectopic induction of *dInR* or *PI3K* is sufficient to bypass the nutritional requirement for autonomous cell growth and DNA replication in starved larvae, 3) PI3K activity is down regulated in response to deprivation of dietary protein and amino acids, and 4) hyperactivation of *dInR* and *PI3K* led to reduced feeding and wandering (Britton et al., 2002). This study also showed that hyperactivation of this pathway increased nutrient storage in the fat body, whereas inhibition of PI3K activity depleted stored nutrients from the fat body, mimicking starvation (Britton et al., 2002). This and a more recent study point to the fat body in *Drosophila* as a key tissue that monitors nutrients through an amino acid transporter and the TOR pathway and controls growth remotely through effects on this pathway in peripheral tissues (Colombani et al., 2003). Autophagy of organelles and proteins in the fat body of *Drosophila* is induced by starvation or metamorphosis, and *g*enetic analysis has shown that

signalling through PI3K and TOR blocked starvation- or ecdysteroid-induced autophagy (Rusten et al., 2004;Scott et al., 2004). Together these findings show that nutrient intake leads to the activation of this pathway and in turn promotes anabolic metabolism, cell growth, and development, in general; whereas, nutrient deprivation has an opposing effect.

Reproduction

The presence of putative insulin receptors in the ovaries of lepidopteran species (Fullbright et al., 1997) hints at a role for ILPs in the reproductive physiology of insects, and studies of dipterans offer more evidence. Female *Drosophila* with genetically ablated DILP MNCs are smaller than wild one and have greatly reduced fecundity (Broughton et al., 2005;Ikeya et al., 2002). Dwarf *Drosophila* females with mutant *dInR* expression have ovaries arrested at the previtellogenic stage, but treatment with the juvenile hormone (JH) analog, methoprene, restores vitellogenesis (Tatar et al., 2001). In many insects, JH is the primary effector of reproduction, and this result may link these two endocrine pathways. In addition, *chico* mutation results in sterile females (Bohni et al., 1999) and impairs the proliferation of ovarian follicle cells and blocks egg chamber progression into vitellogenesis, even when females had abundant food (Drummond-Barbosa and Spradling, 2001). Mutation of *Akt*, however, causes a cell-autonomous reduction in the size of ovarian follicle cells but does not affect cell proliferation (Cavaliere et al., 2005). Ovarian ecdysteroids are the primary effectors of vitellogenesis and egg maturation in many dipterans. As shown for the mosquito *Aedes aegypti*, bovine insulin stimulates ecdysteroid production by ovaries *in vitro*, and specific inhibitors of insulin receptor and PI3K activity blocked this stimulation (Riehle and Brown, 1999). Ovaries from the blowfly, *Phormia regina*, were similarly stimulated *in vitro* with bovine insulin and a *Bombyx mori* ILP (Maniere et al.,

2004), but isolated ovaries from long-lived *dInR*-mutant *Drosophila* produce little ecdysone relative to those from wild ones (Tu et al., 2002). Another neuropeptide, ovary ecdysteroidogenic hormone, originating from brain MNCs, stimulates the same process in ovaries of *A. aegypti* (Brown et al., 1998). This peptide is structurally related to neuroparsins in locusts that appear to be members of a superfamily, which includes IGF binding proteins in vertebrates (Claeys et al., 2003). Although ecdysteroid hormones may be required for egg maturation in these dipterans, heterozygotic mutations in the ecdysteroid receptor increased life-span of *Drosophila* with no reduction in fertility (Simon et al., 2003), contradicting the often postulated tradeoff between reproduction and longevity.

Longevity

The concept that ILPs acting through the conserved pathway stimulate growth and reproduction in organisms has the consequence of a shorter life can be drawn from an early study of an insect. Vertebrate insulins when injected into diapausing pupae of the butterfly, *Pieris brassicae*, induced diapause termination, synchronous adult development, and an apparent *de novo* synthesis of ecdysteroids (Arpagaus, 1987). Now, many studies of *Drosophila* and other animal models offer more support for this concept (Tatar et al., 2003;Tatar, 2004). Flies bearing a hypomorphic mutation in *dInR* or null mutations in *chico* display up to an 85% extension of life span (Clancy et al., 2001;Tatar et al., 2001). Interestingly, the CA isolated from *dInR* mutant flies show decreased JH biosynthesis, and treatment of the long-lived *dInR* flies with a JH analog restored normal life expectancy. In many adult insects, JH stimulates reproduction and inhibits diapause, but in its absence, adult life span may be extended. Because of this connection, it has been conjectured that DILPs acting though this pathway may directly regulate JH synthesis

(Tatar, 2004). More recently, insulin signalling is implicated in the deterioration of heart function in aging *Drosophila*. Flies with genetically ablated DILP MNCs (Broughton et al., 2005) or mutations in *dInR* and *chico* had minimal heart deterioration over time and a longer life span (Wessells et al., 2004). Down regulation of this pathway exclusively in the heart, by overexpressing *PTEN* or *FOXO*, also abolished age-dependent decline in heart function; but lifespan was normal. Together, these results show that this pathway has the potential to affect life span and organ senescence in *Drosophila*. These life-extending effects are comparable to those elicited by diet restriction but are not manifested in reduced metabolic rates (Hulbert et al., 2004).

Potential role of insulin in feeding control

Growing evidence from mammals support a novel idea that insulin expressed in the CNS acts as an important neuromodulator controlling physiological functions related to feeding behavior (Kyriaki, 2003). In the fetal and the adult rat brain, mRNA of preproinsulin I and II was detected in the hypothalamus, the cortex, and the hippocampus. Chronic microinfusion of insulin in the rostromedial hypothalamus of rats led to anorexigenic and leptogenic (permanent loss of body weight) effects. In addition, injection of insulin antibodies in the hypothalamus of rats caused moderate hyperphagia and body-weight gain (McGowan et al., 1990;Strubbe and Mein, 1977). Downregulation of insulin receptors in the arcuate nucleus through antisense oligodeoxynucleotide caused hyperphagia and insulin resistance in rats. Furthermore, mice with neuron-specific insulin receptor knockout (NIRKO) showed increased food intake, diet-sensitive obesity characterized with increased body fat and leptin levels, elevated blood insulin levels, and hypertriglyceridemia (Bruning et al., 2000). However, insulin also acts as an anabolic factor;

when administrated systemically, pharmacological insulin levels are associated with increased body weight in human. Recent studies show that small molecule insulin mimetic compounds administrated through either centrally or systemically can effectively reduce body weight and food intake in a dose-dependent manner (Air et al., 2002b). Thus, insulin mimetics may be served as candidate as novel anti-obesity treatment.

It has been predicted that a bidirectional insulin-NPY effect could exist in the brain. A wide variety of hormones and peptides can affect insulin release in the pancreas, including leptin, galanin, NPY, corticosteroids, and glucogon. It is possible that they might also mediate the expression and function of brain insulin. It has been demonstrated that central melanocortin receptors mediate the action of insulin in the brain of rats (Obici et al., 2001). Genetic models had suggested that impairment in central melanocortin receptor signaling (MCR4 knockout mice) not only led to obesity, but also markedly induced hyperinsulinemia. Moreover, the melanocortin system was responsive to the peripheral signals originated from nutrients and known to interact at various levels with other appetite regulators, particularly with NPY and AgRP (Williams et al., 2001). Another study showed that fasted NPY Y1 deficient mice displayed hyperinsulinemia together with increased glucose utilization and glycogen synthesis (Burcelin et al., 2001). Thus brain insulin appears to be under a multifactorial and multilevel regulation. On the other hand, the expression of NPY in arcuate nucleus was reduced after systemic or central administration of insulin and leptin whereas NPY neurons showed elevated activities when the levels of these hormones were downregulated during undernutrition (Air et al., 2002a;Williams et al., 2001). In addition, injection of insulin receptor antisense oligodeoxynucletides in the hypothalamus caused marked reduction of insulin receptors in the neurons containing NPY and AgRP (Obici et al., 2002). It has been implicated that insulin deficiency in the diabetic syndrome leads to an increase

of NPY synthesis and release that is responsible, at least partly, for the hyperphagia. Some pharmacological assays indicated that insulin could suppress the release of NPY from the nerve terminals in the PVN (Sahu et al., 1995). Taken together, the synthesis, release and function of NPY in controlling energy balance might be directed or indirectly modulated by central insulin and/or peripheral insulin transported into the hypothalamus.

OBJECTIVES

As described above, the insulin- and the NPY-like peptides identified in diverse species belong to two distinct families of regulatory peptides that both exert prominent roles in regulating a variety of physiological processes and behaviors. However, the biological significance of NPY and insulin in feeding regulation has been difficult to establish, largely due to the complicated nature of the mammalian nervous system and the potential existence of functionally redundant molecules. In contrast, *Drosophila melanogaster*, a simple and powerful genetic model organism, offers a number of advantages for comprehensive analysis of the molecular and neural mechanisms underlying the action of insulin- and NPY-like signaling pathways on food-related behaviors. The long-term objective is to identify and characterize different neuronal signaling pathways that compose a complete neural circuitry in control of food intake and energy expenditure. In this dissertation, the following three major objectives were set:

1. Characterization of the regulation of neural NPF in relation to the changes of feeding activity and functional relationship between the NPF system and the developmentally programmed transition from feeding to food-aversion that occurs in the middle of the third instar stage. The results of these studies are presented in Chapter Two of this dissertation in manuscript format and have been published in *Neuron* (Wu et al., 2003).

2. Elucidation of the molecular and neural mechanisms of the insulin- and NPY-like systems underlying the control of hunger-adaptive food response. Specifically, through genetic manipulation and functional analyses of the behavioral responses to different quantitative and qualitative food, the following two fundamental questions were addressed: what enables a deprived animal to take on motivated foraging and food acquisition; what prevents a nondeprived animal from doing so. The physiological role of a central nutrient-sensing effector, dS6K, has also been determined by analyzing the food response in larvae with altered dS6K activity. The results of these studies are presented in Chapter Three of this dissertation in manuscript format and have been published in *Proceedings of National Academy of Sciences* (Wu et al., 2005a).
3. Functional characterization of the role of the insulin- and NPY-like signaling systems and the epistatic relationship in regulating the aversive response to noxious foods. By assessing the larval response to foods adulterated with quinine, a model has been established to illustrate how this conserved neuronal circuit coordinately mediates the risk-sensitive food selection. The results of these studies are presented in Chapter Four of this dissertation in manuscript format and have been published in *Nature Neuroscience* (Wu et al., 2005b).

Figure 1.1 The conserved NPY signaling pathway
NPY mediates diverse cellular events through the conserved G protein-coupled receptor (G_i and G_q) and respective intracellular components . Abbrevations: AC, adenylate cyclase; ATP, adenosine triphosphate; cAMP, 3′,5′-cyclic adenosine monophosphate; PLC-β, phospholipase C-β; PIP_2, phosphatidyl inositol 4,5-diphosphate; IP_3, inositol 1,4,5-triphospate; DAG, diacylglycerol; ER, endoplasmic reticulum).

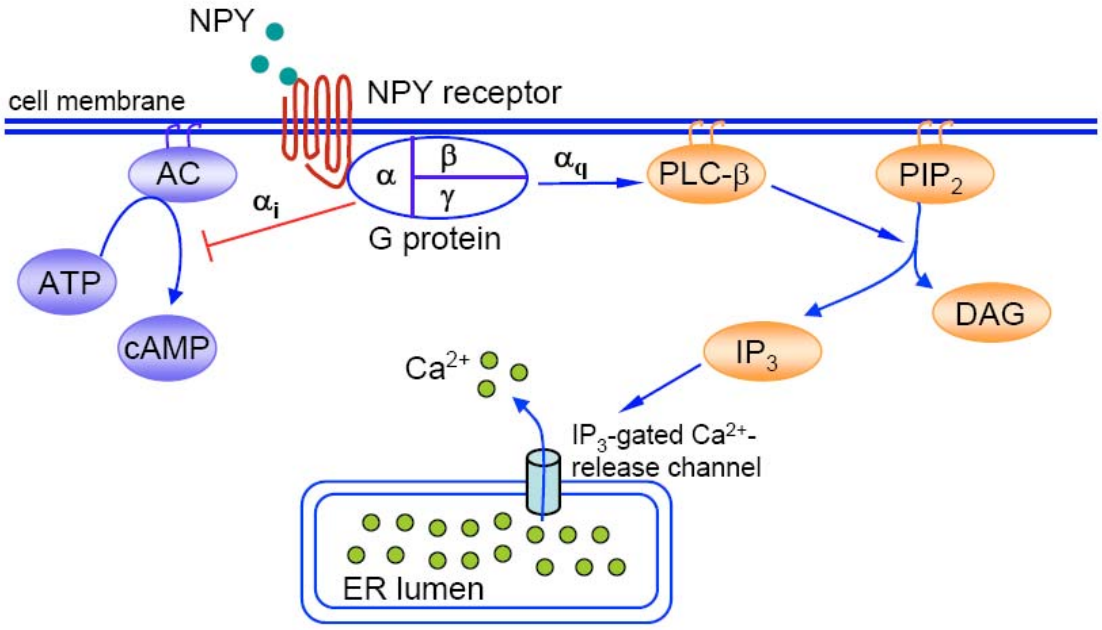

NPY
cell membrane
NPY receptor
AC
α
β
γ
α_q
PLC-β
PIP_2
α_i
G protein
ATP
cAMP
DAG
IP_3
Ca^{2+}
IP_3-gated Ca^{2+}-
release channel
ER lumen

Figure 1.2 Sequence comparisons of *Drosophila* NPF and human NPY family peptides
Drosophila NPF, a 36-amino acids peptide, shares five invariant amino acids with the vertebrate peptides and includes conservative subsititutions of three additional amino acids. Abbrevations: NPF, neuropeptide F; NPY, neuropeptide Y; PYY, peptide YY; PP, pancreatic polypeptide. Multiple sequence alignment analyses shown in Figure 1.2 & 1.3 were performed through ClustalX (Ver. 1.81), and the definitions for the default coloring scheme and the conservation line output at the top row can be found at http://www-igbmc.u-strasbg.fr/BioInfo/.

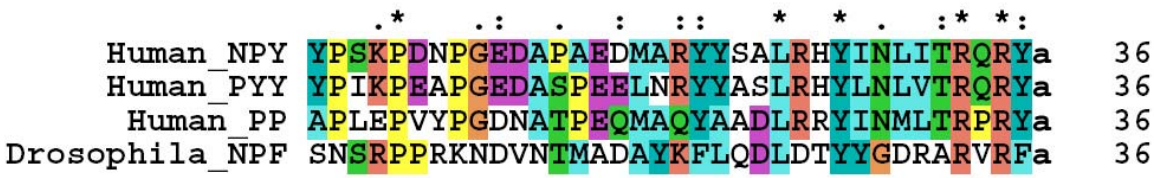

Human_NPY YPSKPDNPGEDAPAEDMARYYSALRHYINLITRQRYa 36
Human_PYY YPIKPEAPGEDASPEELNRYYASLRHYLNLVTRQRYa 36
Human_PP APLEPVYPGDNATPEQMAQYAADLRRYINMLTRPRYa 36
Drosophila_NPF SNSRPPRKNDVNTMADAYKFLQDLDTYYGDRARVRFa 36

Figure 1.3 Multiple sequence alignment of a variety of vertebrate and invertebrate insulin-like peptide precursors
Consensus sequences of the A-chains and the B-chains indicate that a pattern of highly conserved amino acid residues is present. Abbreviations: DILP 1-7, *Drosophila* insulin-like peptide 1-7; LIRP, *Locusta* insulin-like peptide; MIP1, molluskan insulin-like peptide 1 (*Lymnea stagnalis*); IRP2, insulin-related peptide 2.

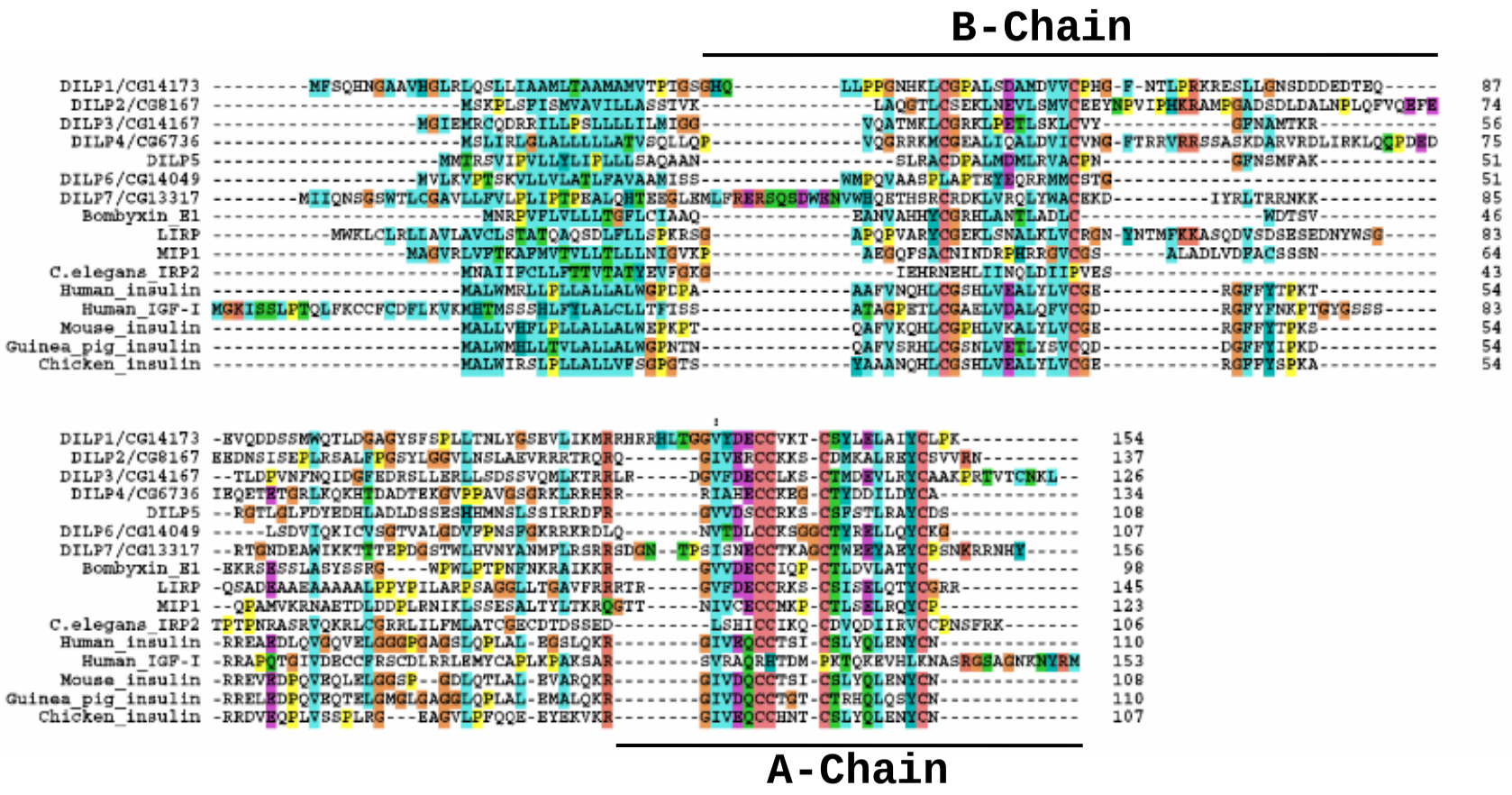
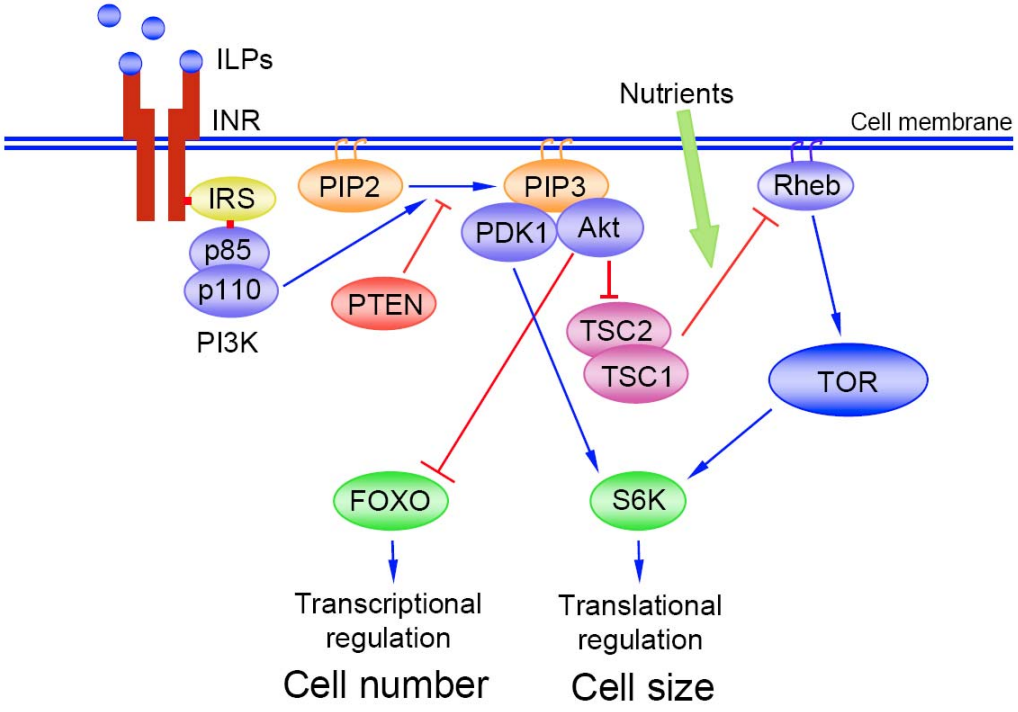

B-Chain
A-Chain

Figure 1.4 Conserved insulin signalling pathway in insects.
Homologs for all the key components illustrated here have been identified and characterized genetically and biochemically in *Drosophila melanogaster*, as well as in other insects. Abbreviations: Akt, protein kinase B; FOXO, forkhead box-containing protein, O subfamily; ILPs, insulin-like peptides; INR, insulin receptor; IRS, insulin receptor substrate; p110, catalytic subunit of PI3K; p85, adaptor subunit of PI3K; PDK1, phosphatidylinositol-dependent kinase 1; PI3K, phosphatidylinositol 3-kinase; PIP2, phosphatidylinositol-4, 5-biphosphate; PIP3, phosphatidylinositol-3, 4, 5-triphosphate; PTEN, phosphatase and tensin homologue; Rheb, Ras-homologue expressed in brain; S6K, p70 ribosomal S6 kinase; TOR, target of rapamycin; TSC, tuberous sclerosis complex. See text for details. (Adapted from Wu and Brown, *Annual Reviews of Entomology*, in press).


ILPs
INR
Nutrients
Cell membrane
IRS
PIP2
PIP3
Rheb
p85
p110
PI3K
PTEN
PDK1
Akt
TSC2
TSC1
TOR
FOXO
S6K
Transcriptional regulation
Translational regulation
Cell number
Cell size

CHAPTER 2

DEVELOPMENTAL CONTROL OF FORAGING AND SOCIAL BEHAVIOR BY THE *DROSOPHILA* NEUROPEPTIDE Y-LIKE SYSTEM[1]

[1]Wu, Q., Wen, T., Lee, G., Park, J.H., Cai, H.N. and Shen, P. 2003. ***Neuron***. 39: 147 – 161. Reprinted here with permission of the publisher.

ABSTRACT

Animals display stereotyped behavioral modifications during development, but little is known about how genes and neural circuits are regulated to turn on/off behaviors. Here we report that *Drosophila* neuropeptide F (dNPF), a human NPY homolog, coordinates larval behavioral changes during development. The brain expression of *npf* is high in larvae attracted to food, whereas its downregulation coincides with the onset of behaviors of older larvae, including food aversion, hyper-mobility and cooperative burrowing. Loss of dNPF signaling in young transgenic larvae led to the premature display of behavioral phenotypes associated with older larvae. Conversely, dNPF overexpression in older larvae prolonged feeding, and suppressed hyper-mobility and cooperative burrowing behaviors. The dNPF system provides a new paradigm for studying the central control of cooperative behavior.

INTRODUCTION

A wide range of behaviors including food response and social interaction are modulated by environmental cues. For example, gustatory stimulation by sugar has been shown to elicit the central excitatory state in flies, and increased hand-mouth contacts in human newborns (Barr et al., 1999;Dethier, 1976;Vargo and Hirsch, 1982). Other chemosensory stimulants such as pheromones are known for their profound effects on social behaviors related to foraging and mating (Wilson, 1975). However, little is known about the genetic and neural mechanisms underlying the central control of such behavioral responses.

Neuropeptides are a group of chemically diverse signal molecules that appear to modulate a broad spectrum of behaviors (Sandman et al., 1999). The genomic sequences of human and *Drosophila* have revealed that the components of a significant number of

neuropeptide signaling pathways are structurally conserved between mammals and insects (Brody and Cravchik, 2000;Hewes and Taghert, 2001). These observations support the use of *Drosophila* as a genetic model for studying the molecular and neural mechanisms underlying the regulation of behavior.

Neuropeptide Y (NPY), a conserved 36-amino acid neuromodulator, is enriched in the hypothalamus that is responsible for the central regulation of feeding in vertebrates (Beck, 2001;Williams et al., 2001). Pharmacological studies have implicated hypothalamic NPY as a prominent stimulator for appetitive behavior. Chronic administration of NPY in the paraventricular nucleus induced uncontrolled food intake in rats, which subsequently developed severe obesity (Stanley et al., 1986). In a leptin-deficient (*ob/ob*) mouse model, loss of NPY was shown to attenuate the obesity syndrome (Erickson et al., 1996b). However, the physiological role of NPY in feeding regulation has been difficult to establish. In most cases, NPY-knockout mice displayed no obvious reduction in food intake under either well-fed or fasting conditions, and had normal body weight (e.g., Erickson et al., 1996a;Qian et al., 2002). Recently, the NPY-deficient mice in a C57BL/6 background were shown to exhibit mild obesity (Segal-Lieberman et al., 2003). Transgenic rodents overexpressing NPY also showed no significant difference in food intake and body weight from the control counterparts (Inui, 2000;Thiele et al., 1998;Thorsell et al., 1998), except in one case where NPY overexpressing mice exhibited mild obesity after 50% sucrose feeding (Kaga et al., 2001). Moreover, the phenotypes of transgenic mice lacking NPY receptor subtypes, Y1, Y2 or Y5, have not provided clear support to a role of the NPY system in promoting food intake and body weight, as predicted by the pharmacological data (Marsh et al., 1998;Naveilhan et al., 1999;Pedrazzini et al., 1998). Thus, much work is still needed to determine the physiological significance of NPY in feeding behavior.

It has been postulated that NPY might play a motivational role in foraging behavior (DiBona, 2002;Tecott and Heberlein, 1998). Genetic and pharmacological studies have provided consistent evidence for a role of NPY in suppressing anxiety, fear and responsiveness to aversive/stress stimuli. (Bannon et al., 2000;El Bahh et al., 2001;Li et al., 2002;Palmiter et al., 1998;Thorsell and Heilig, 2002;Wahlestedt et al., 1993). For example, NPY-knockout mice exhibited less center activity in an open-field test and increased startling response to an acoustic stimulus (Bannon et al., 2000), whereas mice overexpressing NPY showed increased tolerance to stress and lack of fear suppression of behavior (Thorsell et al., 2000). Interestingly, mice injected with NPY were more willing to work for food, and displayed increased tolerance to the aversive taste of quinine-adulterated milk (Flood and Morley, 1991;Jewett et al., 1995). These properties of NPY appear to bode well with its speculated role in promoting food searching and acquisition, especially under adverse conditions.

Drosophila might be a simpler genetic model for studying the physiological role of the NPY system. The *Drosophila* genome contains a single coding sequence for the NPY homolog, dNPF. We have recently characterized the dNPF neuronal network in the central nervous system (CNS) of *Drosophila* larvae (Brown et al., 1999;Shen and Cai, 2001). The dNPF neural system comprises of 4-6 dNPF neurons located in the brain and subesophageal ganglia. In response to chemosensory stimulation by sugar, the dNPF neuronal circuit undergoes long-term, dose-dependent modifications through *npf* activation and an increase in the number of dNPF-positive varicosities. These properties of the dNPF neurons support its potential role in the regulation of food-related behaviors. Although an NPY homolog has not been demonstrated in *C. elegans*, a genetic study has implicated a conserved NPY like signaling system in regulating food-conditioned foraging behavior in the worm (de Bono and Bargmann, 1998). Natural isolates of

the nematode display either solitary or social foraging. The solitary foragers browse slowly across a bacterial lawn, while the social foragers move rapidly toward the edge of a bacterial lawn, and aggregate into clumps. Remarkably, a single nucleotide substitution in a putative NPY receptor-like gene, *npr*-1, is sufficient to account for the two distinct foraging patterns.

We report here that the *npf* gene is highly expressed in larvae attracted to food, but is turned off in older larvae that exhibit food aversion, increased mobility, food-dependent clumping and cooperative burrowing. Transgenic larvae deficient in dNPF signaling precociously exhibited the phenotypes of food aversion and social behaviors normally displayed by older non-feeding larvae. Conversely, dNPF overexpression in the larval CNS prolonged the feeding activity and suppressed the social behavior in older larvae. These results indicate that one of the physiological roles of the NPY-like system is to sustain larval foraging activity, particularly under adverse feeding conditions. Moreover, there is a striking parallel between the food response and social behavior of larvae deficient in dNPF signaling and *C. elegans* lacking an NPY receptor like gene (de Bono and Bargmann, 1998). Our results indicate that the conserved dNPF signaling system is developmentally programmed to modify foraging and social behavior in *Drosophila* larvae.

RESULTS

Developmental regulation of *npf*

We investigated the possible role of dNPF in regulating feeding activity in the third instar larva. Young third instar larvae feed voraciously, but their feeding activity subsides as they mature and become increasingly mobile (Ashburner, 1989). Under controlled growth conditions, the larval transition from feeding to non-feeding is largely completed by the first 24 hours when

larvae are moving away from yeast paste on apple juice-agar. To determine the relationship between the feeding activity and *npf* neural expression, we analyzed the *npf* RNA level in the CNS tissues of feeding and non-feeding larvae by whole-mount *in situ* RNA hybridization using a digoxigenin-labeled antisense *npf* RNA probe. Two-hour old third instars were either harvested immediately or withheld from food for an additional 24 hours before tissue dissection and fixation. In both cases, strong fluorescence staining was detected in the four neurons in the brain lobes (Figure 2.1A,B), indicating that the *npf* RNA level remains high in larvae that are attracted to food regardless of their age and feeding state. Quantification of fluorescence staining in the four neurons showed that the *npf* RNA levels were comparable in both types of tissues ($n = 24$; ANOVA, $p = 0.21$). We also examined the *npf* expression in synchronized 24-h old third instars that were fed continuously. Before tissue collection, the natural cessation of food intake was confirmed by the absence of dyed yeast paste in the gut. The fluorescence staining in the brain was undetectable in most of the non-feeding larvae while the rest showed greatly diminished staining (Figure 2.1C-F; also see Figure 2.10), indicating that the downregulation of *npf* expression in the brain coincides with the cessation of larval feeding activity.

The dNPF system regulates food response

We postulated that an abundance of dNPF in the CNS might promote feeding, whereas *npf* downregulation could facilitate switching to the non-feeding state. To test this hypothesis, we generated transgenic flies deficient in dNPF signaling by using the *Drosophila* GAL4-UAS binary expression system (Phelps and Brand, 1998). The *npf-gal4* constructs with *npf* upstream regulatory sequences of 4.5 or 1 kb, when crossed to a UAS-*GFP* (Green Fluorescent Protein)

reporter line, each directed a GFP expression pattern that reflected endogenous *npf* expression in the CNS of young third instar larvae (74 hours *a*fter *e*gg *l*aying, 74h AEL; Figure 2.2A). Two independent transgenic fly lines containing the 1-kb *npf* promoter (*npf-gal4*-1 and *npf-gal4*-2) were further used to direct the ablation of dNPFergic neurons by crossing them with flies harboring a UAS-*DTI* transgene that encodes an attenuated diphtheria toxin (Han et al., 2000). The larval progeny (74h AEL) from the control cross showed the normal dNPF immunostaining pattern consisting of four protocerebral neurons (Figure 2.2B), whereas the progeny (74h AEL) from crosses between *npf-gal4* and UAS-*DTI* completely lost the dNPF immunoreactivity in the CNS (Figure 2.2C). We also examined dNPF expression in larvae containing UAS-*DTI* driven by either a *Drsophila CCAP or npfr1* promoter *(CCAP-gal4* X UAS-*DTI* and *npfr1-gal4-1* X UAS-*DTI*; Figure 2.3) (Park et al., 2003). Comparable levels of dNPF immunoactivity were observed in these larvae (data not shown and Figure 2.3H). Moreover, the immunostaining of *Drosophila* insulin-like peptides in the CNS of *npf-gal4*, *npfr1-gal4*, *CCAP-gal4* and *y w* X UAS-*DTI* larvae were very similar (data not shown). Thus, there appears to be little or no leaky expression of DTI by the UAS-*DTI* construct alone.

We tested the feeding response of transgenic larvae ablated of the dNPFergic neurons. A solid agar medium containing 10% glucose was used as the food source. We chose 10% glucose for two reasons. First, 10% glucose solution can be utilized by young larvae as the food source. Second, the dNPF neuronal network is known to respond to chemosensory stimulation by 10% glucose (Shen and Cai, 2001). Young third instars normally showed little feeding response to the medium, unless they had been held on water-agar for an extended period. We routinely withheld larvae from food for two hours. Thirty synchronized young third instar larvae (74h AEL) were used in each of the three separate trials. The larvae were transferred onto a glucose-

agar disc (35 mm in diameter) placed on a large food-free agar plate. The feeding assay was designed to allow larvae to forage freely. Approximately 70% or more of control larvae (*y w* X UAS-*DTI*, *y w* and UAS-*DTI*) remained on the disc after a test period of 20 minutes (Figure 2.2D). In contrast, only 5-12% of the larvae from experimental crosses, *npf-gal4*-1 X UAS-*DTI* and *npf-gal4*-2 X UAS-*DTI*, stayed under the same condition. Thus, loss of dNPF neurons caused premature insensitivity in the feeding response. The larvae ablated of dNPF neurons may be less motivated to respond to food that is not readily accessible (e.g., glucose imbedded in agar). We tested this possibility by feeding the larvae with liquid foods (yeast paste or 10% glucose-agarose paste). The assay conditions were the same as above except that a 0.5 ml aliquot of the liquid food was added to the center of the agar disc surface. Upon transferal to the periphery of the yeast or glucose paste, all larvae from both the control and experimental crosses stayed within the food and fed immediately. At the end of 20 minutes, 85-95% larvae still remained inside the food (Figure 2.2E). We have also performed the same assay using older feeding larvae (84h AEL). Again, larvae deficient in dNPF signaling were insensitive to the glucose agar but not to the glucose-agar paste. To rule out the possibility that larvae lacking the dNPF system are deficient in sensing glucose, we tested the ability of experimental (*npf-gal4-1* and *npfr1-gal4-1* X UAS-*DTI*) and control (*y w* X UAS-*DTI*) larvae to discriminate glucose-agar paste from water-agar paste (see Experimental Procedures and Figure 2.3). The two-choice preference test showed that 100% of both control and experimental larvae preferred to feed in 4% glucose-agar over water-agar paste (binomial analysis, $p < 0.001$), and 80% of the larvae fed in 1% glucose-agar ($p < 0.05$). However, the larvae showed difficulty in discriminating 0.25% glucose agar from water agar (60-70% in 0.25% glucose agar, $p > 0.08$). Taken together, these results indicate that the dNPF neural system is not an essential part of the basal feeding

machinery but is crucial for normal food response (motivational feeding) under deprived circumstances.

In mammalian models, feeding motivation has been traditionally determined by measuring animal's willingness to press a lever to obtain food. We designed an assay to quantitatively assess the motivation of dNPF- and NPFR1-neuron deficient larvae to extract embedded nutrients (glucose) from an agar block (see Experimental Procedures for details). Synchronized young third instars (72 hours AEL) were rinsed with water, withheld from food for 3 hours, and subsequently transferred into a plate containing glucose-agar blocks. Before the assay, many small cuts were made in the vertical surface of the agar blocks to accommodate one larva in each crack. Within 10 minutes, about 50% larvae voluntarily crawled into a crack, which invariably triggered larval feeding. The frequency of agar-scraping by larval mouth-hook was determined by counting the number of mouth-hook contraction over a 30-second period (Figure 2.2F). The data showed that larvae deficient in dNPF signaling (*npf-gal4 or npfr1-gal4* X UAS-*DTI*) had much lower frequency of mouth-hook contractions than the *y w* X UAS-*DTI* controls ($p < 0.0001$, ANOVA). However, all three groups of larvae showed similarly high frequency of mouth-hook contraction in liquid food (10%-glucose agar paste), indicating the larvae have no deficits in motions related to food ingestion. In combination, these results strongly suggest that larvae lacking dNPF signaling are less motivated to extract glucose from the agar than normal larvae. We have also compared the growth rate of dNPF-neuron deficient and control larvae under well-fed conditions, and no obvious abnormality in growth and development was observed (data not shown).

Mapping and ablation of dNPF-receptor cells

A *Drosophila* NPY receptor homologue encoded by the *npfr1* gene has been pharmacologically characterized to be a dNPF receptor (NPFR1) (Garczynski et al., 2002). We reasoned that if dNPF-neuron deficient larval phenotypes are due to the specific loss of dNPF action, similar phenotypes might also be expected from larvae lacking NPFR1 cells. We generated two independent transgenic lines containing an *npfr1-gal4* construct, in which a 6.6-kb *npfr1* upstream sequence is fused with *gal4*. To localize the cells that express NPFR1, the CNS tissues of young transgenic third instars (74h AEL) were incubated simultaneously with mouse anti-NPFR1 and rabbit anti-dNPF antiserum. The double immunostaining revealed that in the control larvae (*y w* X UAS-*DTI*), NPFR1 cells are located in the dorsomedial surface of the subesophageal and abdominal ganglia (Figure 2.3A,C,D,F), while the dNPFergic neurons extend their axons into the brain hemispheres as well as along the midline of the ventral nerve cord (VNC, Figure 2.3B,C). Thus, the NPFR1 cells appear to be aligned appropriately for the reception of locally released dNPF in the segmented ventral ganglia. In contrast, the larvae from the experimental crosses (*npfr1-gal4*-1 and -2 X UAS-*DTI*) showed no NPFR1 immunoreactivity (Figure 2.3E,G). The dNPF immunostaining pattern in NPFR1-neuron deficient larvae appeared to be indistinguishable from that seen in normal *y w* X UAS-*DTI* larvae, suggesting that the dNPF secretary circuit remained intact (Figure 2.3F). We have also found that, like dNPF-neuron deficient and control larvae, NPFR1-neuron deficient larvae showed no obvious abnormality in growth and development (data not shown). The feeding response of NPFR1-neuron deficient larvae was performed using the assays as described previously. As we expected, these larvae displayed the altered feeding behaviors similar to those of NPF-deficient larvae (Figure 2.2D, F).

We therefore conclude that the regulatory activity of dNPF in food response is mediated by the NPFR1 neurons, which, in turn, may modulate the activities of head and abdominal muscles involved in larval feeding and foraging (Hartenstein, 1993).

dNPF suppresses hyperexcitation

The dNPF-neuron deficient larvae showed a strong tendency to wander away from the glucose-agar disc. We further examined this behavior by analyzing the effects of the loss of dNPF signaling on larval locomotion in the presence and absence of food. Semi-solid agarose paste was used as the medium that is soft enough to reveal the track of larva movement but hard enough to prevent potential burrowing by larvae. Before the assay, two-hour-old third instars were rinsed with copious amount of water until their bodies were free of visible food particles, and withheld from food for two hours. Each larva was first allowed to crawl on the surface of semi-solid agarose medium for 6 minutes. The same larva was then transferred to a 10% glucose-agarose medium, and allowed to crawl for another 6 minutes. The track lengths of each larva left on the surface of both media were measured (Figure 2.4). About 90% of *y w* or control larvae (*y w X* UAS-*DTI*) crawled more actively on the food-free agarose than on the glucose agarose (Figure 2.4A,B). In contrast, more than 90% of the dNPF-signaling deficient larvae crawled more on the glucose agarose than on the food-free agarose (Figure 2.4C-F). The average of the path lengths of the larvae from the different groups was also calculated. The average crawling distances of the four dNPF- or NPFR1-neuron deficient larvae were shorter than the control larvae on food-free agarose ($p < 0.05$, Student-Newman-Keuls test); when dNPF-signaling deficient larvae were placed on glucose agarose, however, they crawled similar or greater distances than the control larvae (Figure 2.4G). These results revealed that dNPF-

signaling deficient larvae have no general muscle defects for movement; they were less active in foraging than normal larvae on agarose but were hyperactive in locomotion on glucose-agarose medium. The experiments also demonstrate that one of the roles of dNPF in facilitating larval foraging on a poor food source is to suppress the locomotion. The glucose effect on larval locomotion can be quantified more accurately by eliminating variations among individuals. This can be achieved by measuring the ratio of path length on glucose agarose over that on agarose of each larva (defined as *f*ood *r*esponse *i*ndex, FRI). Remarkably, the FRI values for experimental larvae range from 1.7-2.2, which are at least two-fold higher than normal *y w* larvae, while the control larvae showed a FRI value similar to the wild type (Figure 2.4H), indicating that the dNPF system indeed exerts a strong inhibitory effect on larval locomotion in the presence of food. We suggest that glucose elicits a broad central excitation as well as dNPF-mediated neural inhibition that normally counter-balances it, and thus loss of dNPF signaling causes hyper-mobility upon exposure to the glucose-agarose medium.

dNPF controls onset of social behavior

We showed previously that *npf* expression in the CNS was promptly downregulated at the end of the feeding phase when older third instar larvae are moving away from food. We wanted to know whether dNPF signaling plays a role in modifying larval response to food. To investigate this possibility, we placed wild type young (CS72h AEL, n=40) and older third instars (CS98h AEL, n=40), respectively, on a glucose-agar plate (45 mm in diameter) coated with a thin layer of yeast paste. The larval distribution on the plate was recorded after a period of 45 minutes. In a central zone that accounts for 65% of the total area, about 70% of the young third instars were found to browse evenly across the medium surface (Figure 2.5A,E-F). In

contrast, only about 10% of the older larvae remained in the central zone; among those animals (90%) that migrated to the periphery (bordering), 82% of them participated in clumping that involved multiple animals (Figure 2.5B,E-F). Apparently, the older third instar larvae with naturally downregulated *npf* expression in the brain also displayed bordering and clumping phenotypes similar to *C. elegans* social strains. We performed further tests with the young third instar progeny from the control and four experimental crosses (*y w , npf-gal4*-1, -2 and *npfr1-gal4*-1, -*2* X UAS-*DTI*). The control larvae foraged uniformly on the assay plate showing no preference to aggregate (Figure 2.5C,E-F). In contrast, the dNPF- or NPFR1-neuron deficient larvae displayed bordering and clumping phenotypes similar to those of older Canton S larvae (Figure 2.5D-F). Thus, the loss of dNPF signaling is sufficient to cause the premature onset of bordering and clumping behavior. Interestingly, parallel behavioral phenotypes have been reported in *C. elegans* (de Bono and Bargmann, 1998). It has been shown that the loss of a putative NPY receptor homologue is sufficient for switching animals from solitary to social foraging. Unlike solitary foragers that disperse evenly across the food plate, social foragers preferentially accumulate at the edge of the plate, displaying the phenotypes of bordering and clumping.

If the loss of dNPF signaling is sufficient to trigger bordering and clumping in older wild type larvae, can we suppress these phenotypes by overexpressing dNPF? We addressed this question by crossing the UAS-*npf* fly with a *386Y-gal4* enhancer-trap line that drives a broad expression pattern including numerous peptidergic neurons (Brand and Perrimon, 1993;Taghert et al., 2001). The non-feeding third instar progeny (100h AEL) from the cross indeed browsed evenly across the assay plate, while control larvae (*y w X* UAS-*npf,* 100h AEL) showed the phenotypes of bordering and clumping (Figure 2.5E-F). Moreover, the dNPF-overexpressing

larvae remained solitary throughout the third instar life (see below). The suppression of bordering and clumping by dNPF-overexpression cannot be due to locomotory deficits since the path length of *386Y-gal4* X UAS-*npf* larvae crawling on the agarose paste was similar to that of normal larvae (Figure 2.11). Therefore, we conclude that the dNPF signaling system is programmed to both repress and activate the onset of social behavior during larval development.

The time course of larval behavioral changes

Our previous results showed that the dNPF signaling system appears to play a central role in regulating the developmental switch from feeding to food aversion in third instar larvae. To provide further support to the notion that larvae deficient in dNPF signaling prematurely display behaviors associated with older non-feeding larvae, we analyzed quantitatively the food response and bordering and clumping behaviors in NPF- or NPFR1-neuron deficient and control larvae at three different ages (72, 84 and 96 hours AEL). The dNPF-signaling deficient larvae (*npf-gal4* and *npfr1-gal4* X UAS-*DTI*) from the three different age groups displayed insensitivity to solid food (10% glucose embedded in agar) as well as strong bordering and clumping phenotypes (Figure 2.6). On the other hand, younger control larvae (Canton S and *y w* X UAS-*DTI*) of 72h and 84h AEL showed sensitive response to the solid food, and displayed neither bordering nor clumping. However, as expected, the control larvae did display food aversion as well as bordering and clumping at 96h AEL. Therefore, these results indicate that the dNPF signaling system is essential for proper temporal regulation of the onset of behaviors associated with older larvae.

Solitary larvae are deficient in burrowing

Evidently, the bordering and clumping behaviors of non-feeding third instar *Drosophila* larvae cannot be associated with foraging or feeding. Then what is the biological significance of the developmental activation of social behavior in *Drosophila* larvae? Third instars exiting the feeding phase often aggregate while burrowing into apple juice-agar. We speculated that the social behavior might be required for larval burrowing activity. A larval burrowing assay was developed to test the hypothesis. Wild type non-feeding third instar larvae (CS98h AEL, n=30) were placed on an apple juice-agar plate, and monitored over a 60-minute period. The larvae dispersed initially, began to swarm toward the periphery, and subsequently burrowed in clumps under lifted agar pieces within 20 minutes (Figure 2.7A). We also tested the burrowing activity of dNPF-overexpressing solitary larvae (*386Y-gal4* X UAS-*npf;* 100h AEL) under the same conditions. In all five trials, the solitary larvae displayed no social interaction and completely failed to burrow into the agar even after one hour (Figure 2.7B); the control larvae (*y w X* UAS-*npf*; 100h AEL), however, always displayed social burrowing and penetrated into the agar in less than 30 minutes (Figure 2.7C). Approximately 70-90% of the social larvae congregated in burrowing sites in each case, whereas no obvious burrowing was observed for the solitary *386Y-gal4* X UAS-*npf* larvae (Figure 2.7D). The lack of burrowing by the solitary larvae is unlikely due to a deficit in agar cutting, since the same larvae, under crowded conditions, were able to cut apple juice-agar into numerous small pieces (data not shown).

To better understand the larval social burrowing behavior, we attempted to determine how social larvae initiate the burrowing process. Thirty larvae from each group were transferred onto the apple juice-agar plate, and monitored for 10 minutes. While the solitary *386Y-gal4* X UAS-*npf* larvae crawled around in a random manner (Figure 2.7E), control larvae (*386Y-gal4* X

y w or *H1-lacZ*) aggregated at one or more sites (Figure 2.7F, G). Social larvae did not show any preference to the area that was just occupied by 15-20 congregating larvae for about 10 minutes until agar digging just began. These results indicate that social larvae were able to aggregate by attracting each other well before a specific digging site(s) had been marked. We observed that within minutes after aggregation, a small number of larvae began to adopt a unique drilling motion; these larvae swung their bodies back and forth in a vertical, upside-down position while digging into the agar (Figure 2.12). More larvae gradually joined in digging, resulting in the lift of an agar piece. The *386Y-gal4* X UAS-*npf* larvae that overexpress dNPF never displayed such drilling motion. Thus, the food-dependent larval aggregation appears to be critical to turn on the drilling motion which is, in turn, required for efficient penetration through the agar. We also determined the minimal time required for the onset of social burrowing (T_{min}) by larvae at the different population densities (Figure 2.7H-J). The onset of burrowing is defined as the time when one or more larvae begin to engage in the drilling motion. For example, about 15 and 48 minutes are needed for detecting social burrowing at densities of 60 and 5 larvae per plate, respectively. These results strongly suggest that the social interaction can effectively induce larvae to initiate digging behavior. The social burrowing behavior allows larvae to penetrate obstacles efficiently, thereby facilitating their escape from feeding sites to desirable locations to form puparia.

Cooperative interaction between solitary and social larvae

We further tested whether solitary larvae overexpressing dNPF and control social larvae can interact with each other. Five solitary larvae (*386Y-gal4* XUAS-*npf*) and five social larvae (*386Y-gal4* X *y w*) were added to each plate, and the T_{min} of the larvae was determined (Figure

2.8A). We found that mixing five solitary and five social larvae together significantly reduced the T_{min} in comparison to that of five social larvae alone, although T_{min} was still much longer than that of ten social larvae together. Therefore, this result suggests that dNPF-overexpressing larvae may be normal in transmitting signals for social interaction, but deficient in receiving the communication from peers. Consistent with this notion, we have also observed that solitary larvae failed to display bordering and clumping in the presence of social larvae (Figure 1.8B-F). Interestingly, the study in *C. elegans* showed that solitary worms also failed to group with social worms, suggesting a similar sensory mechanism might be involved in both cases (de Bono and Bargmann, 1998).

dNPF overexpression prolongs the feeding phase

We showed earlier that the loss of dNPF signaling in early third instar larvae leads to motivational feeding deficits and food-conditioned hyper-mobility, suggesting that the dNPF system enhances food response. In earlier experiments, we found that dNPF overexpressing and control larvae (*386Y-gal4* XUAS-*npf* or *H1-lacZ*) developed similarly during the feeding phase, and had similar body sizes at 98h AEL (data not shown). We wondered how dNPF overexpresssion might affect food response in older larvae that normally exhibit food aversion. Synchronized *386Y-gal4* XUAS-*npf* and two control larvae (w^{118} and *386Y-gal4* X *H1-lacZ*) were fed with blue yeast paste, and monitored for their food intake, starting from 92 hours AEL. As expected, control larvae showed a normal feeding profile as described previously and by others (Ashburner, 1989). The third-instar larvae ceased their feeding activity when they reached the age of 1 day old (or about 96 hours AEL), and formed puparia close to the end of the second day (Figure 2.9A-C) However, the *386Y-gal4* XUAS-*npf* larvae overexpressing dNPF

did not stop feeding until 12-24 hours later than the controls. In addition, these larvae were also late to reach puparia by about the same amount of time. Importantly, in the absence of food, the well-fed early third instars (74-hours AEL) of both *386Y-gal4* XUAS-*npf* larvae and *386Y-gal4* X *H1-lacZ* controls developed into puparia at the same rate (Figure 2.9D). Taken together, these results indicate that dNPF overexpression increases larval feeding time, and larvae lacking or overexpressing dNPF display reciprocal phenotypes. The fact that dNPF prolongs feeding in the presence but not the absence of food suggests that dNPF overexpression does not destruct the mechanisms for metamorphoses; rather it appears to delay the onset of larval metamorphosis by maintaining the positive energy flow.

We showed that feeding larvae ablated of dNPF or NPFR1 neurons displayed precocious social behavior, suggesting that two complex behaviors, foraging and social burrowing, are independently regulated by the dNPF system. To further address this issue, we performed a developmental analysis of the impact of dNPF overexpression on the behaviors of older third instar larvae. Thirty non-feeding larvae overexpressing dNPF (120 hour AEL) were placed on an apple juice-agar plate. These larvae dispersed randomly across the agar surface, displaying neither food aversion nor social burrowing (Figure 2.9E), indicating that suppression of social behavior can be regulated independently regardless whether they are feeding or not. We also evaluated how the loss of food aversion and social burrowing might impact the selection of pupation sites. We found that the vast majority of the *386Y-gal4* XUAS-*npf* larvae formed puparia on the moist food-containing agar surface, whereas most control larvae picked dry plastic surface for pupariation (Figure 2.9F vs. 2.9G). *Drosophila* pupae, which remain immobile for 4-5 days at the ambient temperature, are highly susceptible to the killing by mold and bacterial overgrowth. Since a moist food-containing environment is much more conducive to

mold and bacterial growth than a dry and food-free environment, the dNPF-overexpressing flies are likely to be disadvantaged in its fitness to survive. Thus, the developmental downregulation of dNPF signaling at the end of the larval feeding phase probably has a critical biological function: it initiates both aversive response to food and cooperative burrowing activity, thereby greatly increasing the odds of larval survival into the adulthood.

DISCUSSION

Targeted ablation of dNPFergic and NPFR1 neurons using an attenuated diphtheria toxin (DTI) has proven to be effective for the dissection and functional characterization of the dNPF neuropeptide signaling cascade. To further verify our results, we have also compared the behavioral phenotypes between larvae deficient in NPF- or NPFR1-neurons and larvae that selectively express tetanus-toxin light chains in dNPF neurons or double-stranded *npfr1* RNA in NPFR1 neurons. Blocking of neurotransmission by dNPF neurons or disrupting *npfr1* function through RNA interference each altered foraging and social behaviors in patterns similar to those of dNPF or NPFR1 neuron deficient larvae (data not shown).

We have shown here that dNPF signaling is developmentally regulated to switch on/off two opposing complex behaviors related to food, foraging and food aversion. The *npf* expression in the brain is strong in feeding larvae, and the loss of dNPF signaling leads to the phenotypes of premature insensitivity in feeding response, food-conditioned hyper-mobility and precocious social interaction. These behavioral phenotypes display a striking resemblance to those of *C. elegans* social strains lacking an NPY receptor like gene (de Bono and Bargmann, 1998). Conversely, in older non-feeding larvae, the brain expression of *npf* is developmentally downregulated, and ectopic expression of a *npf* cDNA can delay larval entry into the non-feeding

phase and suppress the food aversion and cooperative burrowing behaviors normally displayed by these older larvae. These results demonstrate that the conserved NPY signaling system modulates foraging and social behavior in flies and most likely in worms as well. Interestingly, *Drosophila* rover larvae, which have elevated activity of a *for*-encoded cGMP-dependent protein kinase, also exhibit similar behavioral responses to food (Osborne et al., 1997). Like dNPF-signaling deficient larvae, the rovers show no reduction in locomotion on a food surface than on a food-free surface. It is possible that the *for* product might be a component of the dNPF signaling pathway. In this regard, it would be interesting to know if *for* is expressed in NPFR1 neurons. It was recently reported that the increase of *for* expression is associated with honeybee transition from hive work to foraging activity (Ben Shahar et al., 2002). We suggest that the conserved NPY system may regulate foraging and social behavior in many different animals.

The burrowing behavior of non-feeding *Drosophila* larvae is genetically programmed, and is often unique to different species (Ashburner, 1989;Wong et al., 1985). Our study shows that individual larvae of *D. melanogaster* work cooperatively to dig through apple juice-agar in search for food-free sites suitable for pupation. Cooperative social behaviors have been observed across diverse species, which provide the members of an animal group unique superiorities in foraging, feeding, self-defense and aggression that are otherwise impossible to achieve by one or a few animals (Wilson, 1975). The dNPF system provides an excellent model for studying the central control of cooperative social behavior within an animal group.

It is revealing to compare how *Drosophila* and *C. elegans* have exploited the use of the conserved NPY signaling system to their respective advantages. In *Drosophila* larvae, dNPF signaling is dynamically regulated during development, thereby providing a temporal mechanism to restrict the onset of social behavior in non-feeding larvae. The downregulation of *C. elegans*

NPY signaling was achieved through a more permanent mechanism by genetically mutating a putative NPY receptor-like gene. Furthermore, in *Drosophila* larvae, the increased social interaction can lead to more effective burrowing, and therefore a better chance of survival to the adult stage. However, the social variants of *C. elegans* appear to have adopted the social behavior as apart of an evolutionarily advantageous feeding strategy. We suggest that the NPY signaling system may regulate innate social behaviors associated with different biological functions on either short- or long-term basis in diverse organisms. How does the dNPF system regulate larval social behavior? It is possible that the loss of dNPF signaling might lead to the synthesis of a chemotactant(s) that is secreted by larvae while burrowing or crawling on food. Alternatively, the dNPF signaling could suppress larval response to the chemical cue(s) that triggers larval social interaction. We found that solitary larvae overexpressing dNPF could facilitate cooperative digging by social larvae; in contrast, solitary larvae were unable to display bordering and clumping behaviors even in the presence of social larvae. These observations appear to support a role of dNPF in blocking larval response to social signals.

The food-conditioned aggregation by *C. elegans* has been shown to involve nociceptive neurons that detect an aversive signal(s) from bacteria (food to the worm) as well as other antagonistic sensory neurons (Coates and de Bono, 2002;de Bono and Bargmann, 1998). Our results suggest that similar neuronal circuits could also operate in *Drosophila*. The social behavior of *Drosophila* larvae is food-dependent. On water-agar surface, social larvae do not display the bordering and clumping phenotype. Moreover, the social behavior is normally turned on in older third instar larvae exiting the feeding phase and seeking a food-free surface. Thus, like in *C. elegans*, an aversive stimulus from food appears to be needed to initiate the social behavior. We have provided evidence that the activity of the dNPF system is necessary and

sufficient to suppress the onset of food aversion and social interaction by *Drosophila* larvae. In mammals, NPY exerts neuronal inhibitory activity, and reduces sensitivity to nociceptive stimulation (El Bahh et al., 2001;Erickson et al., 1996a;Li et al., 2002). We suggest that dNPF may also exert inhibitory effects on neurons in the sensory circuits that transduce aversive/stress stimuli and chemical cues for triggering social interaction. Further work will be needed to determine whether the regulation of social behavior in *Drosophila* larvae involves a complex network of sensory neuronal pathways similar to that in *C. elegans*.

We have provided evidence for the physiological role of dNPF in regulating food related behaviors. The larvae lacking or overexpressing dNPF displayed two reciprocal feeding behavioral phenotypes. *Drosophila* larvae deficient in dNPF signaling displayed normal baseline feeding, similar to NPY knockout mice. The intake rate of liquid food (e.g., yeast paste) was also similar between dNPF-neuron deficient and control larvae (data not shown). These observations indicate that the NPY system is not an essential component of the basal feeding machinery in both vertebrates and invertebrates. However, fasting larvae ablated of dNPF neurons did exhibit deficits in foraging; these larvae were much less motivated in extracting food from solid agar than their control counterparts. Although the role of mammalian NPY in motivational feeding is still controversial, two recent reports suggested that NPY knockout mice in a C57BL/6 background also had reduced feeding after prolonged fasting (Bannon et al., 2000;Segal-Lieberman et al., 2003). The mouse NPY receptor, Y1, is widely distributed in the brain, and has been implicated in fast-induced hyperphagia (Pedrazzini et al., 1998). In this work, we demonstrate that the activity of NPFR1 neurons is essential for motivational feeding under deprived conditions. These observations suggest that NPFR1 may have a role parallel to the mammalian Y1 receptor. Some important neurological deficits of mice lacking NPY activity

include increased anxiety and seizure susceptibility (El Bahh et al., 2001;Erickson et al., 1996a;Wahlestedt et al., 1993). The glucose-induced hyperactivity of dNPF-neuron deficient larvae may also be caused by the loss of dNPF-mediated neuronal inhibition. Consistent with this notion, dNPF overexpression suppresses the food aversion behavior normally associated with non-feeding larvae, in which the *npf* expression is downregulated. In summary, there is now substantial evidence for the functional conservation of the NPY signaling system in *Drosophila* and mammals, further validating the use of *Drosophila* as a model for studying molecular and neural mechanisms underlying behavior control.

The larvae deficient in dNPF signaling offered a unique opportunity to examine how sugar impacts the nervous system. It is somewhat puzzling that, although dNPF-neuron deficient larvae were hyperactive on solid glucose-agarose medium, they did feed normally on glucose-containing liquid food. However, these apparently conflicting observations can be best explained by the fact that dNPF-neuron deficient larvae still have a dNPF-independent mechanism that can effectively suppress larval locomotion while engaging in food uptake. Previous reports by others indicate that sugar can induce a central excitatory state in flies that enhances their feeding activity (Dethier, 1976;Vargo and Hirsch, 1982). In dNPF-neuron deficient larvae, however, the excitatory effect of glucose is excessive and detrimental to food intake. Apparently, the dNPF mediated neural inhibition is also glucose-dependent, since dNPF-neuron deficient larvae behaved normally on food-free agar. The dNPF action may be essential to refine and limit food elicited excitatory effects to intended action sites (e.g., muscles required for food intake). Such excitation-inhibition interplay is perhaps a general mechanism underlying the neural control of feeding responses in metazoan.

EXPERIMENTAL PROCEDURES

Larvae, adult flies and media

Conditions for raising adult flies and egg collection were described previously (Roberts, 1986;Shen and Cai, 2001). Synchronization of third instar larvae was achieved by collecting eggs in a short time interval, and was confirmed by the synchronized molting after the second instar period. The molting time at the end of the second instar period was also used for more accurate determination of the age of third instar larvae. Unless stated otherwise, larvae were continually fed with a regular diet (yeast paste on apple juice-agar), and grown to desired ages at 25 ^{o}C. The larvae were housed in an incubator with a transparent door that provides exposure to natural lighting. For food deprivation, well-fed young third instars were repeatedly rinsed by bathing the larvae in copious amount of water until free of visible food particle, and starved for desired time periods on water-agar plates before use. Fly strains and transgenic lines include Canton S, y^{1} w^{67c23}, w^{1118}, *w*;UAS-*DTI*, and *w*;*386Y-gal4* (Han et al., 2000;Phelps and Brand, 1998;Taghert et al., 2001). The *npf-gal4*, *npfr1-gal4*, UAS-*npf* and UAS-*GFP* are in the y^{1} w ^{67c23} background (also see below).

In Situ RNA hybridization and immunostaining

The tissues for *in situ* hybridization were dissected and fixed according to a published procedure with some modifications (Brown et al., 1999). The 4% paraformaldehyde was freshly made in phosphate-buffered saline (PBS) solution, and tissues were fixed for 40 minutes at room temperature. The digoxigenin-labeled dNPF RNA probe synthesis and the procedure for *in situ* RNA hybridization has been described previously (Shen and Cai, 2001). The fluorescence staining of the tissues was performed according to a published protocol using a tyramide signal

amplification kit (TSA Cyanine 3 System, NEN Life Science) (Wilkie and Davis, 2001). The immunofluorescence staining protocol was also described previously (Shen and Cai, 2001). The mouse anti-NPFR1 sera were raised against two synthetic peptides (NKLKSRITVVAVQASSAQRK and VYKELINTDTPALLQQIGLQ) derived from the variable regions of the receptor, and diluted 200-fold before use. The rabbit anti-dNPF serum was diluted 2000-fold and pre-absorbed against a C8 peptide corresponding to the C-terminal sequence of dNPF (Shen and Cai, 2001). Secondary antibodies include Alexa 488-conjugated anti-mouse IgG and Alexa 568 conjugated anti-rabbit IgG (Molecular Probes, OR), which were diluted 2000-fold. Unless stated otherwise, more than 12 tissues from at least two separate experiments were examined for each group of larvae, and the consensus patterns reported were derived from at least 80% of the examined tissues.

Transgenic constructs and fly transformation

The *npf* and *npfr1* sequences were obtained from Berkeley *Drosophila* Genome Project (www.fruitfly.org). A 4.5-kb *Mun* I fragment containing the 5' regulatory region and part of the first exon was subcloned from a genomic PCR fragment into the *Eco*RI site of a pCaSpeR-based fly transformation vector that contains a promoterless *gal4* coding sequence. The 1 kb fragment was cloned separately using genomic PCR. It contains the 5' regulatory region and part of the first exon, and was subcloned into the pPTGAL4 vector (Park et al., 2000). The 5' extreme of the *dnpf* transcription unit was independently determined by RACE experiment. For the *npfr1-gal4* construct, a 6.6-kb fragment, including *npfr1* upstream sequence and the first exon, was subcloned from a genomic PCR product, and inserted into the *BamH*I site in the same *gal4*-containing transformation vector. The UAS-*npf* construct was made by inserting a full-length *npf*

cDNA downstream of the UAS promoter in the pUAST vector (Phelps and Brand, 1998). More detailed information on cloning and vector sequences are available upon request. The npf-gal4, npfr1-gal4 and UAS-npf lines were obtained through the P-element mediated transformation of $y^{1}\ w^{67c23}$ flies, using standard procedures (Roberts, 1986).

Food response assays

Synchronized young third instars were withheld from food for 2 hours before use. For larval response to solid food medium, a 10% glucose-agar disc (45 mm in diameter) was placed in the center surface of a large agar slab (20 X 30 cm). Thirty larvae were placed in the center region of the disc and the number of larvae remaining was scored after 20 minutes. The assay conditions for larval response to liquid food (yeast paste or 10% glucose-agarose paste, each of which can sustain larval growth) were identical except for the addition of 0.5 ml of food paste to the center of the disc before larval transfer. The yeast paste contained 0.5 g of yeast powder per ml water, and the glucose paste is composed of 1 g glucose, 1.5 g agarose and water in a10 ml final volume. Quantification of the food effects on larval locomotion was performed on a semi-solid agarose paste plate (87 mm in diameter) containing1.3 g agarose powder in 8.7 ml water, to which 1 g glucose may be supplemented as needed. Larvae were rinsed repeatedly to remove visible food particles. A single larva was allowed to crawl on the surface of water-agarose first, and subsequently transferred to 10% glucose agarose paste. The track length of each larva was manually traced and recorded. Statistical analyses, including binomial analysis, t-tests and ANOVA, were performed using Microsoft Excel. The Student-Newman-Keuls test was performed using the SAS software.

Glucose sensing and motivational feeding Assays

The larva's ability to sense glucose was assayed by allowing an individual to choose between two agarose pastes containing either 10% glucose solution or water only. 0.1 g of agarose paste was used to make a small dome of 1.3 cm in diameter on the surface of a food-free agarose plate, and two neighboring domes either with or without glucose were joined at the edge. Larvae were individually placed in between the agarose domes, and the number of larvae that burrowed into either dome was scored, and statistically analyzed using the binomial test. Second instars (66-72h AEL) or early third instars were placed in a dish with a piece of water-saturated paper for 5 hours before the assay. The test for motivational feeding was performed in a 10% glucose-agar plate. About 15 synchronized third instar larvae (74h AEL) were collected from apple juice-agar plates with yeast paste. After repeated washing with copious amount of water, they were transferred into a plate containing a piece of water-saturated paper for 3 hours. Subsequently, larvae were placed on glucose agar in a 35-mm plate that was pre-cut into four equal quarters. The pre-cut glucose agar plate was dried at room temperature for one day before use to eliminate surface condensation. About 30 minutes after larval transfer, about 16 small cuts (about 2-3 mm deep) in the vertical surfaces of agar pieces were made to entice the larva to crawl in. About 50% of the larvae voluntarily burrowed in within 10 minutes, and they would usually stay and attempt to feed on the glucose-agar. The number of attempts to scrape the agar (as indicated by the frequency of mouth-hook contractions) was counted in a 30-second interval. The food ingestion by the larvae was confirmed by the presence of colored food in the larval gut. In addition, the larvae were also allowed to feed in liquid food (glucose agar paste containing 10% glucose and 10% agar powder). Individual larvae were buried separately into the agar paste

in a 45-mm plate. Larvae were allowed to settle down for five minutes, and their mouth-hook contractions were counted by viewing from the reverse side of the plate.

Social behavior and burrowing assays

The assay for bordering and clumping was performed on plates (45 mm in diameter) containing glucose agar (3% agar in a 10% glucose solution), which was coated with a thin layer of yeast paste (0.5 g yeast powder in 10% glucose solution). Larvae did not burrow into the agar medium under these conditions. Forty third instar larvae of 2 hours old were allowed to crawl on a plate for 45 minutes in the dark, and plates were photographed and counted immediately using a Sony DSC-S75 digital camera. The third instar progeny (74h AEL) were collected from experimental and control crosses for the assay. Canton S larvae of 72 and 98 hours AEL were used as controls. The larvae overexpressing dNPF and controls were collected at 100 hours AEL. For the burrowing assay, 35-mm plates containing apple juice-agar were used instead. The apple juice-agar is made from 2.8 g agar (ABI, Niagara, New York), 20 ml concentrated apple juice and 70 ml water. The agar concentration may need slight adjustment when different quality agar is used. Thirty larvae were placed on each plate, and monitored for an hour. The burrowing activity of wild-type larvae was assayed using non-feeding larvae (98h AEL). Larvae may be fed with yeast paste containing 0.14% bromophenol blue (optional), which aids the monitoring of food intake (Dubreuil et al., 1998). The larvae were kept in the dark during the assay except for the short exposure periods during photography.

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Figure 2.1 Regulation of *npf* RNA in the CNS of larvae that are either attracted to or avoid food

All tissues are positioned anterior top. The dNPFergic neurons, indicated by arrows, are located to the dorsal surface of the brain lobes. **A**) The CNS tissue isolated from a young third instar Canton S larva fed for two hours; **B**) same as in **A** except that the larva was starved for an additional 24 hours on water-agar. Four protocerebral dNPFergic neurons are strongly stained in both cases. In contrast, synchronized 24-hour-old third instars entering the non-feeding phase showed no or diminished staining of the same dNPFergic neurons (**C**-**F**). The numbers indicate that, among a total of seven intact CNS tissues recovered in a representative experiment, four tissues showed no staining (**C**), and one tissue each showed weak staining in a two-, three-, or four-cell pattern (**D**-**F**). We examined 28 tissues in total. Again, about 60% tissues showed no staining, while 14%, 19% and 7% tissues showed the two-, three- or four-cell pattern, respectively. The fluorescence staining in the non-feeding larval tissues was at least 3-4 fold lower than the tissues from 2-hour feeding third instars (ANOVA, $P < 0.0001$). The scale bars in all figures represent 50 μm.

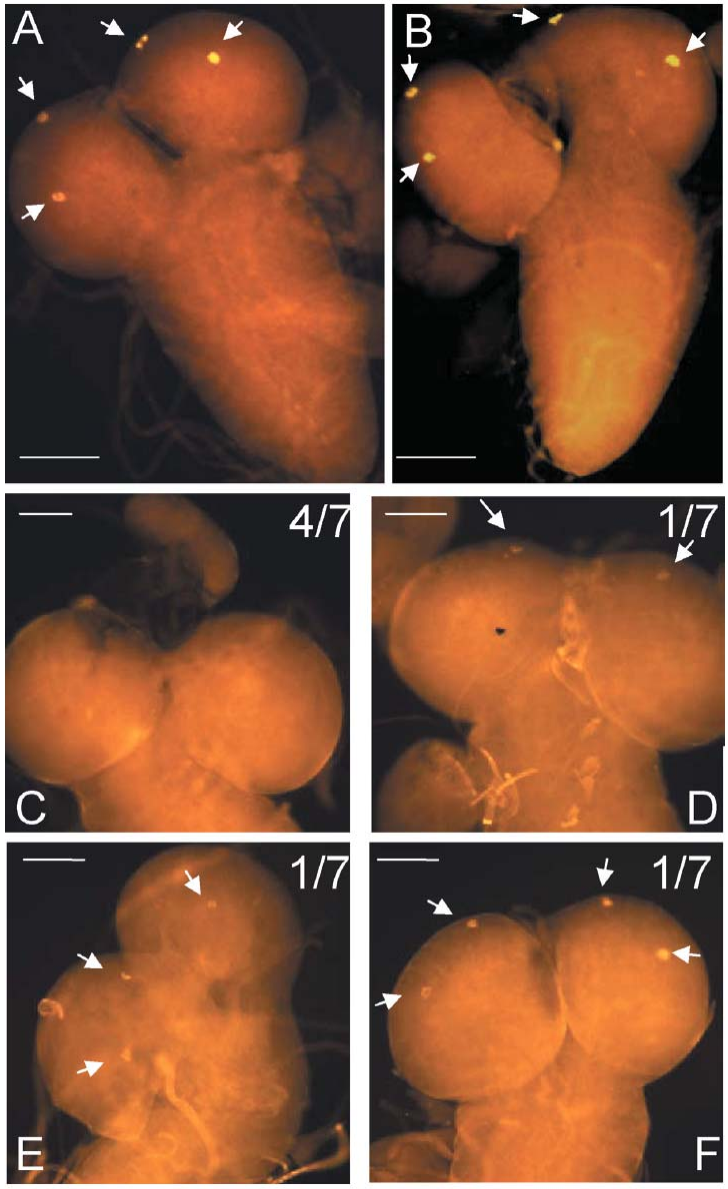

A
B
4/7
C
1/7
D
1/7
E
1/7
F

Figure 2.2 Ablation of dNPFergic neurons and their role in food response
The genomic organization of the *npf* gene and the structure of DNA constructs containing the *npf* promoter are shown at the top. The brain hemispheres of the young third instars (74h AEL) are shown with the anterior top. Arrowheads indicate dNPFergic neurons. GFP expression in the CNS of larval progeny of the cross, *npf-gal4*-1 X UAS-*GFP*, is shown (**A**). The GFP reporter does not reveal all the axonal arbors of dNPFergic neurons (see below). The dNPFergic neurons were detected immunocytochemically in the CNS tissues ($n > 20$) from the progeny of the control cross, *y w X* UAS-*DTI* (**B**), but not in those from the cross of *npf-gal4*-1 X UAS-*DTI* or *npf-gal4*-2 X UAS-*DTI* (**C**). Ten tissues for each group were randomly picked for the quantification. All *y w* X UAS-*DTI* larval CNS tissues showed a four-neuron immunostaining pattern, while all *npf-gal4* X UAS-*DTI* were negative (ANOVA, $p < 0.0001$). The panels **D** and **E** show the food response of 2-hour-old third instar progeny from five crosses: the control larvae, *y w X* UAS-*DTI*; dNPF-neuron deficient larvae *npf-gal4*-1 and *npf- gal4*-2X UAS-*DTI*; NPFR1-neuron deficient larvae, *npfr1- gal4*-1 and *npfr1-gal4*-2 X UAS-*DTI* (see Figure 3 for *npfr1-gal4* lines). The *npf-gal4* and *npfr1-gal4* lines are in the *y w* background. Two additional controls, *y w* and homozygous UAS-*DTI* larvae, were also tested in **D**. The columns show the percentage of larvae remaining on the 10% glucose-containing solid medium after 20 minutes (**D**). The asterisk indicates the groups with significantly fewer remaining larvae than the *y w* X UAS-*DTI* control larvae after 20 minutes (ANOVA, $P < 0.0001$). The percentages of larvae that burrowed into liquid foods (yeast paste or 10% glucose agar paste) at 20 minutes are shown in **e**. The number of mouth-hook contraction of larvae feeding in either solid glucose agar or glucose-agar paste was counted in a 30-second period (**F**). We have further tested the food response in a variety of transgenic larvae that are competent in dNPF signaling (Figure 2.11). The results showed that the genetic variations of the control larvae exhibited at most minor influence on larval food response. Error bars are the standard error of mean (SEM) in all figures.

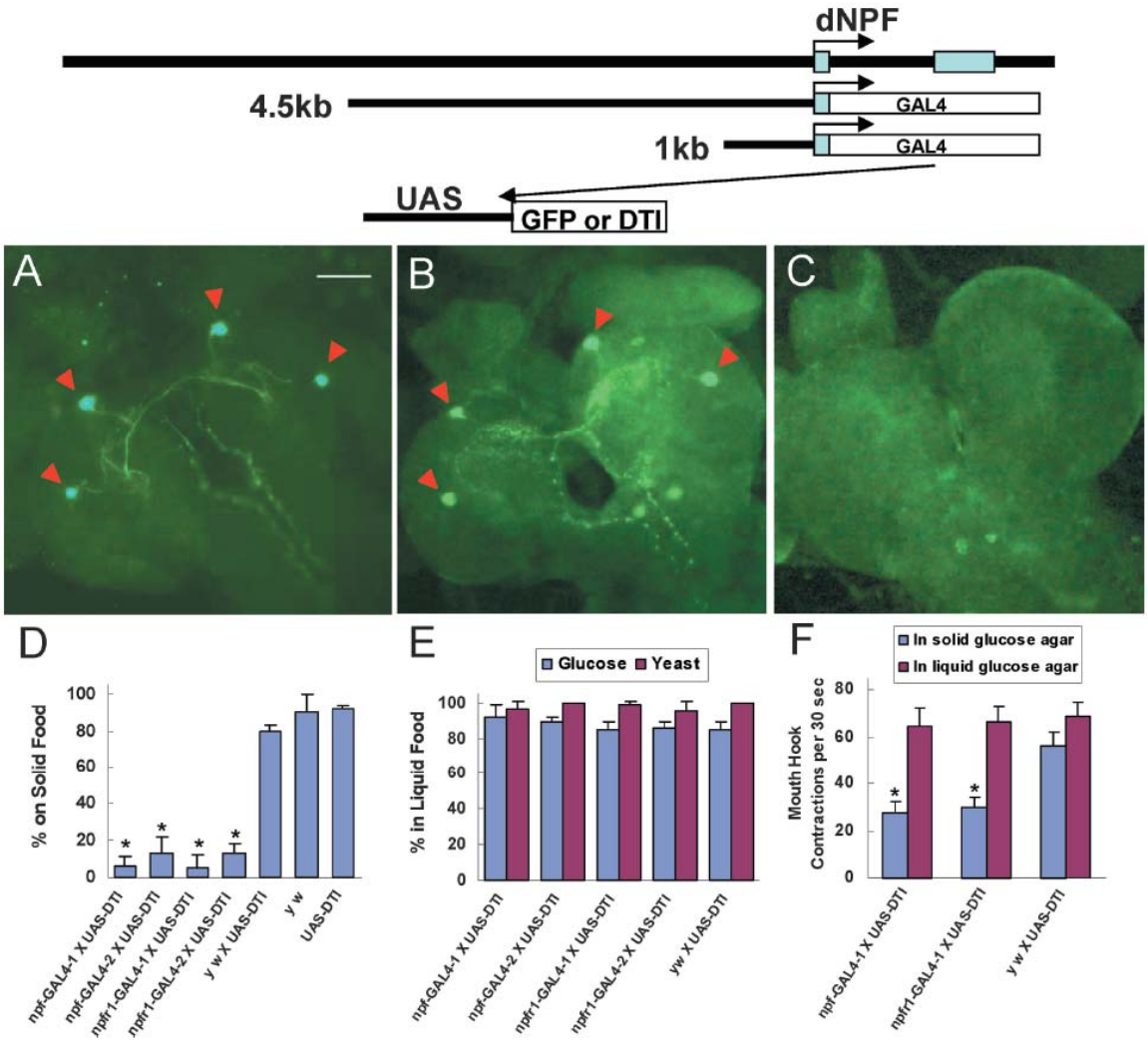

dNPF
4.5kb
GAL4
1kb
GAL4
UAS
GFP or DTI
A
B
C
D
% on Solid Food
100
80
60
40
20
0
npf-GAL4-1 X UAS-DTI
npf-GAL4-2 X UAS-DTI
npfr1-GAL4-1 X UAS-DTI
npfr1-GAL4-2 X UAS-DTI
y w X UAS-DTI
y w
UAS-DTI
E
Glucose
Yeast
% in Liquid Food
100
80
60
40
20
0
npf-GAL4-1 X UAS-DTI
npf-GAL4-2 X UAS-DTI
npfr1-GAL4-1 X UAS-DTI
npfr1-GAL4-2 X UAS-DTI
yw X UAS-DTI
F
In solid glucose agar
In liquid glucose agar
Mouth Hook Contractions per 30 sec
80
60
40
20
0
npf-GAL4-1 X UAS-DTI
npfr1-GAL4-1 X UAS-DTI
y w X UAS-DTI

Figure 2.3 Immunolocolization and Ablation of NPFR1 neurons
The diagram at the top shows the genomic organization of the *npfr1* gene and the structure of the *npfr1* promoter. Immunofluorescence staining of NPFR1 neurons in the CNS tissues ($n > 17$) of the control larvae, *y w* X UAS-*DTI* (**A-D, F**), and in larvae from the crosses of *npfr1-gal4*-1 or *npfr1-gal4*-2 X UAS-*DTI* (**E**, **G**). The double immunostaining of the young third instar CNS is shown in **A** (green, NPFR1), **B** (red, NPF) and **C** (merge); anterior top. The boxed areas in **A** are shown in high magnification in **D** and **F**. NPFR1 neurons are indicated by arrowheads. Eight intact larval tissues from each group were randomly picked for quantification. The *y w* X UAS-*DTI* larval CNS tissues showed a cluster of NPFR1-positive neurons along the midline of the ventral nerve cord, while all *npfr1-gal4* X UAS-*DTI* were negative (ANOVA, $p < 0.0001$). The *npfr1-gal4* X UAS-*DTI* larvae showed a normal dNPF immunostaining pattern in the CNS (**H**).

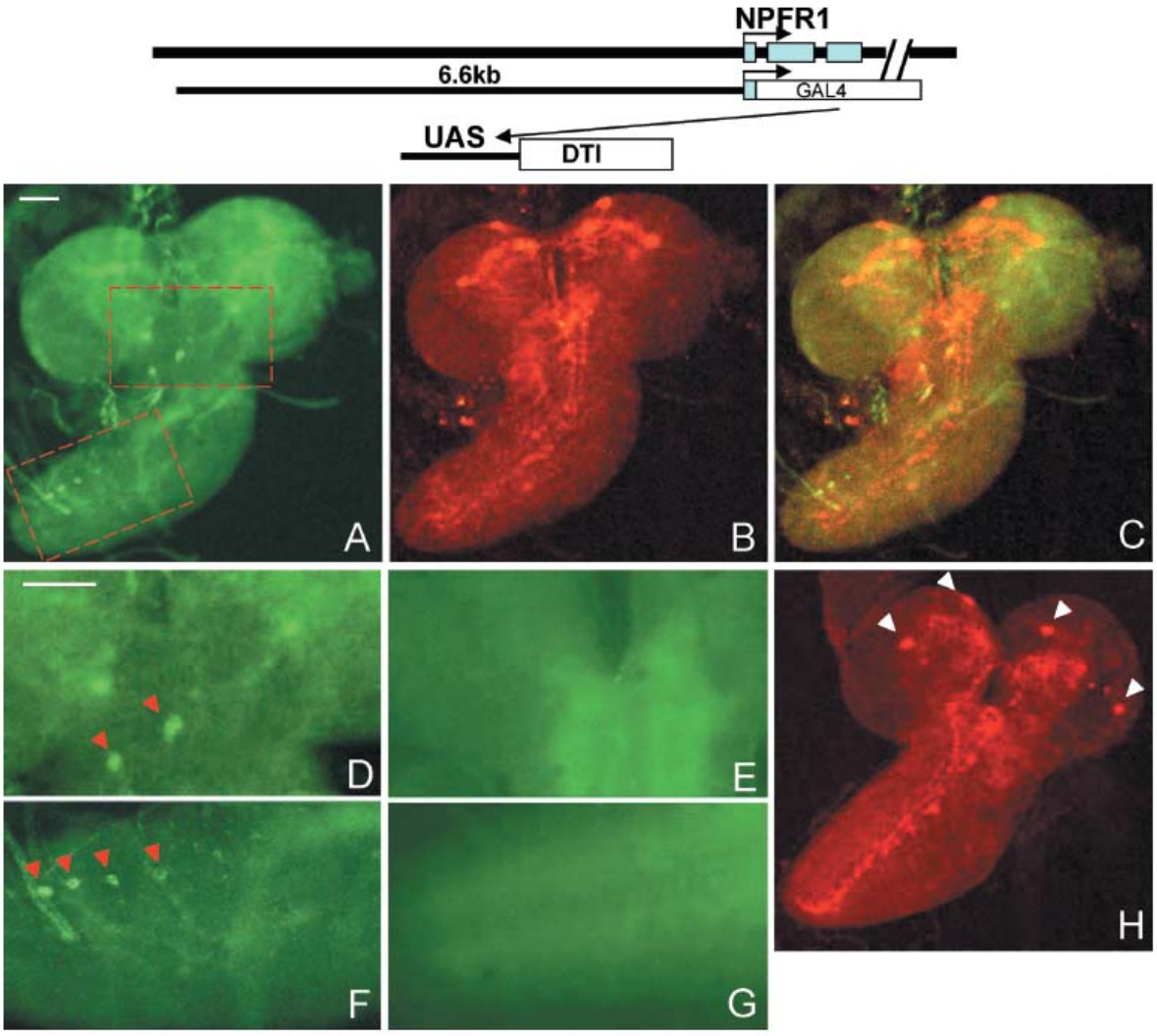

NPFR1
6.6kb
GAL4
UAS
DTI
A
B
C
D
E
F
G
H

Figure 2.4 Quantification of Behavioral Response to Food
Synchronized 2-hour-old third instar larvae were used for the assay. Each dot represents the measurement of the path length of an individual larva crawling first on food-free and then on glucose-containing semi-solid agarose surface in a 6 minute period. **A**) *y w* control larvae (n = 10); **B**) larval progeny from the control cross, *y w X* UAS-*DTI* (n = 27) ; **C**-**D**) *npf- gal4*-1 X UAS-*DTI* and *npf- gal4*-2 X UAS-*DTI* progeny (n=16 and 17); **E**-**F**) *npfr1-gal4*-1 X UAS-*DTI* and *npfr1-gal4*-2 X UAS-*DTI* progeny (n= 18 and 19). **G**) The average path lengths for six larval groups on agarose or glucose-agarose; larvae deficient in dNPF signaling displayed shorter path lengths on food-free agarose surface (Student-Newman-Keuls test with log transformation, $p < 0.05$), but not on glucose-agarose surface. **H**) Food Response Index (FRI) is defined as L_{glc}/L_{agr} where L_{glc} is the path length of a single larvae on glucose-agarose and L_{agr} is the path length on agarose. The average of FRI values from each group was analyzed using ANOVA. The asterisk indicates significantly higher values than that of the *y w* X UAS-*DTI* control group ($P < 0.0001$).

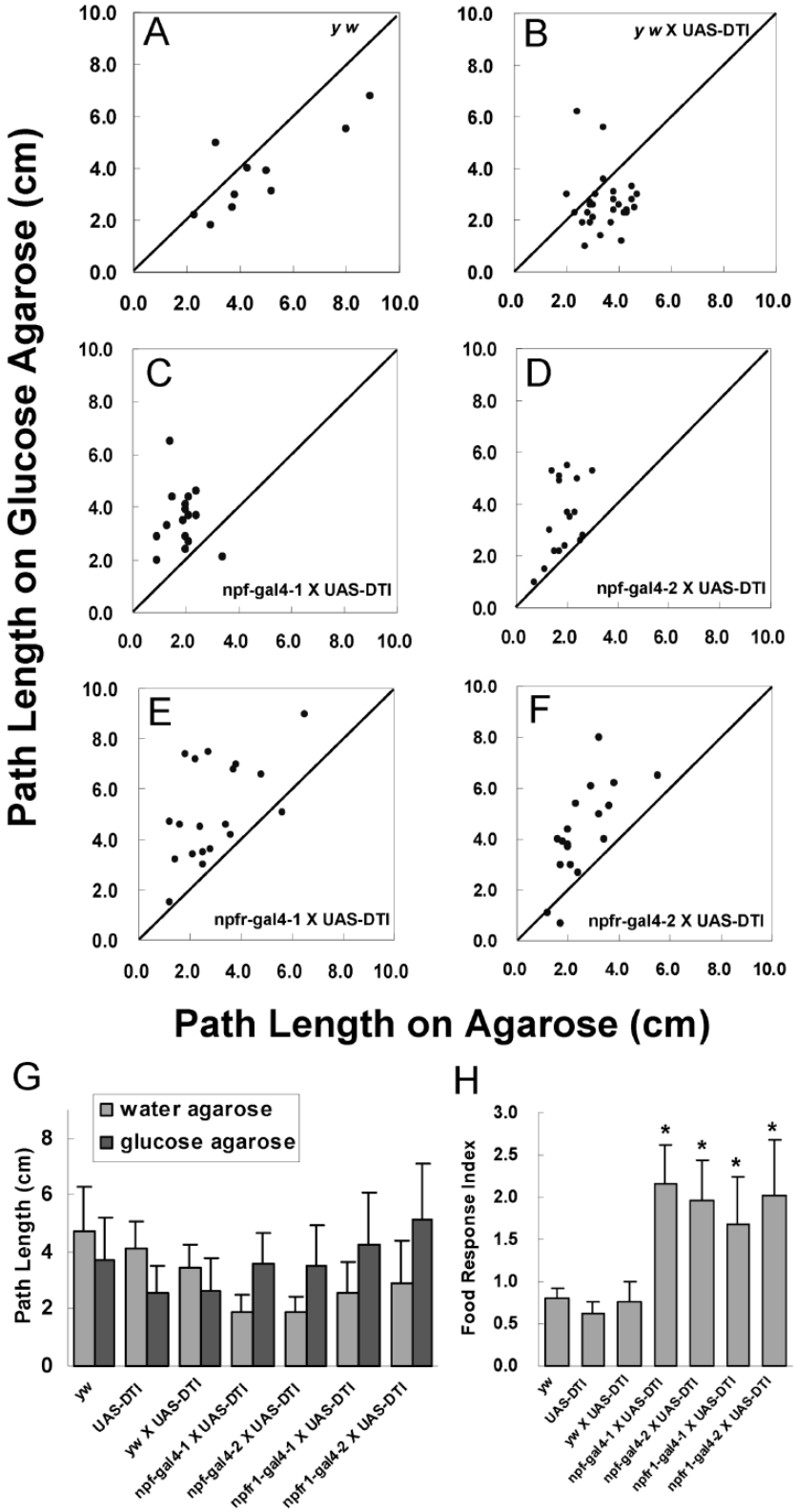
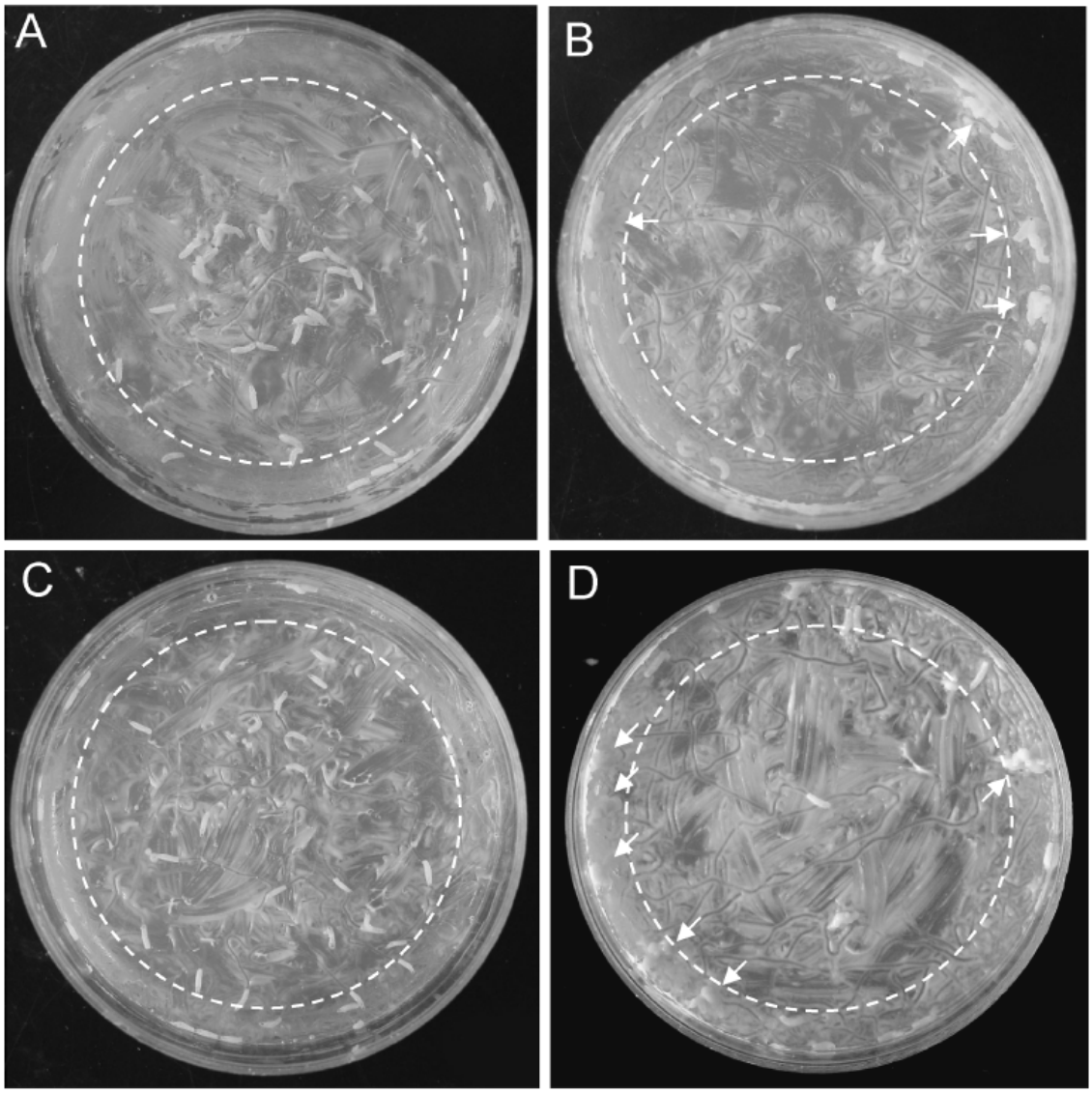
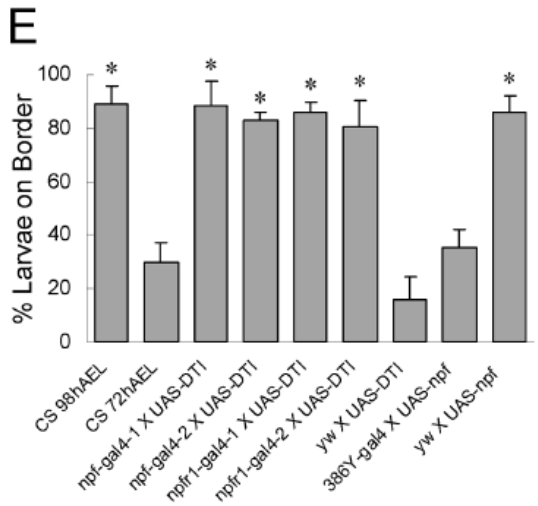

A
y w
B
y w X UAS-DTI
C
npf-gal4-1 X UAS-DTI
D
npf-gal4-2 X UAS-DTI
E
npfr-gal4-1 X UAS-DTI
F
npfr-gal4-2 X UAS-DTI
Path Length on Glucose Agarose (cm)
Path Length on Agarose (cm)
G
water agarose
glucose agarose
Path Length (cm)
yw
UAS-DTI
yw X UAS-DTI
npf-gal4-1 X UAS-DTI
npf-gal4-2 X UAS-DTI
npfr1-gal4-1 X UAS-DTI
npfr1-gal4-2 X UAS-DTI
H
Food Response Index

Figure 2.5 dNPF controls social behavior

The social behavior of third instar larvae was assayed on 10% glucose-containing solid medium coated with a thin layer of yeast paste. The foraging patterns of Canton S feeding larvae (72h AEL), non-feeding larvae (98h AEL), control (*y w X* UAS-*DTI*, n = 40) and dNPF-neuron deficient (*npf-gal4*-1 X UAS-*DTI*, n = 40) larvae (74h AEL) are shown in panels **A**-**D**, respectively. The bordering and clumping of larvae that are wild type (CS72h AEL and 98h AEL), dNPF-neuron deficient (74h AEL), NPFR1-neuron deficient (74h AEL), or dNPF-overexpressing (100h AEL) were quantified (**E**, **F**). Larvae remaining in the peripheral zone (approximately the outer one-fifth of the radius) are considered to display the bordering phenotype. The central zone, indicated by a dashed circle, occupies 65% of the total medium surface. The percentage of the same larvae involved in clumps (defined as three or more larvae in contact along >50% of the body length (de Bono and Bargmann, 1998)(de Bono and Bargmann, 1998)) was also calculated. The larvae remained stably in the clumps. 120-160 larvae were assayed for each group. The asterisk indicates significant differences between the experimental larvae and control larvae (CS72hAEL and *y w X* UAS-*DTI*; ANOVA, $P < 0.0001$).


A
B
C
D


E
% Larvae on Border
100
80
60
40
20
0
CS 98hAEL
CS 72hAEL
npf-gal4-1 X UAS-DTI
npf-gal4-2 X UAS-DTI
npfr1-gal4-1 X UAS-DTI
npfr1-gal4-2 X UAS-DTI
yw X UAS-DTI
386Y-gal4 X UAS-npf
yw X UAS-npf

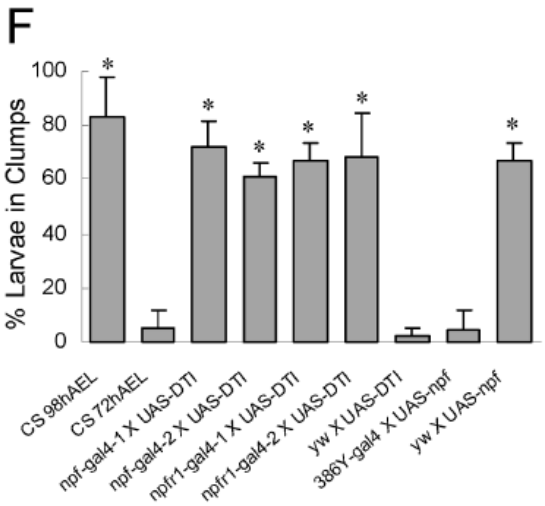

F
% Larvae in Clumps
100
80
60
40
20
0
CS 98hAEL
CS 72hAEL
npf-gal4-1 X UAS-DTI
npf-gal4-2 X UAS-DTI
npfr1-gal4-1 X UAS-DTI
npfr1-gal4-2 X UAS-DTI
yw X UAS-DTI
386Y-gal4 X UAS-npf
yw X UAS-npf

Figure 2.6 The time course of behavioral changes in NPF-, NPFR1-neuron deficient and control larvae

Synchronized third instars of three different ages (72h, 84h and 96hAEL) are included. **A**) quantification of larval response to 10% glucose agar (solid food); **B**) quantification of larval bordering behavior; **C**) quantification of larval clumping behavior. The results support the conclusion that larvae deficient in dNPF signaling prematurely display the behaviors associated with older non-feeding larvae. Also see Figures 2 and 5 for related information.

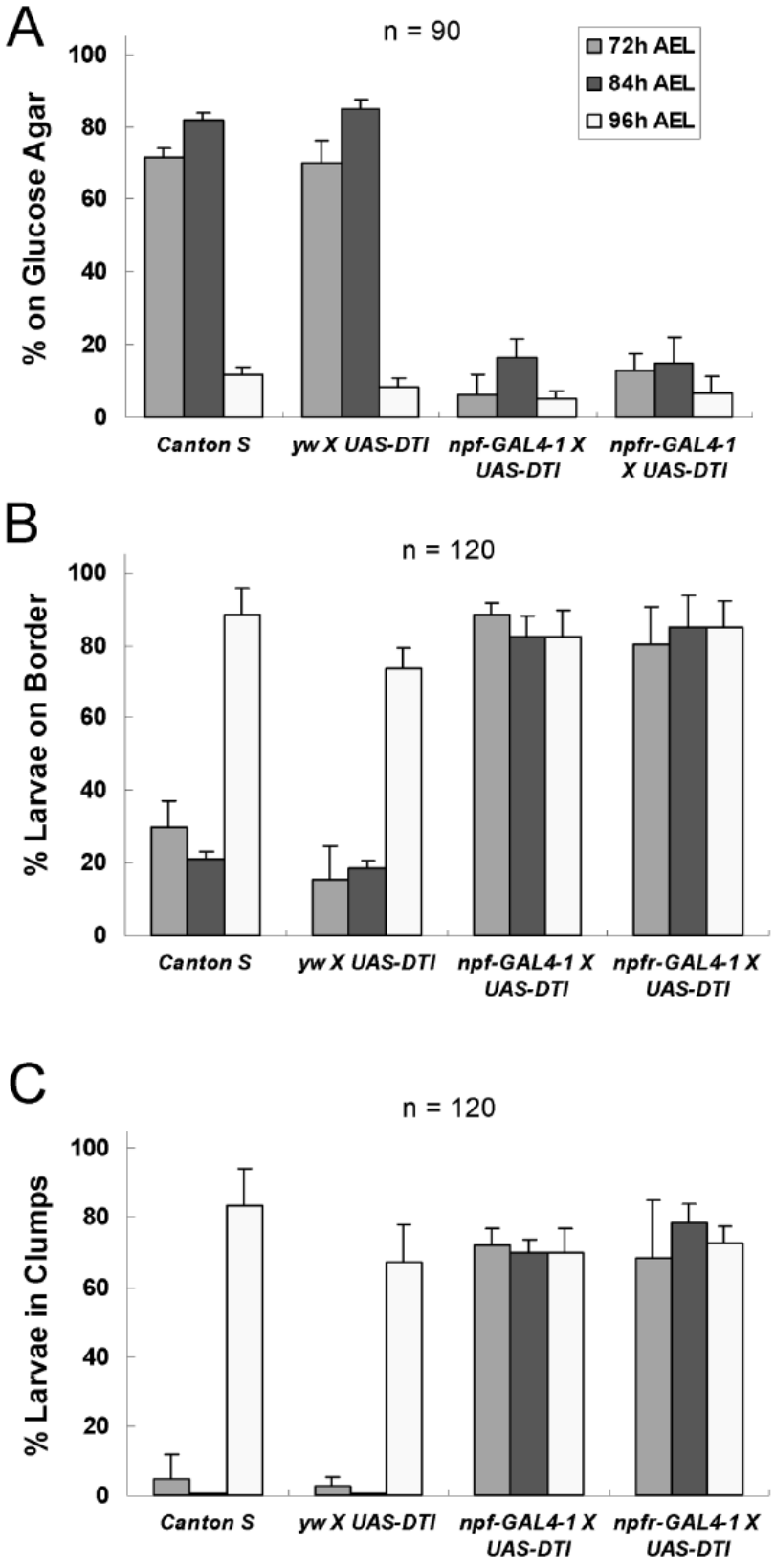
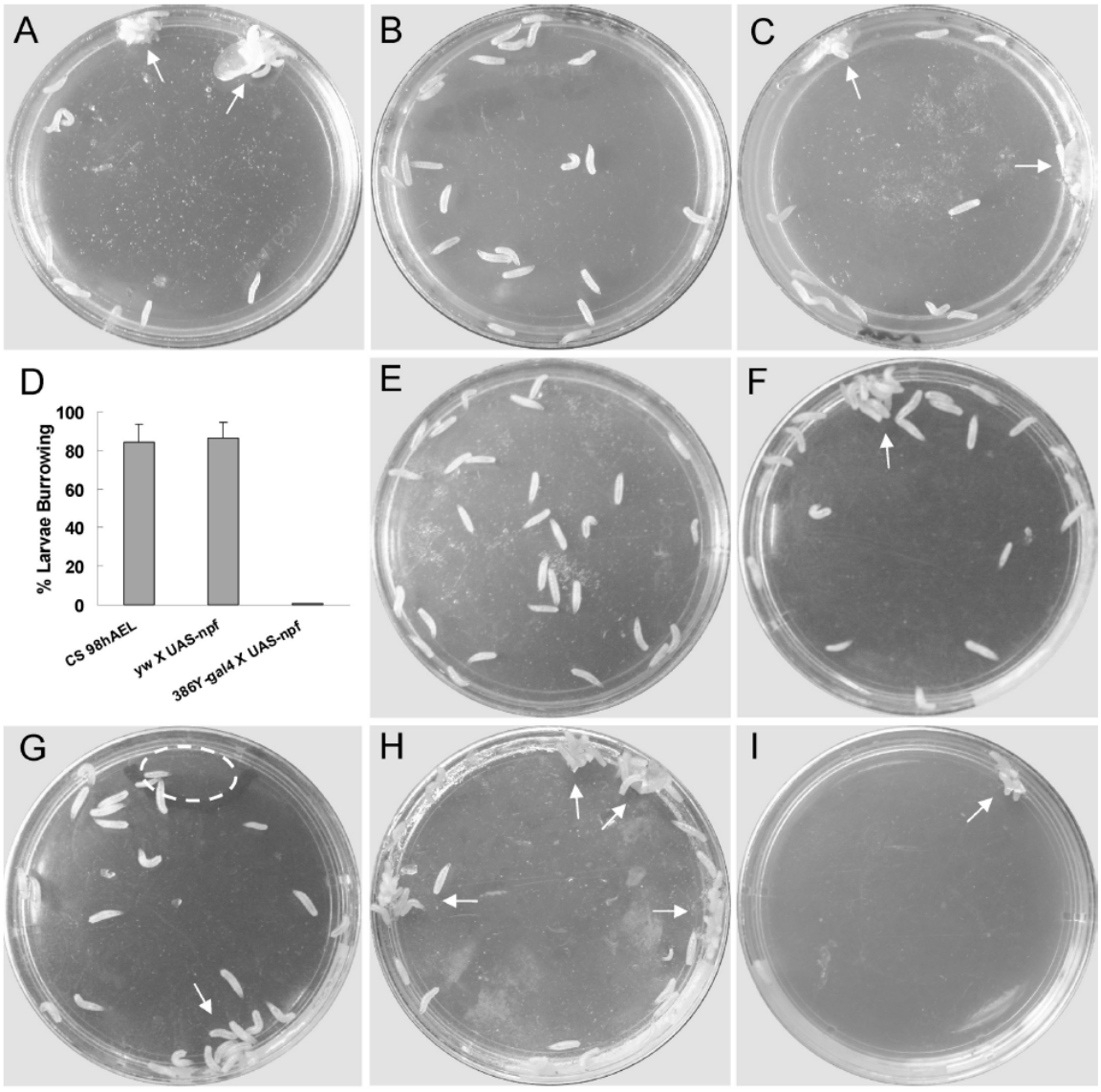

A
n = 90
72h AEL
84h AEL
96h AEL
% on Glucose Agar
100
80
60
40
20
0
Canton S
yw X UAS-DTI
npf-GAL4-1 X UAS-DTI
npfr-GAL4-1 X UAS-DTI
B
n = 120
% Larvae on Border
100
80
60
40
20
0
Canton S
yw X UAS-DTI
npf-GAL4-1 X UAS-DTI
npfr-GAL4-1 X UAS-DTI
C
n = 120
% Larvae in Clumps
100
80
60
40
20
0
Canton S
yw X UAS-DTI
npf-GAL4-1 X UAS-DTI
npfr-GAL4-1 X UAS-DTI

Figure 2.7 dNPF suppresses social burrowing

Non-feeding Canton S larvae (98 h AEL) displayed social burrowing behavior. Thirty larvae were transferred to the center of an apple juice-agar plate (35-mm in diameter). Larvae dispersed initially, subsequently swarmed toward each other, and burrowed into clumps under lifted agar pieces within 30 minutes. About 70-90% larvae participated in each test (**A**). The larvae (100hAEL) that overexpress dNPF (*386Y-gal4* X UAS-*npf*) remained solitary and showed no burrowing even after 1 h (**B**), while the control (*y w X* UAS-*npf*) displayed social burrowing similar to the wild type larvae (**C**). The arrows indicate the sites where agar was cut by larvae within 20 minutes. Five separate tests were performed for each group of larvae, and in all cases, the wild type and control larvae were able to burrow whereas *386Y-gal4* XUAS-*npf* larvae failed to do so. The quantification of the larval social burrowing activities is shown in panel **D**. In all five trials, no clumping or burrowing activity was detected among the *386Y-gal4* X UAS-*npf* solitary larvae, while about 80% of the wild type or control larvae displayed social burrowing activity in every test. To further investigate the mechanism underlying initial larva aggregation, the behaviors of *386Y-gal4* XUAS-*npf* and *386Y-gal4* X *y w* larvae within the first 10 minutes were compared. The *386Y-gal4* X UAS-*npf* larvae (100h AEL) remained solitary (**E**), whereas *386Y-gal4* X *y w* control larvae swarmed towards one another, displaying social burrowing (**F**). The social larvae did not show any preference to an area (circled) that was just occupied by a clump of 15-20 larvae for about 10 min; the larval aggregate was quickly removed at the onset of larval display of the drilling motion (**G**). The efficiency of social burrowing is dependent on the population density (**H**-**J**). For example, Canton S larvae (98 AEL) at the density of three per plate showed no social burrowing during a 90-min assay, indicated as $t > 90$.


A
B
C
D
% Larvae Burrowing
100
80
60
40
20
0
CS 98hAEL
yw X UAS-npf
386Y-gal4 X UAS-npf
E
F
G
H
I

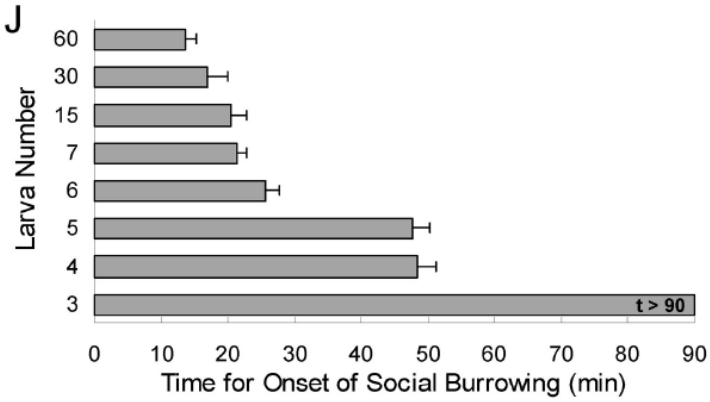

J
Larva Number
60
30
15
7
6
5
4
3
t > 90
0
10
20
30
40
50
60
70
80
90
Time for Onset of Social Burrowing (min)

Figure 2.8 Analysis of cooperative interaction between social and solitary larvae
A) Newly emerged non-feeding larvae from crosses *386Y-gal4* X *y w* and *386Y-gal4* X UAS-*npf* were used for the assay. Solitary larvae showed no social burrowing during the 90-min assay, indicated as $t > 90$. Social larvae were fed with yeast paste containing red food color before use. They displayed cooperative burrowing into apple juice-agar. T_{min} is the minimal time required for the onset of social burrowing by larvae. **B-D**) The same groups of larvae (n =40 per plate) were assayed separately or in mix for bordering and clumping. Arrows indicate social larvae in clumps. **E-F**) Quantification of the results from the bordering and clumping assays.

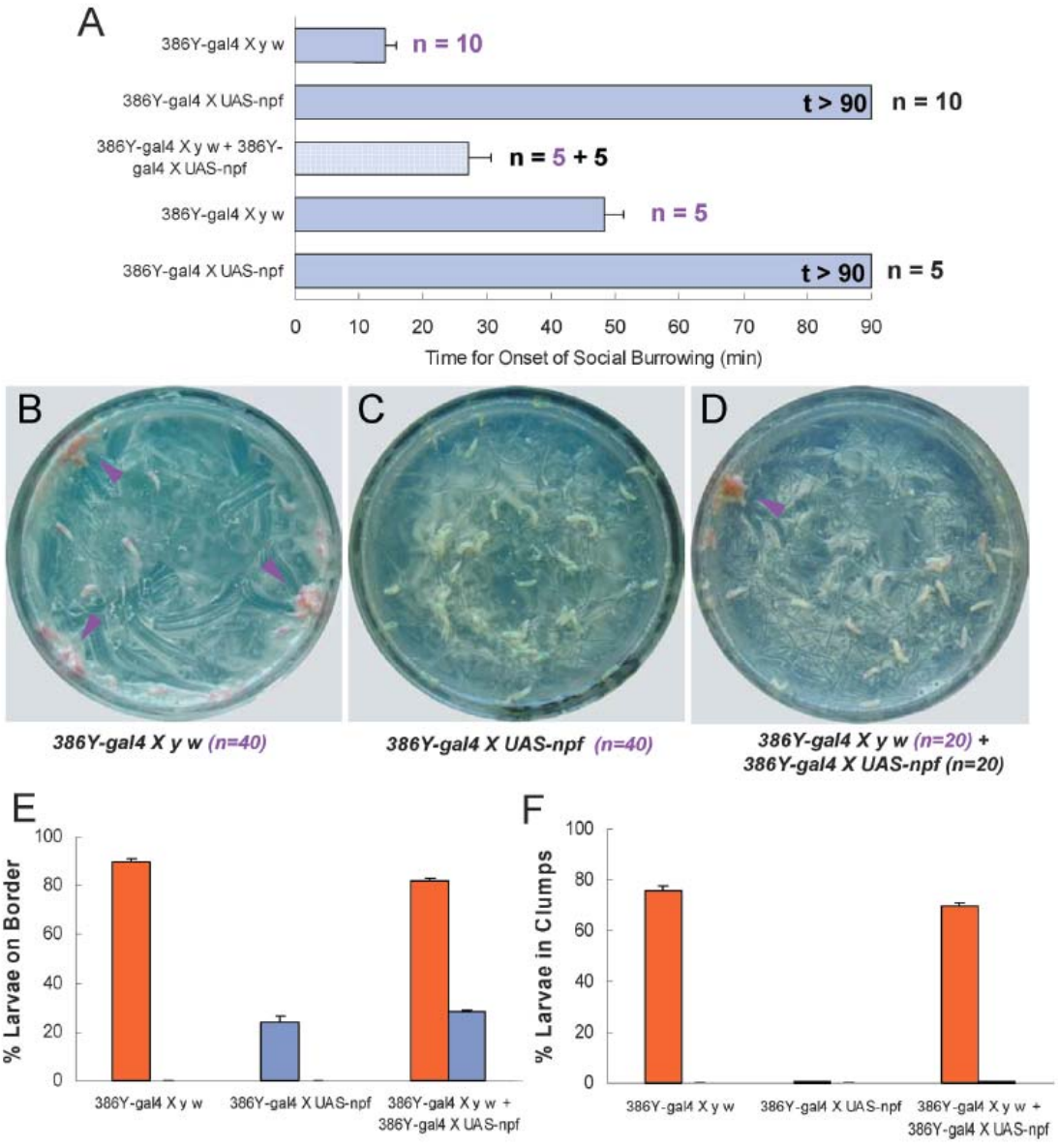

A
386Y-gal4 X y w
n = 10
386Y-gal4 X UAS-npf
t > 90
n = 10
386Y-gal4 X y w + 386Y-gal4 X UAS-npf
n = 5 + 5
386Y-gal4 X y w
n = 5
386Y-gal4 X UAS-npf
t > 90
n = 5
0 10 20 30 40 50 60 70 80 90
Time for Onset of Social Burrowing (min)
B
C
D
386Y-gal4 X y w (n=40)
386Y-gal4 X UAS-npf (n=40)
386Y-gal4 X y w (n=20) +
386Y-gal4 X UAS-npf (n=20)
E
% Larvae on Border
100
80
60
40
20
0
386Y-gal4 X y w
386Y-gal4 X UAS-npf
386Y-gal4 X y w +
386Y-gal4 X UAS-npf
F
% Larvae in Clumps
100
80
60
40
20
0
386Y-gal4 X y w
386Y-gal4 X UAS-npf
386Y-gal4 X y w +
386Y-gal4 X UAS-npf

Figure 2.9 Developmental analysis of foraging and social behavior in older third instar larvae overexpressing dNPF

The feeding activity of third instar larvae overexpressing dNPF was analyzed starting from 92 hours AEL. Blue yeast paste containing bromophenol blue was used to monitor food intake. **A-C**) 70% of the control larvae (w^{1118} and *386Y-gal4* X *H1-lacZ*) showed no blue yeast in the gut at the 98 hour AEL; the other 30% showed relatively faint blue color in the gut, indicating a low level of food intake. About 40% of the control larvae had turned into puparia at 110 hour AEL, and about 90% reached puparia or pupae at 120 hour AEL. In contrast, 85% of the dNPF-overexpressing larvae (*386Y-gal4* X UAS-*npf*) showed strong blue colors in the gut at 98 hour AEL, and the feeding continued at least until the 110 hour AEL. These *386Y-gal4* X UAS-*npf* larvae showed no intake of blue yeast paste at the 120 hour AEL, and about 90% reached puparia or pupae at 132 hour AEL. A small number of larvae in each group died probably due to injuries during the experiment. The data is compiled from separate experiments. Each data point is derived from at least two independent measurements ($n > 30$). **D**) The dNPF-overexpressing and control larvae were fed with yeast paste on apple juice-agar until in liquid food (10%-glucose agar paste), reaching early third instars (74 hour AEL), and subsequently transferred onto water-agar plates. In the absence of food, both dNPF-overexpressing and control larvae developed in a similar manner. **E**) Non-feeding *386Y-gal4* X UAS-*npf* larvae (120h AEL) remained solitary, and showed no burrowing activity on apple juice-agar. Three separate trials were performed ($n = 90$) **F**) Forty *386Y-gal4* X UAS-*npf* or *y w* X UAS-*npf* control larvae were placed onto a 10% glucose-agar plate coated with a thin layer of yeast paste with 10% glucose. About 80% of the *386Y-gal4* X UAS-*npf* pupae were on the surface of food-containing agar (mostly in the central zone); **G**) In contrast, about 20% of the *386Y-gal4* X *y w* control pupae remained on the agar surface (mostly at the edge of the agar). For the rest of the pupae, they were either on the plastic wall or lid (not shown in the picture). h) quantification of larval behavioral differences in pupariation is shown for *386Y-gal4* X UAS-*npf* and *386Y-gal4* X *y w* larvae, which are isogenic. At least three separate trials were performed ($n > 120$). ANOVA, $p < 0.0001$. These results suggest that dNPF overexpression suppresses aversive response to food by wandering larvae.

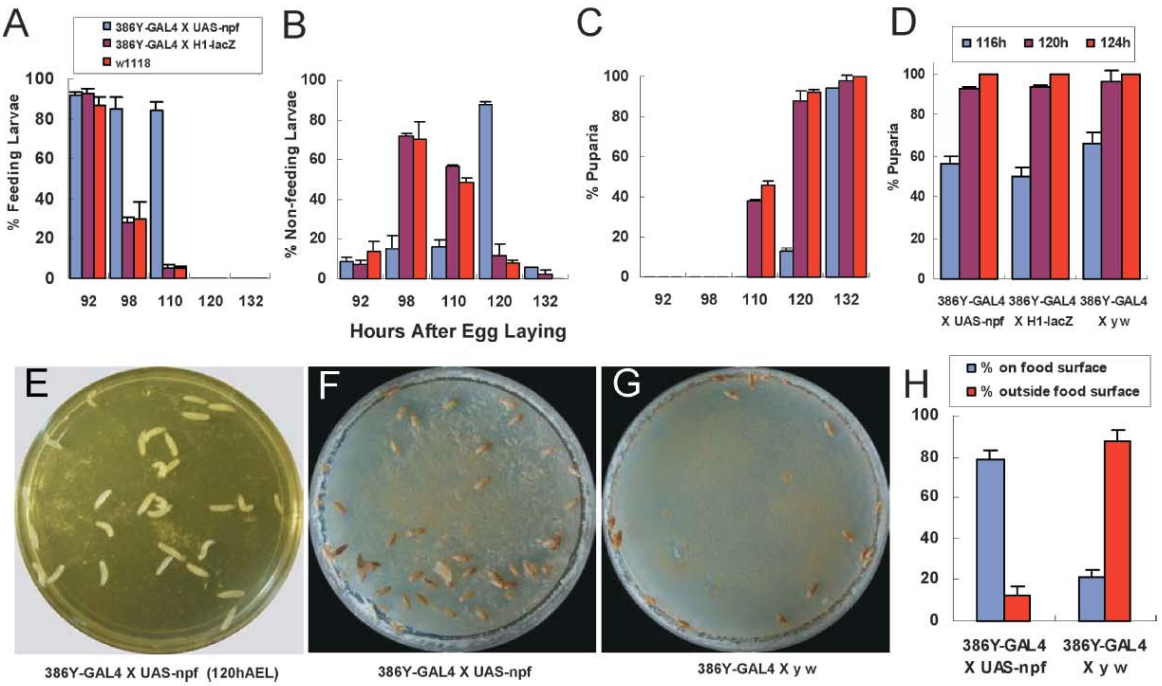

A
386Y-GAL4 X UAS-npf
386Y-GAL4 X H1-lacZ
w1118
% Feeding Larvae
B
% Non-feeding Larvae
Hours After Egg Laying
C
% Puparia
D
116h
120h
124h
% Puparia
386Y-GAL4 X UAS-npf
386Y-GAL4 X H1-lacZ
386Y-GAL4 X y w
E
386Y-GAL4 X UAS-npf (120hAEL)
F
386Y-GAL4 X UAS-npf
G
386Y-GAL4 X y w
H
% on food surface
% outside food surface
386Y-GAL4 X UAS-npf
386Y-GAL4 X y w

Figure 2.10 Quantification of the in situ RNA analysis of *npf* expression in the larval CNS
Three groups of wild-type Canton S larvae are included here: 72hAEL larvae, 72hAEL larvae starved for 24 hours and 96h AEL larvae (also see Figure 2.1 and the associated text). All well-fed 72hAEL larvae showed strong staining in a four-cell pattern. All 72hAEL larvae after starvation showed the similar expression pattern and staining intensity (ANOVA, $P = 0.21$). About 60% 96hAEL larvae shown no npf expression, and 7% of the group showed weak staining in the four NPF neurons (3- to 4-fold reduction in staining intensity).

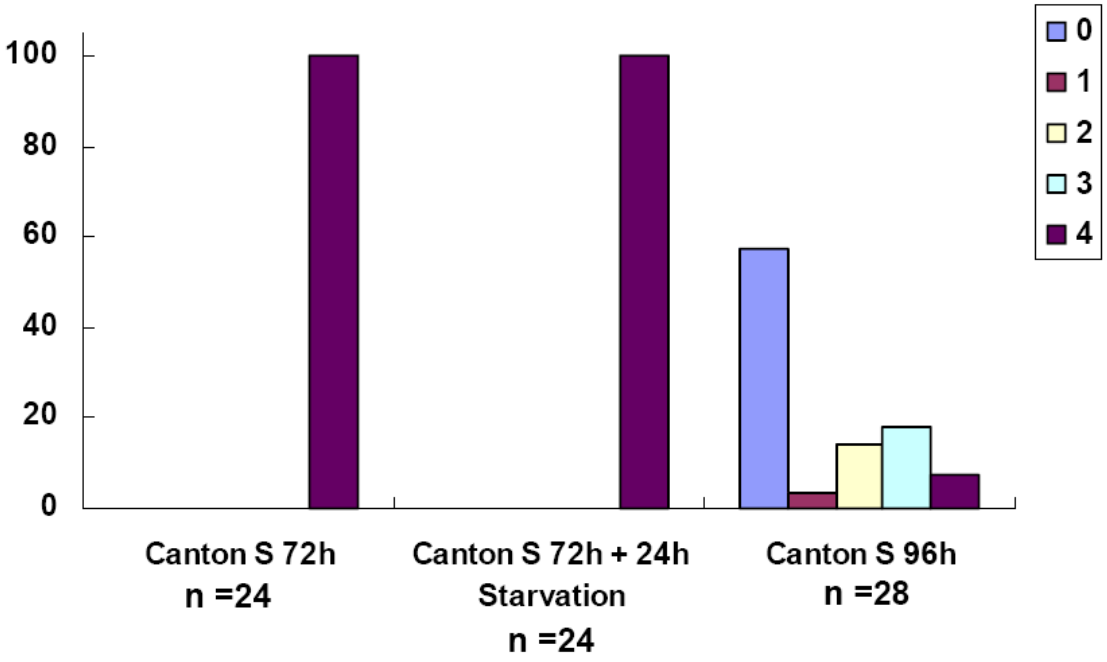
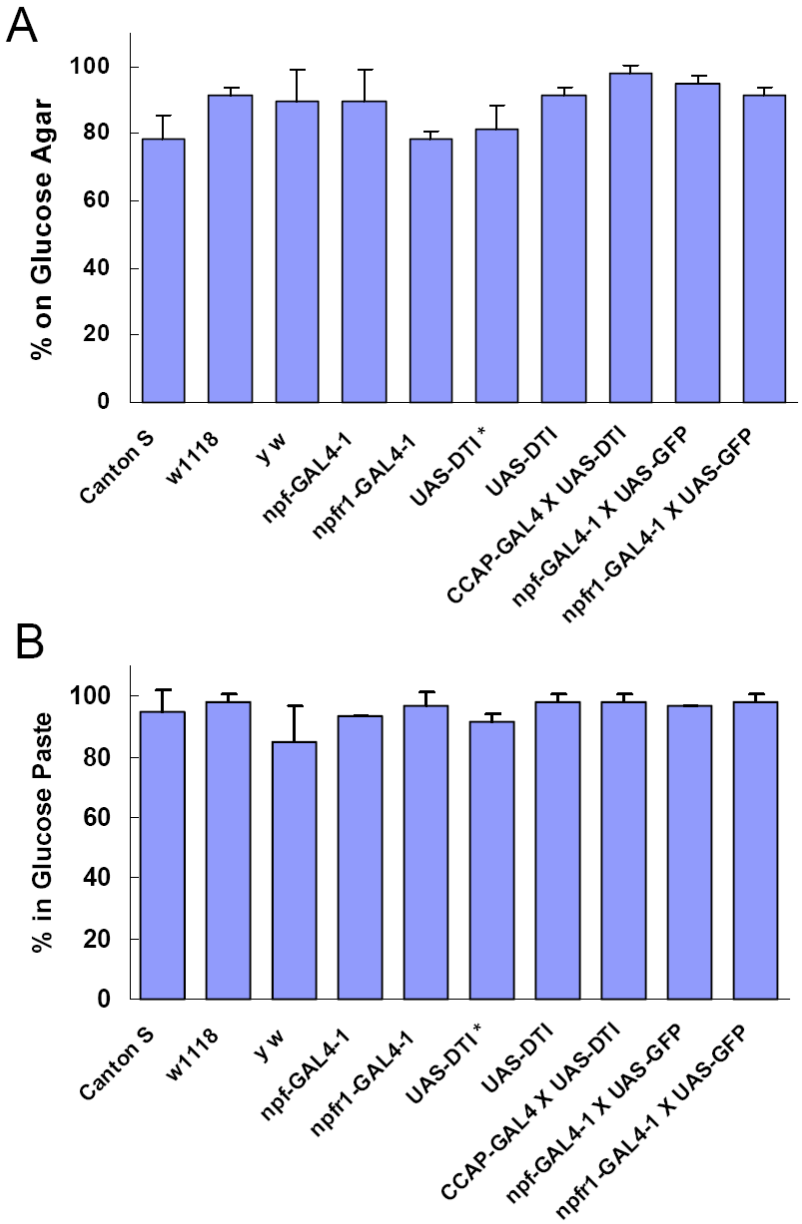
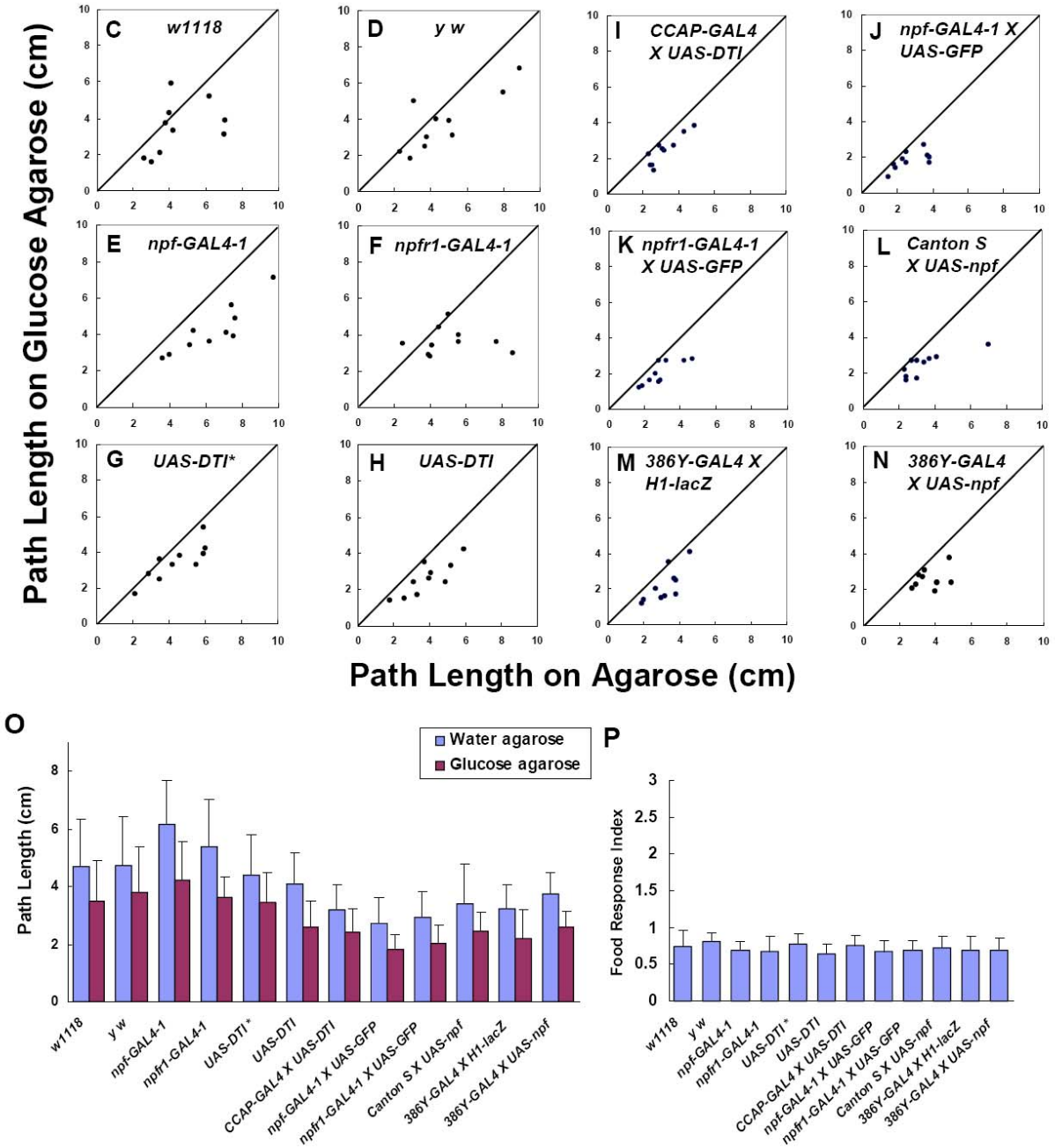
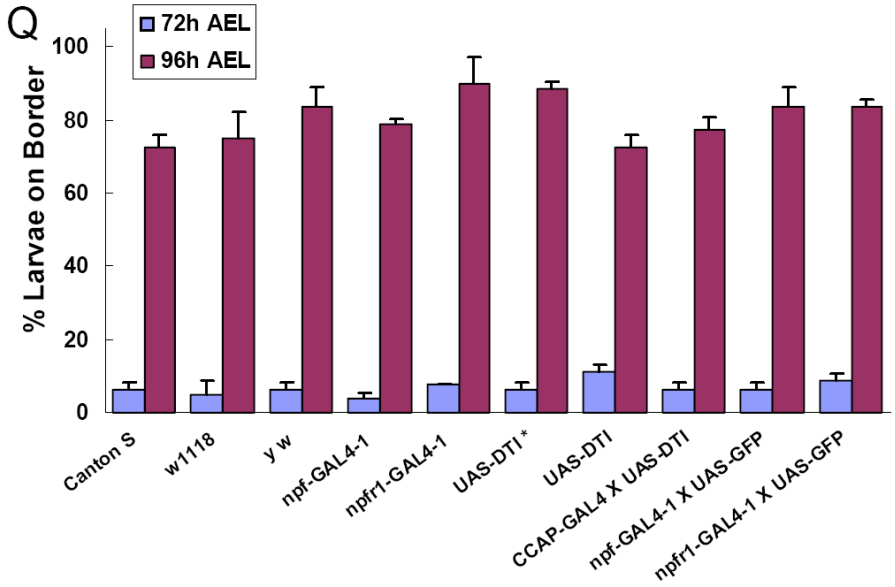

100
80
60
40
20
0
0
1
2
3
4
Canton S 72h
n =24
Canton S 72h + 24h
Starvation
n =24
Canton S 96h
n =28

Figure 2.11 Food Response and Bordering and Clumping Behaviors of Control Larvae
Larval responses to glucose agar (solid food) and glucose-agarose paste (liquid food) are shown in (A) and (B), respectively. All control larvae showed normal response to both solid and liquid food similar to wild-type Canton S larvae. At least 80% larvae remained in the food. n = 60 for each group. *UAS-DTI* * indicates larvae hemizygous for the UAS-DTI transgene (balanced over TM6). (C–N) Synchronized third instars larvae (72hAEL) were used for the assay. Each dot represents the measurement of the path length of an individual larva crawling first on food-free and then on glucose-containing semisolid agarose surface in a 6 min period (n = 10). For larvae expressing or overexpressing dNPF, most individuals showed shorter path length s on glucose-agarose (L_{glc}) surface than th ose on agarose surface (L_{agr}; [O and P]). Food Response Index = L_{glc}/L_{agr}. The assay conditions for bordering and clumping are the same as described for Figure 2.5. (Q) 72hAEL larvae; (R) 96hAEL larvae. All the controls showed bordering and clumping phenotypes similar to the wild-type Canton S larvae. The results indicate that the behavioral phenotypes under this study are insensitive to variations in the genetic background found in commonly used lab strains. n = 120.


A
% on Glucose Agar
100
80
60
40
20
0
Canton S
w1118
y w
npf-GAL4-1
npfr1-GAL4-1
UAS-DTI*
UAS-DTI
CCAP-GAL4 X UAS-DTI
npf-GAL4-1 X UAS-GFP
npfr1-GAL4-1 X UAS-GFP
B
% in Glucose Paste
100
80
60
40
20
0
Canton S
w1118
y w
npf-GAL4-1
npfr1-GAL4-1
UAS-DTI*
UAS-DTI
CCAP-GAL4 X UAS-DTI
npf-GAL4-1 X UAS-GFP
npfr1-GAL4-1 X UAS-GFP


C w1118
D y w
I CCAP-GAL4 X UAS-DTI
J npf-GAL4-1 X UAS-GFP
E npf-GAL4-1
F npfr1-GAL4-1
K npfr1-GAL4-1 X UAS-GFP
L Canton S X UAS-npf
G UAS-DTI*
H UAS-DTI
M 386Y-GAL4 X H1-lacZ
N 386Y-GAL4 X UAS-npf
Path Length on Glucose Agarose (cm)
Path Length on Agarose (cm)
O
Water agarose
Glucose agarose
Path Length (cm)
w1118
y w
npf-GAL4-1
npfr1-GAL4-1
UAS-DTI*
UAS-DTI
CCAP-GAL4 X UAS-DTI
npf-GAL4-1 X UAS-GFP
npfr1-GAL4-1 X UAS-GFP
Canton S X UAS-npf
386Y-GAL4 X H1-lacZ
386Y-GAL4 X UAS-npf
P
Food Response Index


Q
72h AEL
96h AEL
% Larvae on Border
100
80
60
40
20
0
Canton S
w1118
y w
npf-GAL4-1
npfr1-GAL4-1
UAS-DTI*
UAS-DTI
CCAP-GAL4 X UAS-DTI
npf-GAL4-1 X UAS-GFP
npfr1-GAL4-1 X UAS-GFP

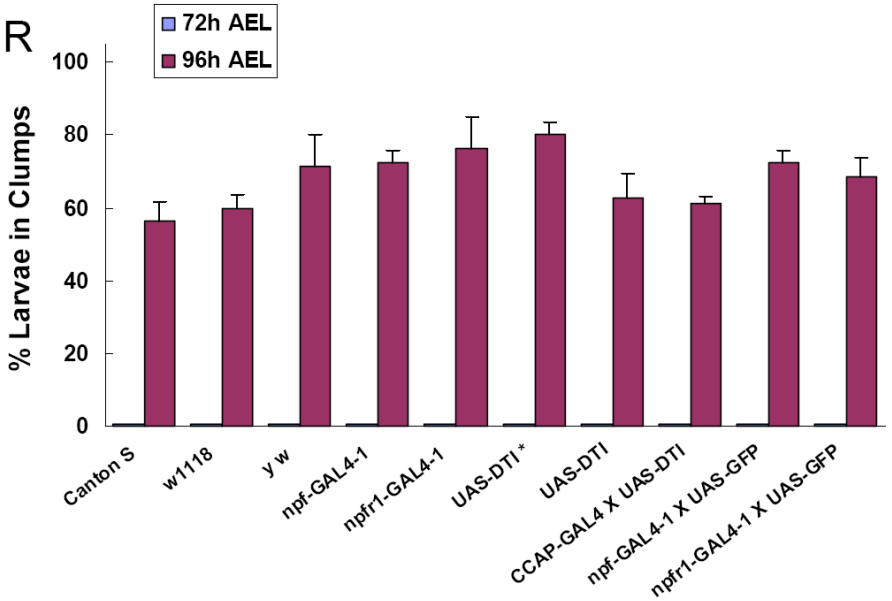
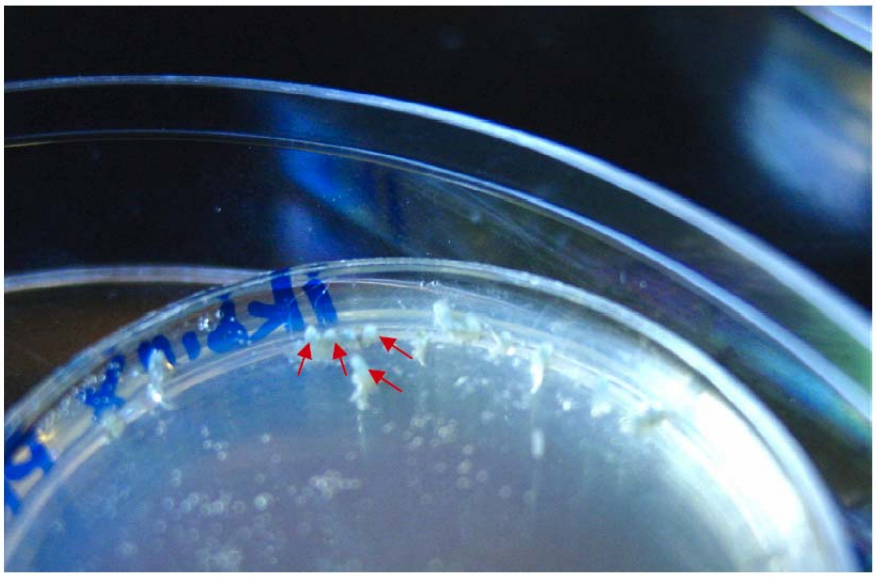

R
72h AEL
96h AEL
% Larvae in Clumps
100
80
60
40
20
0
Canton S
w1118
y w
npf-GAL4-1
npfr1-GAL4-1
UAS-DTI*
UAS-DTI
CCAP-GAL4 X UAS-DTI
npf-GAL4-1 X UAS-GFP
npfr1-GAL4-1 X UAS-GFP

Figure 2.12 Swarming of Social Larvae Induces a Unique Drilling Motion
Social larvae aggregated shortly after placed onto food agar (see Figures 2.7 and 2.8). Subsequently, some displayed the drilling motion (indicated by arrows) by swinging the body violently while stood on the head. S uch drilling motion leads to the lift of agar in the same area. The dNPF-overexpressing solitary larvae never displayed such drilling behavior.



CHAPTER 3

REGULATION OF HUNGER-DRIVEN BEHAVIORS BY NEURAL RIBOSOMAL S6 KINASE IN *DROSOPHILA*[1]

[1]Wu, Q., Zhang, Y., Xu, J. and Shen, P. 2005. ***Proc. Natl. Acad. Sci. U.S.A.*** 102: 13289 – 13294. Reprinted here with permission of the publisher.

ABSTRACT

Hunger elicits diverse yet coordinated adaptive responses across species, but the underlying signaling mechanism(s) remains poorly understood. Here we report that the function and mechanism of the *Drosophila* insulin-like system in the central regulation of different hunger-driven behaviors. We found that overexpression of *Drosophila* insulin-like peptides (DILP)s in the nervous system of fasted larvae suppressed the hunger-driven increase of ingestion rate and intake of non-preferred foods (e.g., a less-accessible solid food). Moreover, upregulation of dS6K activity in DILP neurons led to attenuated hunger response by fasted larvae, whereas its downregulation triggered fed larvae to display motivated foraging and feeding. Finally, we provide evidence that neural regulation of food preference but not ingestion rate may involve direct signaling by DILPs to neurons expressing NPFR1, a receptor for NPY-like neuropeptide F (NPF). Our study reveals a prominent role of neural dS6K in the modulation of hunger response by insulin- and NPY-like signaling pathways.

INTRODUCTION

Most animals including humans have evolved a highly optimized foraging strategy to cope with wide fluctuations in food availability. The state of food depletion exerts a systemic influence that initiates a broad array of adaptive behaviors. For example, deprived animals are generally more tolerant of stressful foraging conditions, and less inhibited in engaging in aggressive behaviors (Cabanac, 1985;Dethier, 1976). They also show increased appetite and a lower threshold of food acceptance to accommodate less palatable energy sources (Carr, 1996;Wong, 1995). In humans, feeding mechanisms evolved for protecting against starvation are now thought to be major causal factors for the widespread problem of obesity in affluent

societies (Berthoud, 2002). It remains poorly understood as to what and how signaling systems are responsible for integrating hunger stimuli and organizing diverse adaptive behaviors.

The prominent feeding control mechanisms in vertebrates have been mapped to the regions of the central nervous system, especially the hypothalamus also known as the "feeding center" (ANAND and BROBECK, 1951;Hillebrand et al., 2002). This was most strikingly demonstrated by the observation that electrical stimulation of the lateral parts of the hypothalamus was sufficient to cause self-sustained food intake (Hess, 1954). Pharmacological studies have implicated a variety of brain chemicals including bioactive peptides in promoting/inhibiting food intake. Several hypothalamic neuropeptides including agouti-related protein (AgRP) and neuropeptide Y (NPY) are thought to be potent feeding stimulants (Beck, 2001). On the other hand, peptides such as insulin, leptin and melanocortins suppress food intake (Porte, Jr. et al., 2002). However, the elucidation of the physiological roles of these molecules and their sites of action has been difficult largely because of the complexity of vertebrate models.

Drosophila genome has seven insulin-like genes, *dilp1-7*, and at least one insulin receptor-like gene (*dInR*) (Brogiolo et al., 2001;Oldham and Hafen, 2003;Rulifson et al., 2002). Insulin/insulin-like peptides and their receptors are widely distributed in the central nervous system (CNS), and they have been implicated in the control of body weight and reproduction, learning and memory, and axon pathfinding (Bruning et al., 2000;Gerozissis, 2003;Song et al., 2003). An NPY-like signaling pathway, comprising neuropeptide F (NPF) and its receptor NPFR1, has also been identified in *Drosophila* (Brown et al., 1999;Garczynski et al., 2002). We recently found that this NPY-like signaling pathway developmentally regulates larval feeding and social migratory behavior (Shen and Cai, 2001;Wu et al., 2003). Interestingly, both fly NPF

and mammalian NPY appear to promote feeding response in fasted animals but not those fed *ad libitum* (Bannon et al., 2000;Segal-Lieberman et al., 2003;Wu et al., 2003).

We have used the relatively simple *Drosophila* larva as a genetic model to elucidate molecular and neural mechanisms underlying hunger regulation. Our main objective is to define and characterize different neuronal signaling pathways that constitute a complete central feeding apparatus. In this report, we address two fundamental questions: what enables a deprived animal to take on motivated foraging and food acquisition, and what prevents a nondeprived animal from doing so. We show that dS6K is a key organizer of different hunger-driven behaviors in *Drosophila* larvae. Downregulation of the DILP-neuronal dS6K activity in fed larvae is sufficient to trigger hunger-driven foraging and feeding behaviors, whereas its upregulation suppresses the normal onset of such motivated behaviors in fasted animals. We also identified a neural pathway involving neurons expressing DILP, NPF or NPFR1 that differentially regulates food preference but not ingestion rate. Our study reveals a molecular signaling cascade that mediates the action of DILP-neuronal dS6K in overriding a forager's normal feeding response. The conserved S6K signaling pathway may be crucial for regulating hunger-driven behaviors in diverse animals.

METHODS

Flies and Media

Fly rearing and the collection of eggs and larvae were described previously (Wu et al., 2003). The UAS-$npfr1^{dsRNA}$ flies are in a w^{1118} background. The UAS-shi^{ts1} , UAS-*dilp* flies and UAS-*ANF-GFP* (also named UAS-*preproANF-EMD* containing a GFP-tagged atrial natriuretic factor fusion) were kindly provided by T. Kitamoto, E. Hafen and D. Deitcher, respectively (Ikeya et

al., 2002;Kitamoto, 2002a;Rao et al., 2001). The UAS-*ANF-GFP*, UAS-*npfr1*, UAS-*dilp2*, UAS-*dilp3*, UAS-*dilp4* lines are in a *y w* background. The *elav-gal4*, *appl-gal4*, *MHC82-gal4*, UAS-shi^{ts1}, UAS-$S6K^{DN}$, UAS-$S6K^{ACT}$, UAS-$dInR^{DN}$, UAS-$dInR^{ACT}$, UAS-*dp110*, UAS-$dPI3K^{DN}$ and UAS-*dPTEN* are in a w^{1118} background (Barcelo and Stewart, 2002;Davis et al., 1998;Gao et al., 2000;Leevers et al., 1996;Lin and Goodman, 1994;Parks, 2004;Torroja et al., 1999). The UAS-$S6K^{DN}$ construct encodes a dominant negative form of S6K with the substitution of a lysine (K_{109}) with glutamine (Q). The UAS-$S6K^{ACT}$ encodes a constitutively active form of S6K with two substitutions: S_{418} to D and T_{422} to E (26). Other fly lines were described before (Wen et al., 2005;Wu et al., 2003).

Molecular cloning and analysis

The cloning of *npfr1* cDNA was described previously (Garczynski et al., 2002), and its coding sequence was subcloned into the downstream of the UAS promoter in the pUAST vector. For making the *dilp2-gal4* construct, a 2-kb fragment of the *dilp2* promoter fragment was obtained through genomic PCR using a pair of forward and reverse primers (GGCCATGGCGATGGCGATGA and GAGATCTTTACGATCAA ATGGATTA), and was cloned in front of the *gal4* coding sequence. More detailed information is available upon request.

Food Response Assays

The quantification of intake rate, as indicated by the frequency of mouth hook contractions, of solid and liquid foods was performed as described before(Wu et al., 2003). Briefly, for the liquid food assay, larvae were transferred to a 35mm petri-dish containing 2g of glucose/agar paste, prepared by mixing 45ml 10% glucose solution with 5.5g agar powder (ABI, Niagara,

New York). For the solid food assay, each 35mm petri-dish contains 2.5ml of melted glucose agar (10g glucose and 2.3g of agar per 100 ml). Solidified agar was cut into four quarters, and air dried for 1 day. On the next day, multiple small cuts were made on the vertical surfaces of the agar pieces before adding larvae. Larvae displayed virtually identical feeding responses to 10% glucose, apple juice or 10% glucose/yeast containing liquid and solid foods under deprived and non-deprived conditions (Figure 3.6).

For those assays that involve *shi*^{ts1}-expressing larvae, the permissive and restrictive temperatures were set at 23 and 30^{o}C, respectively. Larvae were reared and held at 23 ^{o}C. To measure the intake of solid and liquid foods, larvae were held in 30 ^{o}C water for 15 min, transferred to glucose-agar block or paste and quantified for the frequency of mouth hook contraction by individual larva at 30 ^{o}C. Typically, the assay time is about 20 min. The contraction frequencies of individual larva remained consistent throughout the assay. To measure the recovery of food response of larvae expressing *shi*^{ts1}, larvae were returned from 30 ^{o}C to 23 ^{o}C, and measured for food response at 25 and 50 min afterwards. At least three separate trials were performed for each assay. Statistical analyses were performed using one-way ANOVA.

Analysis of larva growth and development

For each control and experimental group, eggs collected within a 2-hour interval were left on an apple juice-agar plate with yeast paste. The total numbers of hatched larvae were counted. The larvae were fed continuously on the same plate, and monitored for growth. Fresh yeast paste was supplemented for third instars when necessary to ensure that they were well nourished. At

the 120 hr AEL, the number of puparia in and around the plate was recorded. The results of the analyses are presented in Table 3.1 and Figure 3.7.

RESULTS

Quantitative assessment of larval behavioral responses to prolonged deprivation

The relatively simple *Drosophila* larva offers a genetically tractable model to define and characterize different neuronal signaling pathways that constitute a complete central feeding apparatus. Younger third instar larvae forage actively, and use their mouth hooks for food intake. Larvae normally feed on liquid food, and their food ingestion can be quantified by measuring the contraction rate of the mouth hooks (Wu et al., 2003). We examined how food deprivation affects larval feeding response to a liquid (e.g., 10% glucose-agar paste) and less-accessible solid food (e.g., 10% glucose agar blocks). To extract embedded glucose from the solid food, larvae have to pulverize the food by scraping agar surface with mouth hooks. Unless stated otherwise, synchronized third instar larvae (74 hr *a*fter *e*gg *l*aying, 74 hr AEL) were used for the assays.

When fed *ad libitum*, normal larvae (w^{1118}) displayed significant feeding activity in the liquid food with an average mouth-hook contraction frequency of about 30 times in a 30 s test period; in contrast, these larvae declined the solid food (Figure 3.1A). However, larvae withheld from food (on a wet tissue) for 40 or 120 min displayed increased intake of both liquid and solid foods. For example, larvae fasted for 120 min showed a 100% and > 500% increase in mouth-hook contraction rate in liquid and solid food, respectively. Thus, deprivation not only enhances feeding rate in a graded fashion, but also triggers motivated foraging on the less-accessible food normally rejected by fed larvae. In addition, larvae display virtually identical feeding responses to liquid and solid foods containing 10% glucose, apple juice or 10% glucose/yeast under

deprived and non-deprived conditions (Figure 3.6). Therefore, these paradigms appear to provide a general assessment of larval feeding response.

DILP-neuronal dS6K mediates hunger regulation of food response

dS6K is a cell-autonomous effector of nutrient-sensing pathways (Thomas, 2002). We investigated a possible role of neural dS6K in coupling peripheral physiological hunger signals and neuronal activities critical for hunger-driven behaviors. The transcripts of *dilp1, 2, 3* and *5* are predominantly expressed in two small clusters of medial neurosecretory cells that project to the ring gland, the fly heart and the brain lobes (Brogiolo et al., 2001;Rulifson et al., 2002;Wu et al., 2003). We generated a *gal4* driver containing a 2-kb fragment from the *dilp2* promoter (*dilp2-gal4*) that directs the specific expression of a GFP reporter in those cells (Figure 3.1B and Figure 3.8). Using *dilp2-gal4*, we expressed two transgenes, UAS-$dS6K^{DN}$, encoding a dominant-negative, and UAS-$dS6K^{ACT}$, a constitutively active form of dS6K (Barcelo and Stewart, 2002). When fed *ad libitum*, control larvae (w X UAS-$dS6K^{DN}$ or UAS-$dS6K^{ACT}$) behaved like *w* larvae (Figure 3.1C). However, *dilp2-gal4* X UAS-$dS6K^{DN}$ larvae displayed a 50% increase in the rate of liquid-food intake, and significant feeding of the solid food. Conversely, fasted larvae overexpressing dS6K activity *(dilp2-gal4* X UAS-$dS6K^{ACT}$) showed attenuated feeding response to both liquid and solid foods (Figure 3.1D). These findings reveal that dS6K in DILP neurons mediates hunger regulation of approaching/consumptive behaviors, controlling both quality and quantity of food for ingestion. We also measured the body size as well as the developmental rate of all four groups of larvae, and no significant differences were detected (Figure 3.7 and Table 3.1).

Overexpression of DILPs suppresses hunger-driven behaviors

DILPs act as neurohormones in *Drosophila* larvae (Rulifson et al., 2002). Downregulation of dS6K activity in DILP neurons may reduce DILP release, thereby promoting increased food intake that is normally triggered only by hunger. A corollary of this interpretation is that overproduction of DILPs in the nervous system should interfere with hunger response by deprived animals. To test this, a neural-specific *elav-gal4* driver was used to direct *dilp* expression in the larval nervous system. Three UAS-*dilp* lines (UAS-*dilp2,3* and *4*) were chosen for the analysis (Ikeya et al., 2002). The *elav-gal4* X UAS-*dilp2* and UAS-*dilp4* larvae displayed normal feeding response when fed *ad libitum* (data not shown). However, the same larvae fasted for 120 min displayed significantly attenuated feeding rates, similar to those of *dilp2-gal4* X UAS-$dS6K^{ACT}$ larvae (Figure 3.2). For example, the comparative analysis of the *elav-gal4* X UAS-*GFP* control and *elav-gal4* X UAS-*dilp2* and UAS-*dilp4* experimental larvae showed that the latter were about 30% and 33-45% lower in the ingestion rate of the liquid and solid food, respectively; surprisingly, *elav-gal4* X UAS-*dilp3* and UAS-*GFP* larvae showed virtually identical feeding responses. Therefore, DILP2 and 4 negatively regulate hunger-driven feeding activities. Taken together, our results suggest that a high level of dS6K activity in DILP neurons may suppress hunger response by reducing DILP release.

dInR and dS6K in NPFR1 cells differentially regulate food preference

We sought to delineate the signaling mechanism that couples the dS6K activity in DILP neurons with its broad impact on hunger-driven feeding activities. Our previous study showed that fasted larvae ablated of neuropeptide F (NPF) or its receptor (NPFR1) neurons are deficient in motivated feeding of the less-preferred solid food but normal in feeding of richer liquid food

(Brown et al., 1999;Garczynski et al., 2002;Shen and Cai, 2001;Wu et al., 2003). We wondered whether the NPF/NPFR1 neuronal pathway might be one of the downstream effectors of the DILP pathway. To test this, we analyzed the function of three components of the dInR signaling pathway in NPFR1 neurons: insulin-like receptor (dInR), phosphatidylinositol 3,4,5-trisphosphate 3-phosphatase (dPTEN) and phosphatidylinositol 3-kinase (dPI3K) (ref. Oldham and Hafen, 2003;Thomas, 2002). Five different transgenes were used: UAS-$dInR^{ACT}$ and UAS-$dInR^{DN}$ encode a constitutively active and a dominant-negative form of dInR, respectively (Parks, 2004); UAS-*Dp110* and UAS-$dPI3K^{DN}$ encode a catalytic subunit and a dominant-negative form of dPI3K, respectively (Leevers et al., 1996); UAS-*dPTEN* encodes a functional enzyme (Gao et al., 2000). When fed *ad libitum*, *npfr1-gal4* X UAS-$dInR^{DN}$, UAS-*dPTEN* or UAS-$dPI3K^{DN}$ larvae displayed hyperactive feeding of the solid food, similar to *w* larvae deprived for 40 min (Figure 3.3A). In contrast, fasted larvae overexpressing dInR or dPI3K *(npfr1-gal4* X UAS-$dInR^{ACT}$ or UAS-*Dp110*) displayed attenuated feeding response to the solid food (Figure 3.3B). Importantly, larvae with up- or down-regulated dInR signaling in NPFR1 neurons did not exhibit significant changes in the intake rate of the richer liquid food relative to the paired controls. Taken together, these findings suggest that the dInR pathway negatively regulates the activity of NPFR1 neuron, and mediates the DILP-regulated change in food preference but not ingestion rate. Furthermore, the results suggest that NPFR1 neurons are the direct targets of DILPs.

We also evaluated a possible role of dS6K in hunger regulation of the functioning of NPFR1 neurons, by expressing UAS-$dS6K^{DN}$ and UAS-$dS6K^{ACT}$using *npfr1-gal4* (Wu et al., 2003). When fed *ad libitum*, *npfr1-gal4* X UAS-$dS6K^{DN}$ larvae displayed hyperactive feeding of the solid food, similar to *npfr1-gal4* X UAS-$dInR^{DN}$ larvae (Figure 3.3C). However, these larvae, unlike *dilp2-gal4* X UAS-$dS6K^{DN}$ animals, displayed no increases in the ingestion rate of the

richer liquid food. Conversely, fasted larvae overexpressing dS6K (*npfr1-gal4* X UAS-$dS6K^{ACT}$) displayed attenuated feeding response to the solid food (Figure 3.3D). These findings suggest that dS6K also negatively regulates the activity of NPFR1 neurons in food preference, but does not mediate the regulation of feeding rate by DILP signaling.

Overexpression of NPFR1 promotes hunger-adaptive change in food preference

We also examined the food response of the solid and liquid food by larvae overexpressing an *npfr1* cDNA under the control of an *npfr1-gal4* driver. In the presence of the liquid food, both experimental (*npfr1-gal4* X UAS-*npfr1*) and control larvae (e.g., *npfr1-gal4* X UAS-*ANF-GFP*), fed or fasted, showed similar intake rates as well as comparable increases in feeding response to hunger (Figure 3.4A-C, $p > 0.25$). However, when forced to feed on the solid food, fed experimental larvae exhibited significant intake of the solid food (30 times per 30 s, $p < 0.001$), while fed controls rejected the same food. Thus, NPFR1 overexpression selectively promotes change in food preference without increasing ingestion rate. We also observed that the feeding responses of NPFR1 overexpressing larvae and controls fasted for 120 min were indistinguishable (Figure 3.4C). Thus, the effect of NPFR1 overexpression on food preference is only detectable in fed or mildly-fasted larvae, suggesting hunger-activated NPFR1 signaling approaches to a plateau in severely fasted animals.

Neural NPFR1 is responsible for hunger-regulated food selection

We also selectively knocked down *npfr1* activity by expressing *npfr1* double-stranded RNA (dsRNA) in the nervous system. The UAS-$npfr1^{dsRNA}$ lines were previously used to functionally disrupt *npfr1* activity (Wen et al., 2005). We found that 120-min fasted larvae expressing *npfr1*

dsRNA in NPFR1 or the nervous system (*npfr1-gal4*, *elav-gal4* or *appl-gal4* X UAS-*npfr1*^{dsRNA}) were deficient in motivated feeding of the solid but not liquid food (Figure 3.4D). In contrast, all control larvae including those expressing *npfr1*^{dsRNA} in muscle cells (*MHC82-gal4* X UAS-*npfr1*^{dsRNA}) showed normal feeding responses. These results indicate that neural NPFR1 mediates hunger regulation of food selection.

The NPF/NPFR1 pathway acutely regulates food selection

A potential problem of the previous transgenic studies is that NPF/NPFR1 signaling is likely to be disrupted in a relatively early stage of larval development. Conceivably, the NPF/NPFR1 neuronal pathway could be essential for *ad libitum* or hunger-driven feeding of richer liquid foods, but such an activity might be masked by some yet unidentified compensatory mechanism(s) triggered by its early loss. To test this, we attempted to disrupt NPF/NPFR1 neuronal signaling in a temporally controlled manner by expressing a temperature-sensitive allele of *shibire* (*shi*^{ts1}) driven by *npf- or npfr1-gal4*. The *shi*^{ts1} allele encodes a semidominant-negative form of dynamin that blocks neurotransmitter release at a restrictive temperature (> 29 ^{o}C) (ref. Kitamoto, 2002b). At the permissive temperature of 23 ^{o}C, 120-min fasted experimental larvae (*npf-gal4* and *npfr1-gal4* X UAS- *shi*^{ts1}) and paired controls (*y w* X UAS-*shi*^{ts1} and *npf-gal4* and *npfr1-gal4* X *w*^{1118}) displayed normal feeding responses to both liquid and solid foods (Figure 3.4E). However, if larvae were incubated at 30 ^{o}C for 15 min, controls still displayed normal feeding activities, while the experimental larvae showed attenuated feeding response to the solid but not liquid food (Figure 3.4F). Therefore, there was no detectable developmental or physiological compensation for the loss of NPF signaling in *Drosophila* larvae. These results also suggest that the NPF/NPFR1 neuronal pathway is acutely required to initiate

and maintain larval hunger response. We found that the foraging activity of the experimental larvae was completely restored when the assay temperature was reduced to 23 ^{o}C (Figure 3.9), suggesting that the NPF system can modulate the intensity and duration of feeding response.

DISCUSSION

We have shown that dS6K regulates different yet coordinated behaviors controlling quantitative and qualitative aspects of hunger-adaptive food response. We provide evidence that dS6K mediates hunger regulation of two opposing insulin- and NPY-like signaling activities, dynamically modifying larval food preference and feeding rate based on the nutritional state (Figure 3.5). For example, hunger stimuli may cause a reduction of dS6K activity in DILP neurons, resulting in the suppression of DILP signaling which negatively regulates a downstream NPF/NPFR1-dependent and another NPF-independent neuronal pathway. The DILP/NPFR1 neuronal pathway selectively mediates hunger-adaptive change in food preference, possibly by overriding the high threshold of food acceptance set by a separate default pathway, enabling hungry animals to be receptive to less-preferred foods. The NPF/NPFR1-independent pathway promotes a general increase in the ingestion rate of preferred/less-preferred foods, enabling animals to compete effectively for limited food sources. Our study also implicates the presence of a separate default pathway for mediating the selective intake of preferred foods (baseline feeding) in larvae fed *ad libitum*. This default pathway may be largely insensitive to DILP or NPF signaling, since overexpression of dS6K, DILPs or NPFR1 in nondeprived larvae did not affect *ad libitum* feeding in the liquid food (see Figures 3.1 – 3.3, and data not shown). We suggest that the conserved S6K pathway may be critical for regulating behavioral adaptation to

hunger in diverse organisms including humans, and its components are potential drug targets for appetite control.

The functional differences of DILP1-7 had not been reported previously. In this study, we show that *dilp2, 3* and *4* are functionally distinct. DILP2 and 3 are both produced in the same medial neurosecretory cells. However, unlike DILP2, DILP3 is apparently not involved in suppressing deprivation-motivated feeding. It is still unclear whether the differential activities of DILP2 and 3 reflect their structural divergence or are due to the presence of yet unidentified dInR isoforms. DILP4 is not expressed within the two medial clusters of DILP neurons (Brogiolo et al., 2001). Under acute deprivation, the level of *dilp4* transcripts showed a 5-fold reduction in the larval CNS (data not shown). Thus, it is possible that DILP4 may play a localized role in promoting feeding response inside the CNS.

Feeding is a reward-seeking behavior, and deprivation strengthens the reinforcing effect (reward value) of food (Saper et al., 2002). Our studies suggest a previously uncharacterized role of the DILP/dInR signaling pathway in regulating an animal's perception of food quality. The DILP/NPF neural network may regulate an animal's incentive to acquire lower-quality foods by modifying the reward circuit. This hypothesis is interesting in light of the findings that foods and abused substances may act on the same reward circuit, and highly palatable foods can reduce drug-seeking behaviors (Hajnal and Norgren, 2001;Levine et al., 2003;Martel and Fantino, 1996). It is also possible that the DILP/NPF system might represent a specialized neural circuit that positively alters the reward value of lesser-quality foods. Conceivably, a better understanding of the action of this signaling system may provide fresh insights into neural mechanisms for controlling eating and drug-seeking behaviors.

Given its prominent role in behavioral adaptation to hunger, the insulin/NPY-like neural network is likely of primary importance to animal evolution. In addition, insulin and NPY family molecules have been found in a wide range of animals from humans to worms (Larhammar, 1996). Therefore, the insulin/NPY-like network may be a useful model to study comparatively how diverse animals have evolved distinct ways of adapting an ancestral neural system to suit their respective life styles.

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Figure 3.1 The dS6K activity in DILP neurons mediates hunger regulation of both food preference and ingestion rate

Transgenic analysis of dS6K activity was performed using the *Drosophila* GAL4/UAS binary expression system (Brand and Perrimon, 1993). UAS-$dS6K^{DN}$ and UAS-$dS6K^{ACT}$ encode a dominant-negative and constitutively active form of dS6K, respectively. All the fly lines used are in a *w* background. All the controls in this and other Figures are isogenic to the relevant experimental larvae except for the transgene tested. **A**) Quantification of liquid and solid food intake by synchronized w^{1118} larvae (74 hr AEL) in response to increasing food deprivation. The number of larval mouth hook contractions within a 30 s interval was measured. We found that larvae display virtually identical feeding responses to various liquid and solid foods containing 10% glucose, apple juice or 10% glucose/yeast under deprived and non-deprived conditions (see Figure 3.6 in Supporting Materials). **B**) DILP neurons expressing GFP in *dilp2-gal4* X UAS-*GFP* larvae. The *dilp2-gal4* driver selectively directs a GFP reporter expression in the two clusters of medial neurosecretory cells, as indicated by arrowheads. No GFP expression was detected in cells of other tissues including the gut, imaginal discs or salivary glands. **C, D**) Synchronized third-instar larvae were withheld from food for 0 or 120 min before the assay. Control larvae: *w* X UAS-$dS6K^{DN}$; *w* X UAS-$dS6K^{ACT}$. Experimental larvae: *dilp2-gal4* X UAS-$dS6K^{DN}$; *dilp2-gal4* X UAS-$dS6K^{ACT}$. At least 30 larvae per group were assayed in three separate trials. Fed larvae expressing $dS6K^{DN}$ in DILP neurons showed significant increases in the feeding of liquid and solid food relative to controls ($p < 0.0001$), while those overexpressing $dS6K^{ACT}$ showed no changes in the feeding responses ($p > 0.34$). Fasted larvae expressing $dS6K^{ACT}$ in DILP neurons showed attenuated feeding of the liquid and solid food relative to controls ($p < 0.0001$), but those overexpressing $dS6K^{DN}$ showed no altered feeding responses ($p > 0.16$). At least 20 larvae per group were assayed in three separate trials. All statistical analyses were performed using ANOVA. Error bars are the standard error of mean (SEM).

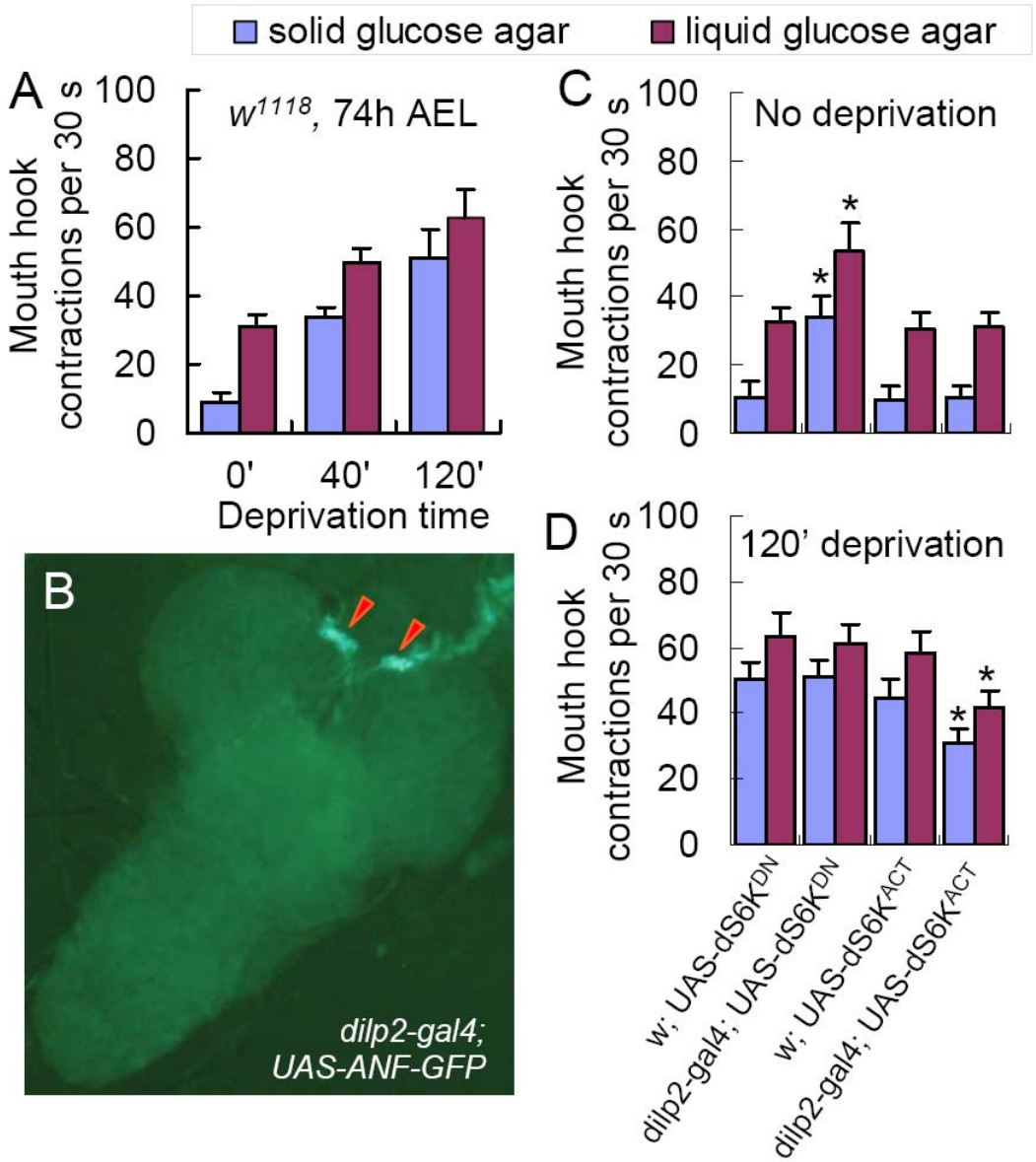
solid glucose agar
liquid glucose agar
A
Mouth hook contractions per 30 s
100
80
60
40
20
0
w1118, 74h AEL
0'
40'
120'
Deprivation time
B
dilp2-gal4;
UAS-ANF-GFP
C
No deprivation
*
*
D
120' deprivation
*
*
w; UAS-dS6KDN
dilp2-gal4; UAS-dS6KDN
w; UAS-dS6KACT
dilp2-gal4; UAS-dS6KACT

Figure 3.2 Overexpression of DILPs suppresses hunger-driven feeding activities
The *elav-gal4* flies are in a *w* background; the UAS-*dilp2*, *3*, and *4* lines are in a *y w* background. Overexpression of DILPs in the larval nervous system was directed by *elav-gal4*. Larvae were fasted for 120 min before the assays. The controls (*elav-gal4* X UAS-*GFP*, w X UAS-*dilp2, 3* and *4*) displayed normal increases in feeding rate and motivated intake of the less preferred solid food. In contrast, the experimental larvae overexpressing *dilp2* or *dilp4* but not *dilp3* showed significantly attenuated hunger responses ($p < 0.0001$ and $p > 0.05$, respectively). It still remains unclear why DILP3, which was shown to increase body size (Ikeya et al., 2002), is ineffective in attenuating feeding response. It is possible that DILP3 may be a weaker ligand to neural dInR. At least 20 larvae per group were assayed in three separate trials.

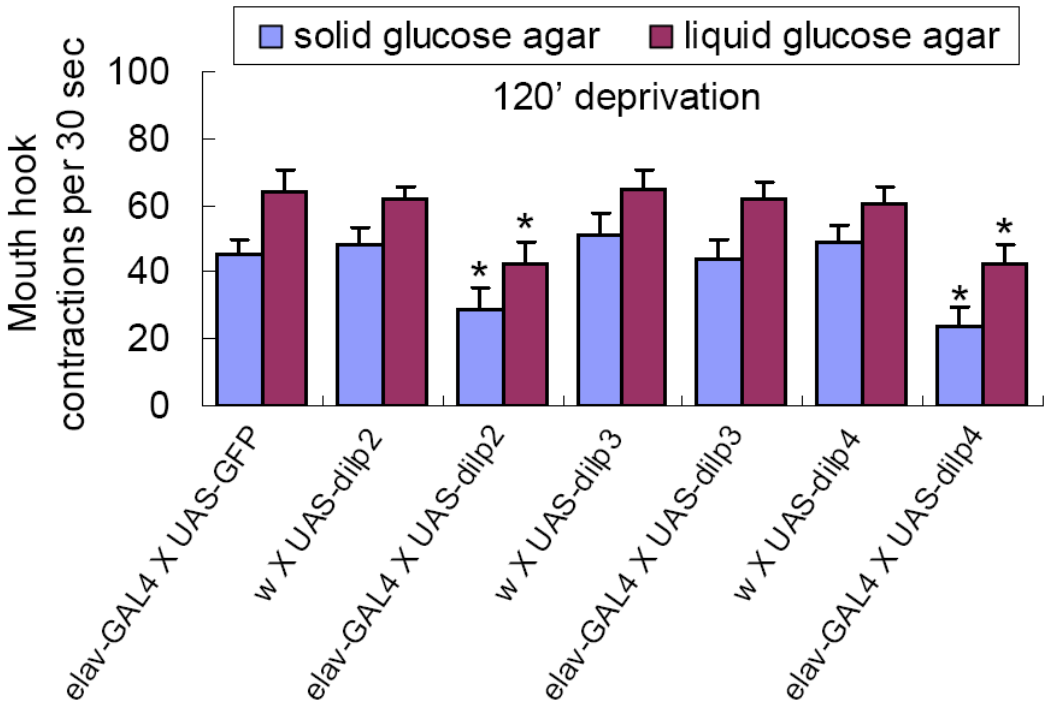
solid glucose agar
liquid glucose agar
120' deprivation
Mouth hook contractions per 30 sec
100
80
60
40
20
0
*
*
*
*
elav-GAL4 X UAS-GFP
w X UAS-dilp2
elav-GAL4 X UAS-dilp2
w X UAS-dilp3
elav-GAL4 X UAS-dilp3
w X UAS-dilp4
elav-GAL4 X UAS-dilp4

Figure 3.3 The dInR and dS6K activities in NPFR1 neurons selectively regulate food selection

The *npfr1-gal4* is in a *y w* background, while all the UAS line are in a *w* background. **A, B**) UAS-$dInR^{DN}$ and UAS-$dInR^{ACT}$ each encodes a dominant-negative and a constitutively active form of dInR. UAS-*dp110* and UAS-$dPI3K^{DN}$ encode a catalytic subunit and a dominant-negative form of dPI3K, respectively. UAS-*dPTEN* encodes wild-type dPTEN. Control larvae: *y w* X UAS-$dInR^{DN}$, UAS-$dInR^{ACT}$, UAS-*dp110*, UAS-$dPI3K^{DN}$ and UAS-*dPTEN*. Experimental larvae: *npfr1-gal4* X UAS-$dInR^{DN}$, UAS-$dInR^{ACT}$, UAS-*dp110*, UAS-$dPI3K^{DN}$ and UAS-*dPTEN*. At least 20 larvae per group were assayed in three separate trials. Fed larvae expressing transgenes ($dInR^{DN}$, $dPI3K^{DN}$ or *dPTEN*) that suppress dInR signaling in NPFR1 neurons showed significant feeding of the solid food ($p < 0.0001$), while deprived larvae expressing transgenes ($dInR^{ACT}$ or Dp110) that enhance dInR signaling displayed attenuated feeding of the solid but not liquid food ($p < 0.0001$). **C, D)** Control larvae: *y w* X UAS-$dS6K^{DN}$, *y w* X UAS-$dS6K^{ACT}$. Experimental larvae: *npfr1-gal4* X UAS-$dS6K^{DN}$, *npfr1-gal4* X UAS-$dS6K^{ACT}$. Larvae expressing $dS6K^{DN}$ in NPFR1 cells showed significant feeding of the solid but not liquid food ($p < 0.0001$) without deprivation, while fasted larvae expressing $dS6K^{ACT}$ displayed attenuated feeding of the solid but not liquid food ($p < 0.0001$).

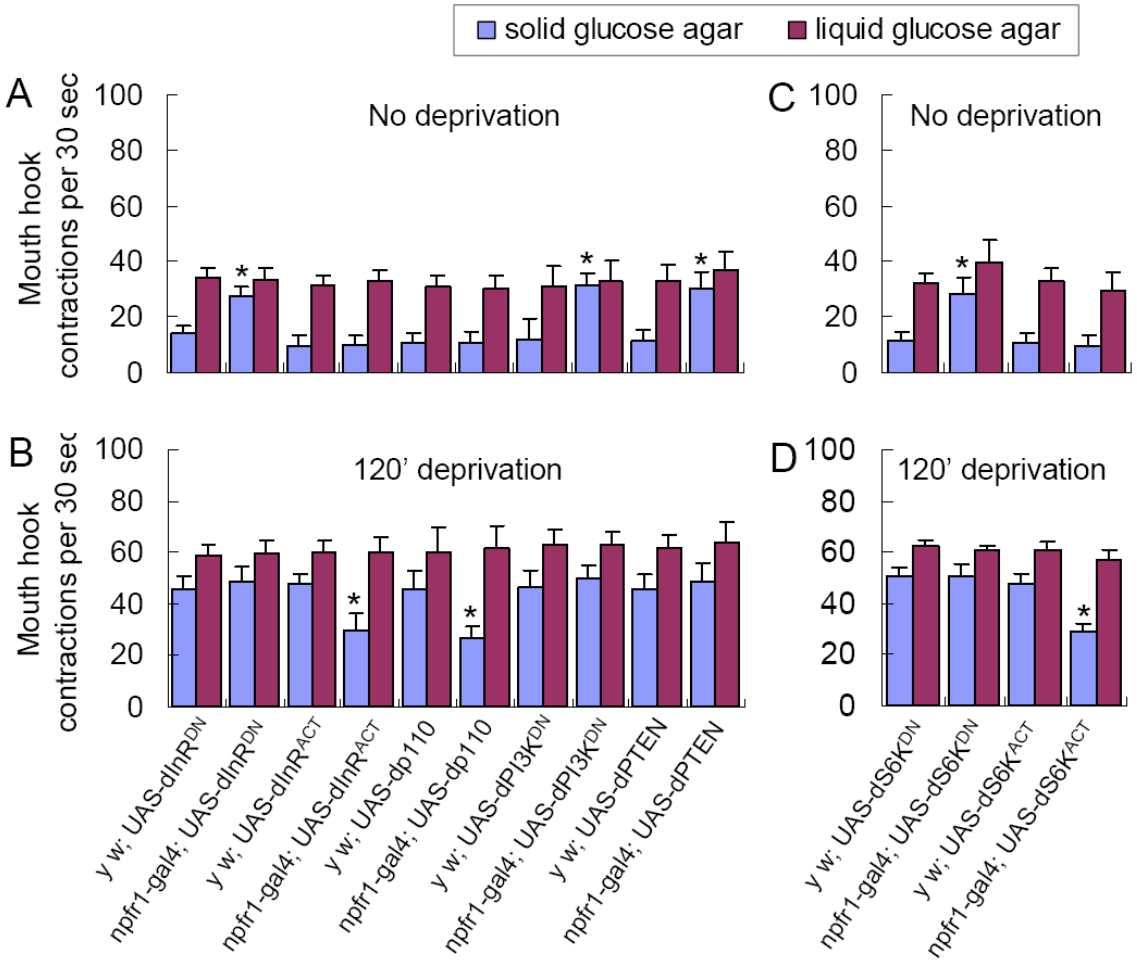
solid glucose agar
liquid glucose agar
A
No deprivation
Mouth hook contractions per 30 sec
100
80
60
40
20
0
C
No deprivation
B
120' deprivation
D
120' deprivation
y w; UAS-dInR^DN
npfr1-gal4; UAS-dInR^DN
y w; UAS-dInR^ACT
npfr1-gal4; UAS-dInR^ACT
y w; UAS-dp110
npfr1-gal4; UAS-dp110
y w; UAS-dPI3K^DN
npfr1-gal4; UAS-dPI3K^DN
y w; UAS-dPTEN
npfr1-gal4; UAS-dPTEN
y w; UAS-dS6K^DN
npfr1-gal4; UAS-dS6K^DN
y w; UAS-dS6K^ACT
npfr1-gal4; UAS-dS6K^ACT

Figure 3.4 The NPF/NPFR1 pathway acutely mediates hunger regulation of food preference

The *UAS-npfr1* and *UAS-ANF-GFP* are in the *y w* background, while the *elav-gal4*, *appl-gal4*, *MHC82-gal4*, UAS-*shi*^{ts1} and UAS-*npfr1*^{dsRNA} are in a *w* background. **A-C**) NPFR1-overexpressing larvae fasted for 0, 30 or 120 minutes were assayed. The rate of mouth hook contractions was scored individually (n = 15-20 per group for each of three trials). Among the fed larvae, the experimental group (*npfr1-gal4* X UAS-*npfr1*) showed significantly higher activity of extracting agar-embedded glucose relative to controls (*npfr1-gal4 X y w, npfr1-gal4 X UAS-ANF-GFP* and *y w X UAS-npfr1*; $p < 0.0001$), while the ingestion rate of the liquid food remained unchanged ($p > 0.25$). The enhancement of motivated intake of the solid food was also observed in NPFR1-overexpressing larvae fasted for 30 minutes ($p < 0.001$), but the difference became insignificant after 120 min ($p > 0.86$). **D**) Larvae expressing *npfr1* dsRNA in NPFR1 cells as well as the nervous system were tested for their feeding responses to the liquid and solid food. Larvae were fasted for 120 min before the assay. The control larvae (w^{1118}, *npfr1-gal4* X *w*, *y w* X *npfr1*^{dsRNA}, *w X* UAS-*npfr1*^{dsRNA}, *elav-gal4 X w* and *MHC-82-gal4 X* UAS-*npfr1*^{dsRNA}) showed enhanced feeding responses, while the experimental larvae (*npfr1-gal4*, *elav-gal4* and *appl-gal4* X UAS- *npfr1*^{dsRNA}) were deficient in feeding response to the solid but not liquid food ($p < 0.0001$; n > 20 per group). The UAS-*npfr1*^{dsRNA} line was previously shown to reduce *npfr1* transcript levels and NPFR1 signaling activity(Wen et al., 2005). **E, F**) UAS-*shi*^{ts1} encodes a semidominant-negative form of dynamin that blocks neurotransmitter release at a restrictive temperature (> 29 ^{o}C). Larvae were fasted for 120 min before the assay (n > 20 per group for each of three trials). At 23 ^{o}C, both experimental larvae (*npf-gal4* and *npfr1-gal4* X UAS-*shi*^{ts1}) and control larvae (*y w* X UAS- *shi*^{ts1}, *npf-gal4* X *w* and *npfr1-gal4* X *w*) displayed normal food response ($p > 0.45$). At 30 ^{o}C , the experimental larvae showed deficits in motivated feeding response to the solid food but not liquid food ($p < 0.0001$), while the controls remained normal. The feeding behaviors of control larvae at both temperatures were also similar ($p > 0.08$).

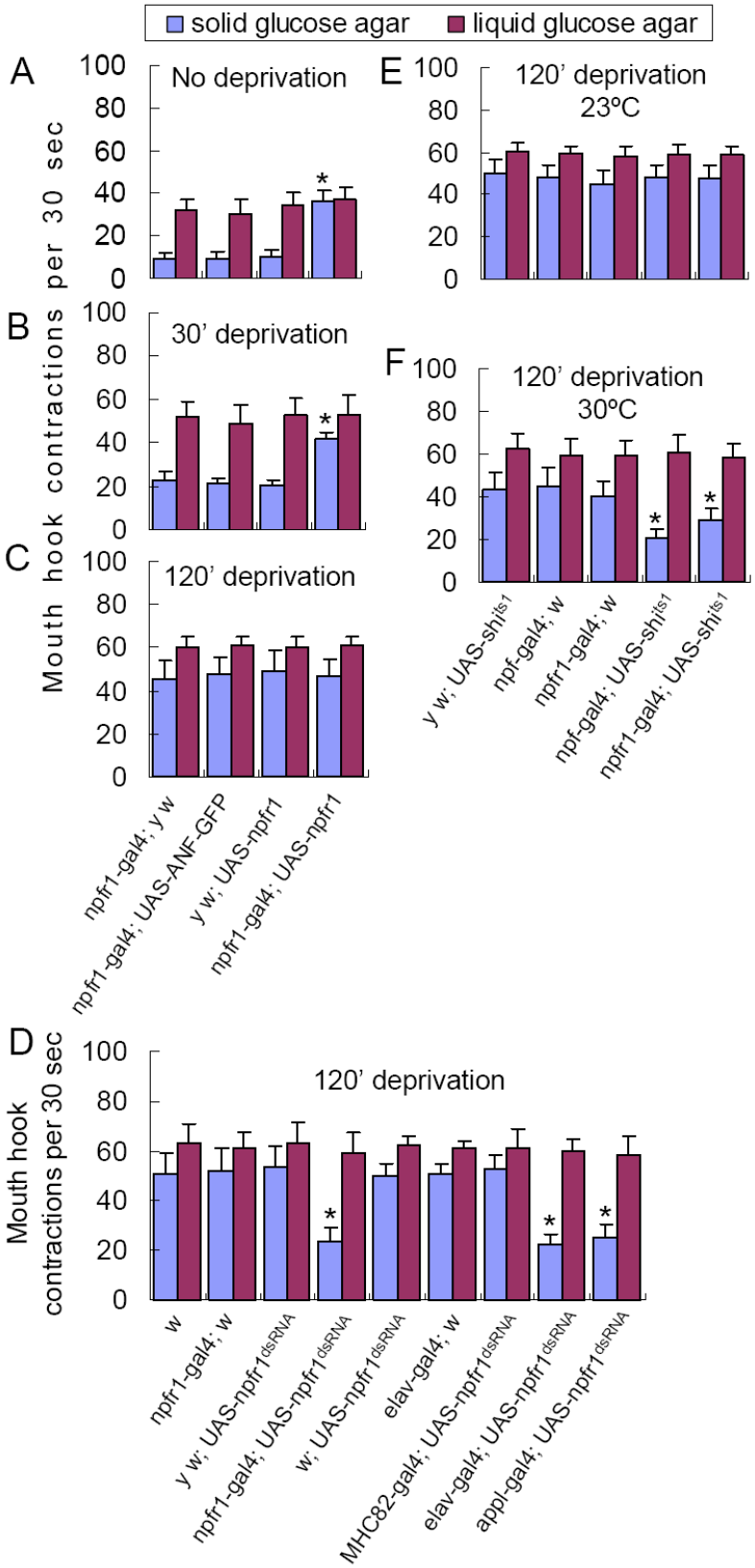
solid glucose agar
liquid glucose agar
A
No deprivation
B
30' deprivation
C
120' deprivation
D
120' deprivation
E
120' deprivation
23ºC
F
120' deprivation
30ºC
Mouth hook contractions per 30 sec
Mouth hook contractions per 30 sec
0
20
40
60
80
100
*
npfr1-gal4; y w
npfr1-gal4; UAS-ANF-GFP
y w; UAS-npfr1
npfr1-gal4; UAS-npfr1
y w; UAS-shi ts1
npf-gal4; w
npfr1-gal4; w
npf-gal4; UAS-shi ts1
npfr1-gal4; UAS-shi ts1
w
npfr1-gal4; w
y w; UAS-npfr1 dsRNA
npfr1-gal4; UAS-npfr1 dsRNA
w; UAS-npfr1 dsRNA
elav-gal4; w
MHC82-gal4; UAS-npfr1 dsRNA
elav-gal4; UAS-npfr1 dsRNA
appl-gal4; UAS-npfr1 dsRNA

Figure 3.5 A model for the hunger regulation of adaptive feeding behaviors in *Drosophila* larvae

Our results suggest that DILP2 neurons and possibly together with DILP4 neurons negatively regulate two downstream hunger-responsive feeding systems: an NPF/NPFR1-dependent pathway specialized for motivated feeding, and an NPF/NPFR1-independent pathway for general enhancement of feeding rate. In DILP neurons, dS6K activity is likely to positively regulate DILP synthesis and/or release. In fed larvae, a relatively high level of DILP signaling suppresses the two downstream pathways. In fasted animals, hunger stimuli downregulate dS6K activity in DILP neurons, which in turn leads to decreased DILP signaling and therefore disinhibition of the two pathways. The former overrides the high threshold set by the default pathway, enabling hungry animals to engage in motivated foraging and food selection. The latter enhances feeding rate, allowing animals to compete effectively for limited food sources. DILPs negatively regulate the NPFR1 pathway through the dInR/dPI3K/dS6K pathway in NPFR1 neurons. Our data also implicate the presence of a separate default pathway for *ad libitum* feeding of higher quality foods (baseline feeding) by fed larvae. This default pathway may be largely insensitive to DILP or NPF signaling, since overexpression of dS6K, DILPs or NPFR1 in fed larvae did not affect baseline feeding in the presence of the liquid food. (see Figures 1 – 3 and data not shown).

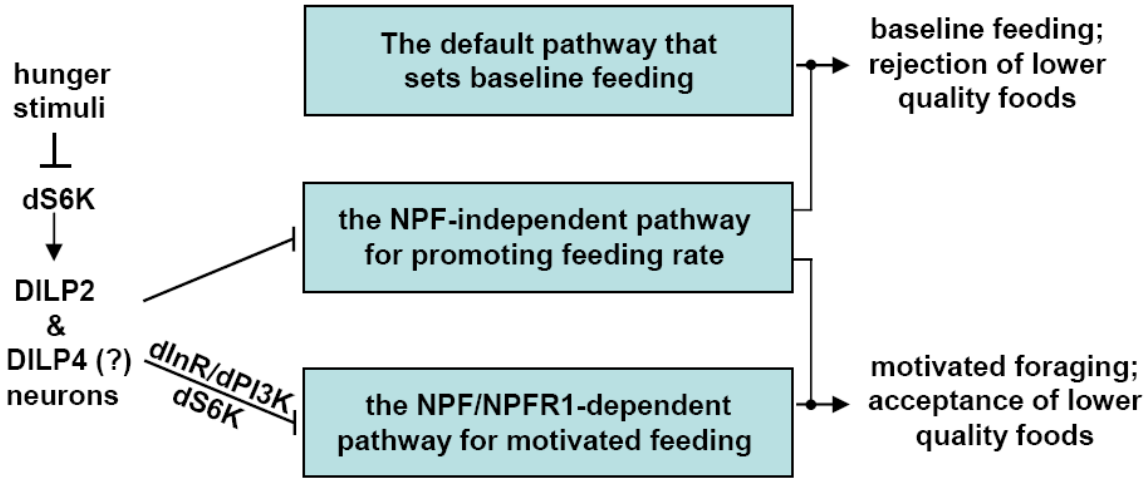
hunger
stimuli
dS6K
DILP2
&
DILP4 (?)
neurons
dInR/dPI3K
dS6K
The default pathway that sets baseline feeding
the NPF-independent pathway for promoting feeding rate
the NPF/NPFR1-dependent pathway for motivated feeding
baseline feeding; rejection of lower quality foods
motivated foraging; acceptance of lower quality foods

Figure 3.6 Comparison of feeding activities of Canton S larvae on different media
Mouthhook contraction rates of WT third instars (Canton S, 74 h after egg laying) deprived for 0, 40, and 120 min were measured in either glucose agar, apple juice agar, and 10% glucose/yeast-containing agar. (A) Solid foods. (B) Liquid foods. Larvae displayed virtually identical feeding activities under all feeding conditions (ANOVA, $P > 0.1$). The apple juice food contained 2.5 ml of concentrated apple juice per 10 ml final volume. The 10% glucose/yeast food contained 1 g of yeast powder per 10 ml final volume.

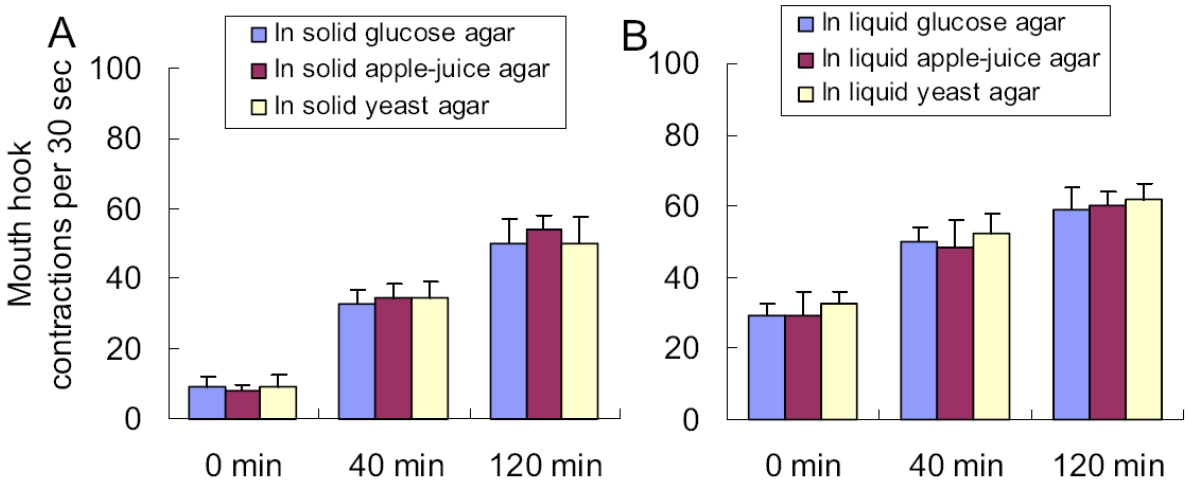
A
Mouth hook contractions per 30 sec
100
80
60
40
20
0
In solid glucose agar
In solid apple-juice agar
In solid yeast agar
0 min
40 min
120 min
B
100
80
60
40
20
0
In liquid glucose agar
In liquid apple-juice agar
In liquid yeast agar
0 min
40 min
120 min

Figure 3.7 The body length of larvae
Third instars (74 h after egg laying) that fed ad libitum on a rich diet (apple juice plus yeast paste) were collected from each group ($n = 15$) and heated briefly in boiling water to fully extend the larval body for measurement. The animal size is not affected by disrupting or overexpressing dS6K activity in DILP2 neurons ($P > 0.16$, ANOVA).

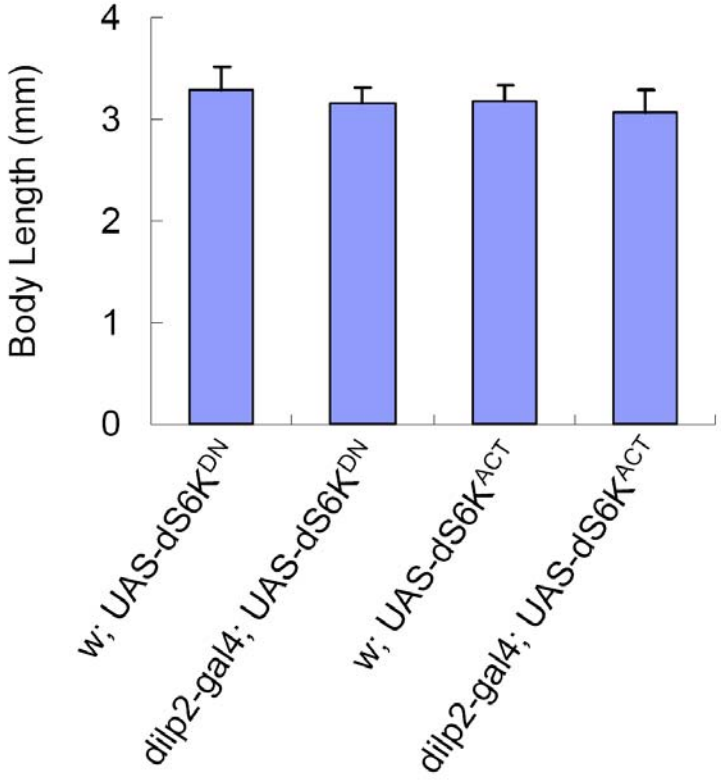
Body Length (mm)
4
3
2
1
0
w; UAS-dS6K^DN
dilp2-gal4; UAS-dS6K^DN
w; UAS-dS6K^ACT
dilp2-gal4; UAS-dS6K^ACT

Figure 3.8 Immunolocalization of DILP2 neurons
A *dilp2* promoter fragment (*dilp2-gal4*) is sufficient to drive GFP expression (*UAS-ANF-GFP*) specifically in a single small cluster of cells in each of the brain hemispheres (arrow heads, A) where the endogenous *dilp2* is expressed (arrowheads, B). These results is consistent with previous published data (Rulifson et al., 2002). (Magnification: x200)

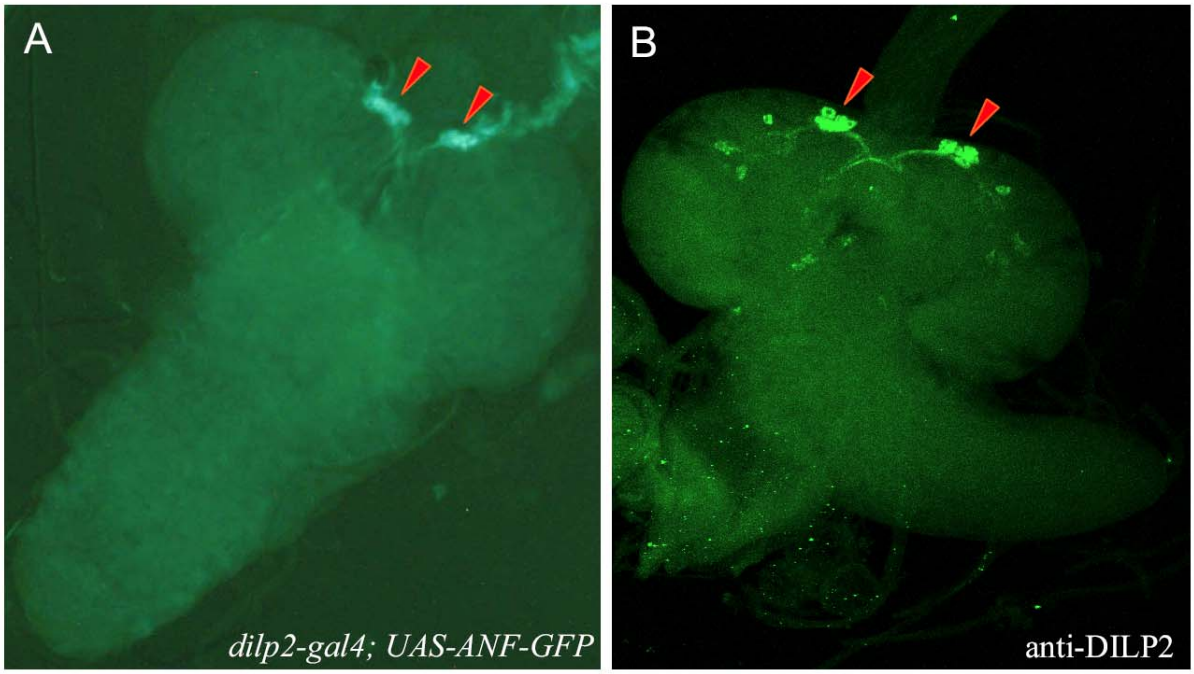
A
dilp2-gal4; UAS-ANF-GFP
B
anti-DILP2

Figure 3.9 **NPF/NPFR1 signaling is tonically required for deprivation-motivated response to solid food**

The assay for free foraging on 10% glucose agar and agar paste has been described (Wu et al., 2003). Larvae were deprived for 120 min before each assay ($n > 60$ from three separate trials). (*A* and *B*) At 23°C, both experimental larvae (*npf-gal4* and *npfr1-gal4* X UAS-shi^{ts1}) and control larvae (*y w* X UAS- shi^{ts1}, *npf-gal4*, or *npfr1-gal4* X *w*) displayed normal food response ($P > 0.45$). At 30°C, the experimental larvae showed deficits in feeding response, whereas the controls remained normal ($P < 0.0001$). The feeding behaviors of control larvae at both temperatures were also similar ($P > 0.08$). (*C*) The foraging activity of the experimental larvae was first assayed at 30°C, and the percentage of remaining larvae was scored after 20 min ($t = 0$ min). The same groups of larvae were recollected immediately and assayed again within 30 min at the bench-top ($t = 25$ min). The foraging activity was partially restored at this time point of the cooling process. The same assay was repeated again, and >85% larvae stayed on the glucose agar ($t = 50$ min). These data suggested that the NPF signaling not only can modulate the intensity and duration of feeding response quantitatively, but also is tonically required to maintain deprivation-motivated feeding.

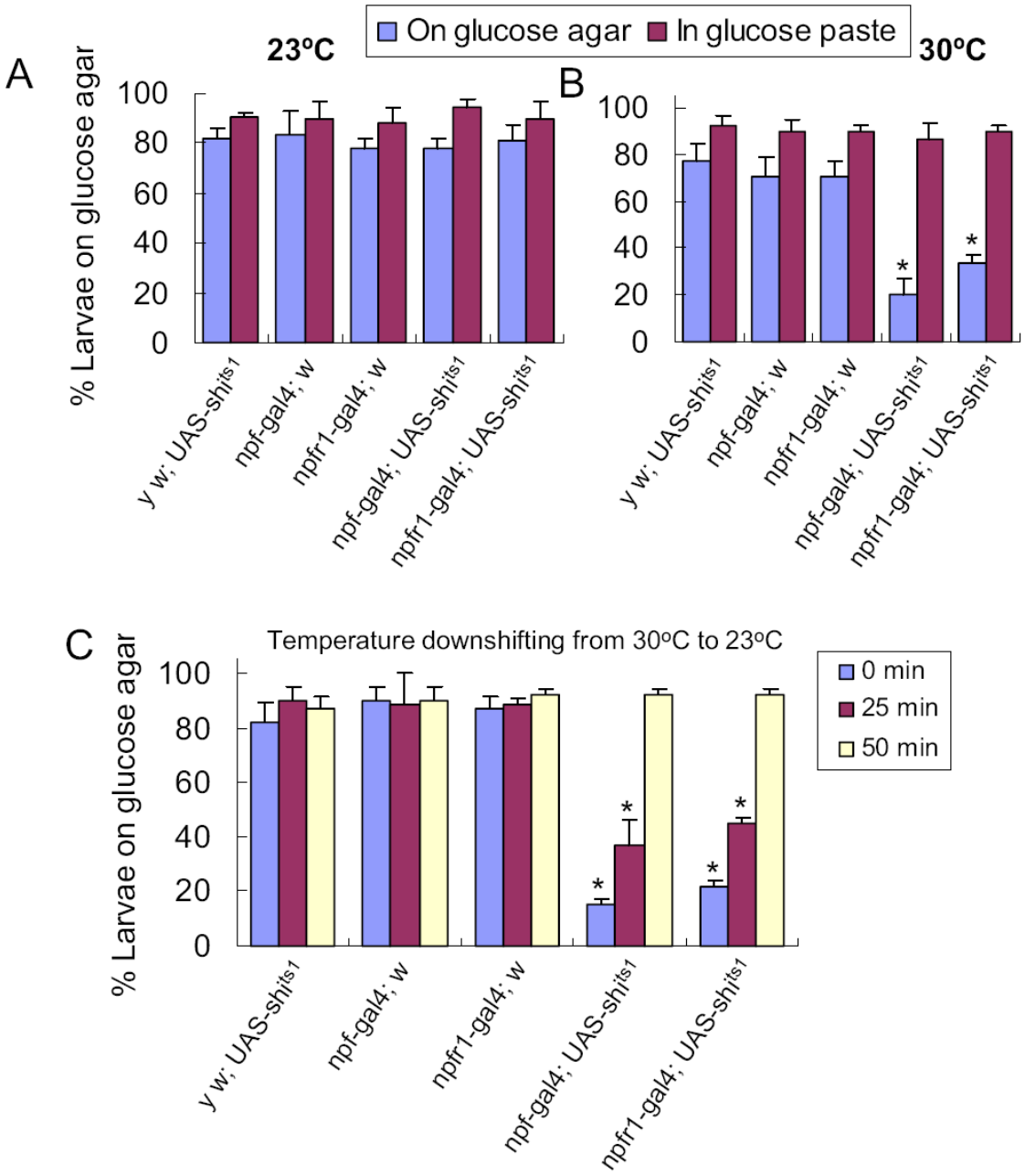
A
23°C
On glucose agar
In glucose paste
30°C
B
% Larvae on glucose agar
100
80
60
40
20
0
y w; UAS-shits1
npf-gal4; w
npfr1-gal4; w
npf-gal4; UAS-shits1
npfr1-gal4; UAS-shits1
*
C
Temperature downshifting from 30°C to 23°C
0 min
25 min
50 min

Table 3.1 Growth and development of larvae expressing dS6K^{DN} or dS6K^{ACT}

Parental genotypes	Embryos that hatch 24 h after egg laying, %	Hatchlings that pupariate 120 h after egg laying, %
w; UAS-dS6K^{DN}	85.3	93.8
dilp2-gal4; UAS-dS6K^{DN}	82.3	90.0
w; UAS-dS6K^{ACT}	83.8	91.2
dilp2-gal4; UAS-dS6K^{ACT}	80.0	90.9

At least 130 synchronized embryos were collected within 2 h for each group in two separate trials. The experimental larvae showed a virtually identical growth profile relative to their paired controls. The larvae and flies were reared with the apple juice/yeast paste diet.

CHAPTER 4

REGULATION OF AVERSION TO NOXIOUS FOOD BY *DROSOPHILA* NEUROPEPTIDE Y- AND INSULIN-LIKE SYSTEMS[1]

[1]Wu, Q., Zhao, Z. and Shen, P. 2003. ***Nat. Neurosci.*** 8, 1350 – 1355. Reprinted here with permission of the publisher.

ABSTRACT

Ominivores including humans display congenital tendency to avoid noxious or unfamiliar foods. Such defensive foraging behaviors are, however, modifiable in response to physiological needs. We describe a paradigm for assessing risk-sensitive food acquisition in *Drosophila*. Fly larvae display increased compromise in feeding of noxious foods (adulterated with quinine) as deprivation prolongs. The neuropeptide F receptor NPFR1, a mammalian neuropeptide Y receptor homologue, centrally regulates response to noxious food. Overexpression of NPFR1 is sufficient to cause nondeprived larvae to display compromised intake of noxious food, whereas loss of NPFR1 signaling lead to the opposite phenotype. Moreover, NPFR1 neuronal activity may be directly regulated by the insulin-like signaling pathway. Upregulation of insulin-like receptor signaling in NPFR1 cells suppresses feeding response to noxious food. Our results suggest that the coordinated activities of the conserved neuropeptide Y- and insulin-like receptor signaling system(s) are essential for the dynamic regulation of noxious food intake under different energy states.

INTRODUCTION

Natural selection acts strongly on the foraging behavior of diverse animals (Charnov, 1976). Since food acquisition is not only essential to all animals but can also be potentially risky, animals must have evolved effective strategies to manage risks (e.g., food poisoning) inherently associated with food acquisition (Stricker, 1990). For example, ominivores, including humans, have an innate ability to avoid noxious or unfamiliar foods. On the other hand, an animal's risk-averse behaviors are subject to physiological regulations. In general, energy-depleted animals are more risk-prone in food acquisition than those on a positive energy budget (Bateson, 2002).

At present, relatively little is known about the genetic and neural mechanisms underlying an animal's innate ability to modulate risk tolerance.

The components of a significant number of neuropeptide signaling pathways are structurally conserved between flies and mammals (Hewes and Taghert, 2001). *Drosophila* has one NPY-like neuropeptide, NPF, and its action is mediated by a G-protein coupled receptor NPFR1 related to mammalian NPY receptors (Brown et al., 1999). We recently found that fly larvae utilize this NPY-like signaling pathway for regulating food response similar to mammals (Wu et al., 2003). Interestingly, both fly NPF and mammalian NPY have been reported to promote feeding response in deprived animals but not *ad libitum* feeding by nondeprived animals (Bannon et al., 2000;Segal-Lieberman et al., 2003;Wu et al., 2003). Thus, the mechanism underlying the action of this conserved system may be complex.

The *Drosophila* genome contains seven insulin-like genes, *dilp1-7* (Brogiolo et al., 2001;Rulifson et al., 2002). Overexpression of each of the seven DILPs promoted larval growth, indicating that they have insulin-like activities (Ikeya et al., 2002). *Drosophila* appears to have a single insulin-like receptor (dInR) that regulates diverse physiological functions including metabolism, growth and size, reproduction and lifespan (Oldham and Hafen, 2003). Insulin/insulin-like peptides and their receptors are widely present in the central nervous system (CNS), but their neural functions remain largely uncharacterized. In a limited number of studies, the brain insulin/insulin-like receptors have been implicated in the control of body weight and reproduction, learning and memory, axon pathfinding and ethanol response (Bruning et al., 2000;Gerozissis, 2003;Moore, 2003;Song et al., 2003).

In this report, we show that NPFR1 is critical for regulating responses to noxious food in *Drosophila* larvae. Fly larvae display increased compromise in feeding of noxious foods (e.g.

adulterated with 0.5% quinine) as deprivation prolongs. We show that overexpression of NPFR1 is sufficient to trigger compromised intake of noxious food in nondeprived animals. Conversely, disruption of neural NPFR1 signaling in deprived animals causes deficits in acquisition of noxious food. We further show that *Drosophila* insulin-like signaling pathway directly modulates NPFR1 neuronal activity. Upregulation of the dInR signaling pathway in NPFR1 cells leads to attenuated feeding responses to noxious food, whereas downregulating the activity of the dInR pathway results in the opposite phenotype. Our study suggests that the conserved neuropeptide Y- and insulin-like systems are critical for regulating noxious food intake in insects and possibly vertebrates as well.

RESULTS

NPF/NPFR1 mediate hunger-driven intake of noxious food

Drosophila larvae are amenable to genetic and neurobiological analyses of various food-related behaviors (Osborne et al., 1997;Wu et al., 2003). Synchronized early third-instar larvae (74 hr *a*fter *e*gg *l*aying, 74 hr AEL) feed voraciously for rapid growth. Nondeprived wild-type larvae display a strong feeding response to a liquid diet containing sugar, yeast paste and green food dyes. However, addition of quinine, a noxious substance, to the diet elicited a strong aversive response (Figure 4.1a). For example, nondeprived larvae largely rejected the 0.5% quinine food that they initially encountered, and continued to search for new food sources (Figures 4.5-4.6). In contrast, a significantly higher number of larvae deprived for 40 or 120 min (about 30% and 80%, respectively) remained feeding in the 0.5% quinine food. Thus, the larval response to noxious food appears to be a useful model for assessing risk tolerance associated with food/reward seeking.

A neuronal circuit consisting of 4-6 neuropeptide F (NPF) neurons and a small number of NPFR1 neurons in the larval central nervous system (CNS; Figure 4.1b,c) displays neural plasticity in response to chemosensory stimuli, and promotes deprivation-motivated intake of less-accessible food (Shen and Cai, 2001;Wu et al., 2003). To test whether NPF and NPFR1 neurons play a role in larval response to noxious food, we performed controlled disruption of NPF/NPFR1 neuronal activities in feeding larvae. Two drivers, *npf-gal4* and *npfr1-gal4*, were used to direct expression of a temperature-sensitive allele of *shibire* (shi^{ts1}), which encodes a semidominant-negative form of dynamin capable of blocking neurotransmitter release at a restrictive temperature (> 29 ^{o}C) (Kitamoto, 2002b). At 23 ^{o}C, nondeprived control larvae (*y w;UAS-*shi^{ts1}, *w;npf-gal4* and *w;npfr1-gal4*) displayed a gradually reduced feeding response as the quinine concentration increased from 0 to 0.05, 0.2 and 0.5%, and their feeding responses to quinine foods remained the same even at 30 ^{o}C (Figure 4.1d,e). Nondeprived experimental larvae (*npf-gal4;UAS-*shi^{ts1} and *npfr1-gal4;UAS-*shi^{ts1}) also behaved like controls except for a small but significant reduction in feeding response by the *npf-gal4;UAS-*shi^{ts1} larvae at 30 ^{o}C ($P < 0.01$), suggesting that the NPF pathway may be weakly active in nondeprived larvae. The control larvae deprived for 120 min exhibited enhanced feeding response relative to the nondeprived counterparts at both 23 and 30 ^{o}C (>70% vs. 0% feeding in the 0.5% quinine diet, Figure 4.1f,g). Such deprivation-induced intake of 0.5% quinine food was drastically reduced in both *npf-gal4;UAS-*shi^{ts1} and *npfr1-gal4;UAS-*shi^{ts1} larvae after 15 min incubation at 30 ^{o}C, but remained normal at the permissive temperature of 23 ^{o}C. These findings suggest that the NPF/NPFR1 neuronal circuit is acutely required to suppress aversive response to noxious foods in deprived animals. Our data also provide functional evidence that NPF release may be coupled to a dynamin-mediated process. Consistent with this notion, dynamin has been implicated in the

formation of transport vesicles from the trans-Golgo network (Cao et al., 2000;Jones et al., 1998;Kreitzer et al., 2000). It has also been reported that pharmacological inhibition of norepinephrine uptake by neurons that co-release NPY and norepinephrine markedly reduces NPY release (Haass et al., 1989).

Role of NPFR1 and dS6K in feeding response to noxious food

To determine whether NPF and NPFR1 directly regulate aversive response to noxious food, we selectively knocked down *npfr1* by expressing its double-stranded RNA (dsRNA) in NPFR1 cells as well as in the nervous system. We used a UAS-*npfr1*^{dsRNA} line that was previously shown to cause reduced *npfr1* expression and NPFR1 signaling deficiency (Wen et al., 2005). Nondeprived experimental larvae (*npfr1-gal4;UAS-npfr1*^{dsRNA}, *elav-gal4;UAS-npfr1*^{dsRNA} or *appl-gal4*;*UAS-npfr1*^{dsRNA}) and controls (e.g., *w;npfr1-gal4* and *y w;UAS-npfr1*^{dsRNA}) showed similar responses to quinine foods (Figure 4.2a). However, after 2-hour deprivation, the experimental, but not the control, larvae displayed attenuated feeding responses, which are virtually identical to those displayed by *npf-gal4;UAS-shi*^{ts1} and *npfr1-gal4;UAS-shi*^{ts1} larvae at 30 ^{o}C (Figure 4.2c). For example, about 80% of the control larvae displayed feeding of 0.5% quinine food, whereas < 40% of the experimental larvae fed on the same diet ($P < 0.0001$). Therefore, these findings further indicate that neural NPFR1 is critical for regulating aversive response to noxious food.

We also tested the potential effect of overexpressing an *npfr1*cDNA in NPFR1 neurons on feeding of quinine-containing foods. Nondeprived control larvae (e.g. *y w;UAS-npfr1*) rejected the 0.5% quinine food. In contrast, at least 50% of the nondeprived experimental larvae (*npfr1-gal4;UAS-npfr1*) displayed active feeding in the same food (Figure 4.2b). Furthermore,

experimental and control larvae deprived for 120 min also showed significant feeding of food containing 0.2 or 0.5 % quinine (Figure 4.2d). However, when the quinine concentration was increased to 0.8%, a significantly higher number of NPFR1 overexpressing larvae (> 50%) remained feeding relative to controls (15 - 20%, $P < 0.001$). Thus, larvae overexpressing NPFR1 displayed feeding behavioral phenotypes opposite to those deficient in NPF/NPFR1 signaling.

We postulated that NPFR1 neurons may centrally mediate deprivation-induced intake of noxious food. To test this, we evaluated the role of *Drosophila* p70 ribosomal S6 kinase (dS6K), a cell-autonomous effector of nutrient-sensing pathways, in NPFR1 neurons (Colombani et al., 2003;Kimball and Jefferson, 2001;Thomas, 2002). We used the *npfr1-gal4* driver to direct two transgenes UAS-$dS6K^{DN}$ and UAS-$dS6K^{ACT}$, each encoding a dominant-negative and constitutively active form of dS6K, respectively (Barcelo and Stewart, 2002). In the absence of deprivation, *npfr1-gal4;UAS-*$dS6K^{DN}$ larvae displayed significant feeding of the 0.5% quinine-adulterated food, similar to *npfr1-gal4;UAS-npfr1* larvae (Figure 4.3a). Moreover, *npfr1-gal4;UAS-*$dS6K^{DN}$ larvae showed significant feeding of 0.8% quinine-containing food under deprivation (Figure 4.3c). In contrast, deprived larvae overexpressing dS6K *(npfr1-gal4;UAS-*$dS6K^{ACT}$) displayed deficits in feeding response to the 0.5% quinine food (Figure 4.3d). Taken together, these results suggest that dS6K is essential for the transduction of hunger stimuli to NPFR1 neurons.

DILP/dInR may directly modulate NPFR1 neuronal activity

It has been shown that dS6K is a downstream effector of the *Drosophila* insulin-like receptor (dInR) pathway (Oldham and Hafen, 2003). We therefore examined whether dInR signaling

regulates NPFR1 pathway. A dominant-negative and a constitutively active form of dInR (dInR^{DN} and dInR^{ACT}, respectively) were expressed under the control of *npfr1-gal4* (Personal communications to Flybase, http://rail.bio.indiana.edu/.bin/fbidq.html?FBrf0178856; also see Figure 4.7). Without deprivation, more than 40% of the *npfr1-gal4;UAS-InR^{DN}* larvae displayed feeding response to the 0.5% quinine food. In contrast, less than 10% of control larvae (e.g., *y w;UAS-InR^{DN}*) showed a feeding response to the same food (Figure 4.3a). Moreover, like controls, the *npfr1-gal4;UAS-InR^{ACT}* larvae displayed no feeding response. Phosphatidylinositol 3-kinase (dPI3K) and phosphatidylinositol 3,4,5-trisphosphate 3-phosphatase (dPTEN) are key components of the *dInR* pathway. Disruption of dInR signaling by overexpressing dPTEN or a dominant-negative form of dPI3K also led to increased feeding response to noxious food by nondeprived larvae. Under deprived conditions, dInR/dPI3K signaling-deficient larvae displayed significantly higher feeding response to 0.8% quinine than control larvae (Figure 4.3c). Conversely, deprived larvae expressing dInR^{ACT} or Dp110 (encoding a catalytic subunit of dPI3K) in NPFR1 neurons exhibited reduced feeding response to 0.5% quinine food relative to their paired controls (*y w;UAS-InR^{ACT}* and *UAS-dp110*, Figure 4.3d). These results suggest that dInR signaling is critical for transducing hunger stimuli in NPFR1 neurons.

Among seven insulin-like genes, *dilp2* is most abundantly expressed in two clusters of neurosecretory cells in the larval CNS (Rulifson et al., 2002). We overexpressed *dilp2* in the nervous system using neural-specific *elav-gal4*. Nondeprived *elav-gal4;UAS-dilp2* larvae displayed a normal response to foods containing various concentrations of quinine (Figure 4.3b). However, the same larvae deprived for 120 min displayed significantly attenuated feeding at 0.5% quinine concentration in comparison with controls (*elav-gal4;UAS-GFP* and *w;UAS-dilp2*,

Figure 4.3d). Taken together, these results suggest that DILP2 negatively regulates the signaling activity of NPFR1 neurons.

dS6K negatively regulates NPFR1 signaling

We have shown that overexpression of NPFR1 or dS6K in NPFR1 cells each exerts a dominant but opposite effect on larval response to noxious food. To delineate the functional relationship between the dS6K and NPFR1 signaling pathways, we simultaneously expressed *UAS-dS6K*^{DN} and *UAS-npfr1*^{dsRNA} using the *npfr1-gal4* driver. The experimental and control larvae were individually assayed for their feeding response to quinine diets under deprived and nondeprived conditions. As expected, about 50% of the *npfr1-gal4;UAS-dS6K*^{DN} larvae showed feeding response to the 0.5% quinine food. In contrast, >80% of the controls (*UAS-dS6K*^{DN} along or *UAS-npfr1*^{dsRNA} and *UAS-dS6K*^{DN} only) as well as larvae coexpressing *dS6K*^{DN} and *npfr1*^{dsRNA} driven by *npfr1-gal4* rejected the same food (Figure 4.4a, $P < 0.001$). Under deprived conditions, the *npfr1-gal4;UAS-dS6K*^{DN} larvae also displayed higher feeding response to 0.8% quinine food than larvae coexpressing *npfr1*^{dsRNA} and *UAS-dS6K*^{DN} in NPFR1 cells (Figure 4.4c).

In parallel, we also coexpressed *UAS-dS6K*^{ACT} and *UAS-npfr1* using the *npfr1-gal4* driver. Under nondeprived conditions, more than 70% of the *npfr1-ga4;UAS-npfr1* larvae as well as larvae that co-express *dS6K*^{ACT} and *npfr1* cDNA in NPFR1 cells displayed significant feeding activity in the 0.5% quinine food (Figure 4.4b, $P < 0.001$); in contrast, virtually all of the control larvae (*y w;UAS-dS6K*^{ACT} and *y w;UAS-npfr1; UAS-dS6K*^{ACT}) as well as *npfr1-gal4;UAS-dS6K*^{ACT} larvae rejected the same food. Therefore, nondeprived larvae overexpressing *npfr1* cDNA in NPFR1 cells alone or together with *dS6K*^{ACT} exhibited similar responses to noxious food. We also tested the same groups of larvae under deprived conditions. About 50% of the

npfr1-ga4;UAS-npfr1 as well as larvae coexpressing *npfr1* cDNA and $dS6K^{ACT}$showed feeding response to 0.8% quinine food, while virtually all of the *npfr1-gal4;UAS-*$dS6K^{ACT}$ larvae rejected the same food (Figure 4.4d). Taken together, these results indicate that NPFR1 activity exerts a dominant effect over that of dS6K in NPFR1 cells.

DISCUSSION

Our study reveals a previously uncharacterized role of the insulin signaling pathway in regulating aversive response to noxious food. We propose a model to illustrate how DILP and NPF neural signaling pathways coordinately regulate feeding of noxious food (Figure 4.8). NPFR1 neurons respond to two extracellular cues, DILPs and NPF. DILP/dInR signaling is likely responsible for transducing hunger stimuli in NPFR1 neurons through dS6K, which in turn negatively regulates the NPFR1 signaling pathway. Furthermore, the observation that NPFR1 overexpression is sufficient to override the inhibitory effect of constitutively active dS6K suggests that dS6K may suppress directly or indirectly the activity of NPFR1.

It remains to be determined how the NPFR1 neurons regulate the tolerance of noxious food. One possibility is that the NPF/NPFR1 system might be part of the neural circuit regulating risky behaviors. Consistent with this, NPY, the mammalian homolog of NPF, plays a role in suppressing anxiety and fear (Thorsell and Heilig, 2002). For example, NPY knockout mice display reduced activities in the center of an open-field and increased startling response to an acoustic stimulus (Bannon et al., 2000;Thorsell et al., 2000). Future experiments will be needed to test whether the NPY and insulin receptor pathways coordinately regulate risk-sensitive foraging behavior in mammals. On the other hand, the NPF/NPFR1 system, which is upregulated in deprived animals, may strengthen the reinforcing effect (reward value) of food,

thereby reducing the avoidance of noxious stimuli (Levine et al., 2003;Levine et al., 2004). For example, in the absence of deprivation, NPFR1 overexpression was sufficient to bypass hunger stimulation to trigger motivated feeding of less-accessible solid medium which is otherwise rejected by normal larvae (Wu et al., 2003).

Insulin and NPY family molecules have been found in a wide range of animals from humans to worms (Larhammar, 1996). Given its prominent role in behavioral adaptation to adverse feeding conditions, the insulin/NPY-like signaling network may be a useful model for studying comparatively how diverse animals have evolved distinct ways of adapting an ancestral neural system to suit their respective life styles.

METHODS

Flies and Media

Fly rearing and the collection of eggs and larvae were described previously (Wu et al., 2003). The UAS-*npfr1*^{dsRNA} and UAS-*npfr1* flies are in the w^{1118} background. The UAS-*shi*^{ts1} and UAS-*dilp2* lines were kindly provided by T. Kitamoto and E. Hafen, respectively (Ikeya et al., 2002;Kitamoto, 2002b). The *elav-gal4*, *appl-gal4*, *MHC82-gal4,* UAS-*S6K*^{DN}, UAS-*S6K*^{ACT}, UAS-*dInR*^{DN}, UAS-*dInR*^{ACT}, UAS-*dp110*, UAS-*dPI3K*^{DN}, and UAS-*dPTEN* are in the w^{1118} background (Barcelo and Stewart, 2002;Davis et al., 1998;Gao et al., 2000;Leevers et al., 1996;Lin and Goodman, 1994;Torroja et al., 1999). The UAS-*ANF-GFP* (Rao et al., 2001) and UAS-*dilp2* are in the *y w* background. Other fly lines were described before (Wen et al., 2005;Wu et al., 2003).

Molecular cloning and analysis

For RNAi analysis, the UAS-*npfr1*^{dsRNA} construct was made by cloning two copies of a 665-bp fragments from the 5' portion of the *npfr1* coding sequence separated by a 619-bp DNA spacer into the downstream of the UAS promoter in the pUAST vector (Piccin et al., 2001). The knockdown of *npfr1* activity in the fly head was assayed by real-time quantitative reverse transcriptase coupled PCR reaction (RT-PCR) according to standard protocols (Invitrogen and Roche). The cloning of *npfr1* cDNA was described previously (Garczynski et al., 2002), and its coding sequence was subcloned into the downstream of the UAS promoter in the pUAST vector. More detailed information is available upon request.

Food Response Assays

The assay for the intake of quinine-adulterated food is as follows. Each ml of the glucose-yeast paste contains 0.4 g granular yeast powder (Labscientific), 0.1 g glucose, and 40 µl green food dye (McCormick). Quinine (Sigma-Aldrich, MO, USA) was supplemented at concentrations from 0 to 0.8 %. Deprived or nondeprived larvae were rinsed with copious amount of water and subsequently transferred into the quinine- containing yeast paste (0.3 ml) placed on the center surface of a 3% agar plate. Our assay essentially measures "free foraging"; larvae could voluntarily burrow into food and feed or declined the food. In fact, larvae largely behaved in a bimodal fashion during a 20-min test period: they either fed persistently and therefore had a substantial amount of green coloring or moved away from food and displayed largely white gut. To objectively measure larval feeding activity, feeding larvae are defined as those containing green food in > 50% linear length of the midgut, and non-feeding larvae as those with little or no green food (< 10% linear length of the midgut). The representative images

of white and green guts are shown in the supporting materials (Figures 4.5 and 4.6). For those assays that involve *shi*^{ts1}-expressing larvae, the permissive and restrictive temperatures were set at 23 and 30^{o}C, respectively. Larvae were reared and held at 23 ^{o}C. For the temperature-shift experiment, larvae were incubated in prewarmed water or liquid food (30 ^{o}C) for 15 minutes before being transferred to the quinine-containing yeast paste (0.3 ml), which is placed on the center surface of a 3% agar plate pre-warmed to 30 ^{o}C. At least three separate trials were performed for each assay. The data were presented as analyzed using one-way ANOVA, followed by the Student-Newman-Keuls *post hoc* analysis.

ACKNOWLEDGMENTS

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Figure 4.1 The NPF/NPFR1 neuronal pathway acutely suppresses aversive response to noxious food in deprived larvae

a) Under nondeprived conditions, wide-type (Canton S) larvae displayed decreased feeding as the quinine concentration increased from 0 to 0.2, 0.5 and 0.8%. In contrast, larvae fasted for 40 or 120 min showed significantly increased intake of quinine-adulterated foods. b) GFP expression in NPF neurons in the larval CNS driven by *npf-gal4*. c) Immunofluorescence staining of NPFR1 in the larval CNS. Arrow heads indicate NPFR1 neurons in the subesophageal and abdominal ganglia. d, e) Nondeprived experimental (*npf-gal4; UAS-shi*^{ts1} and *npfr1-gal4;UAS-shi*^{ts1})and control larvae were tested for their feeding responses to a rich liquid diet containing quinine. At 23 ^{o}C, control and experimental larvae exhibited similar feeding responses to foods containing different concentrations of quinine ($P > 0.13$). At 30 ^{o}C, the control and experimental larvae also showed similar profiles of feeding response to quinine foods ($P > 0.1$), except one group (*npf-gal4;UAS-shi*^{ts1}) which showed a small yet significant reduction ($P < 0.01$). f, g) Larvae were deprived for 120 min before the assay. Control and experimental larvae showed similar increases in feeding response to quinine foods at 23 ^{o}C ($P > 0.21$). However, the experimental but not control larvae displayed attenuated feeding of 0.5% quinine food at 30 ^{o}C ($P < 0.001$). Sixty larvae per group were tested in three separate trials. Feeding larvae showed bright green color in the gut (see Figures 4.5 and 4.6). Scale bar: 50 μm. Error bars, s.e.m.

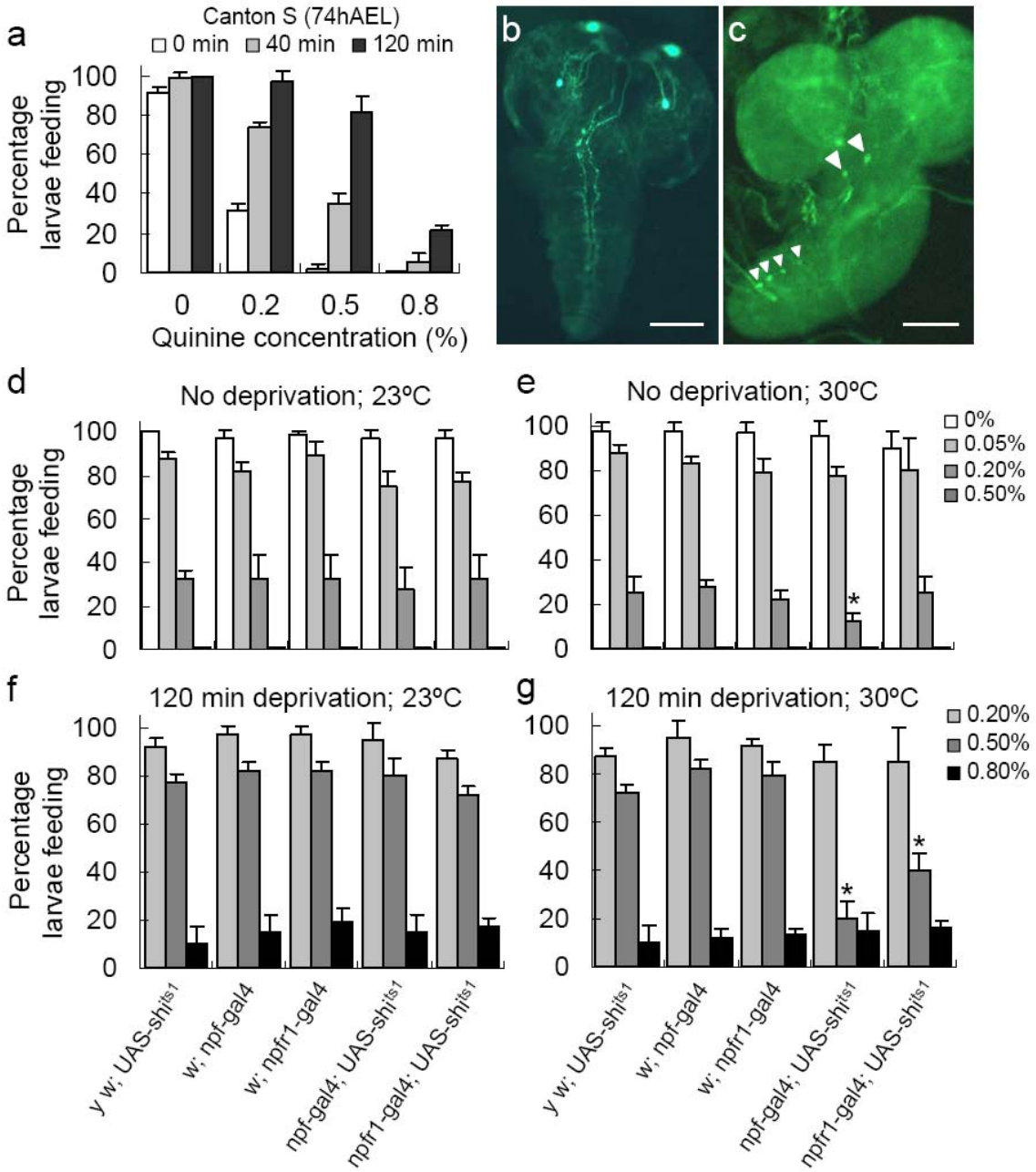

a
Canton S (74hAEL)
0 min
40 min
120 min
Percentage larvae feeding
Quinine concentration (%)
b
c
d
No deprivation; 23ºC
e
No deprivation; 30ºC
0%
0.05%
0.20%
0.50%
f
120 min deprivation; 23ºC
g
120 min deprivation; 30ºC
0.20%
0.50%
0.80%
y w; UAS-shits1
w; npf-gal4
w; npfr1-gal4
npf-gal4; UAS-shits1
npfr1-gal4; UAS-shits1

Figure 4.2 NPFR1 mediates hunger regulation of feeding response to noxious food
a, c) The UAS-*npfr1*^{dsRNA} transgene was selectively expressed in the NPFR1 cells as well as in the nervous system. Nondeprived larvae from experimental and control groups showed similar responses to quinine-containing foods. Deprived experimental larvae (*npfr1-gal4; UAS-npfr1*^{dsRNA}*, elav-gal4;UAS-npfr1*^{dsRNA}, and *appl-gal4;UAS-npfr1*^{dsRNA}) showed impaired feeding of 0.5% quinine food ($P < 0.0001$). In contrast, all control larvae including those expressing *npfr1* dsRNA in muscle cells (*MHC82-gal4;UAS-npfr1*^{dsRNA}) showed normal feeding response. b, d) *UAS-npfr1* encodes a full-length *npfr1* cDNA; *UAS-ANF-GFP* encodes a GFP-tagged preproatrial natriuretic factor (Rao et al., 2001). Larvae expressing NPFR1 (*npfr1-gal4;UAS-npfr1*) showed significant feeding response to 0.2 and 0.5% quinine-containing diet even in the absence of deprivation ($P < 0.01$ and $P < 0.0001$, respectively), as well as to 0.8 % quinine food under 120 min deprivation ($P < 0.001$). At least 45 larvae per group were assayed in three separate trials.

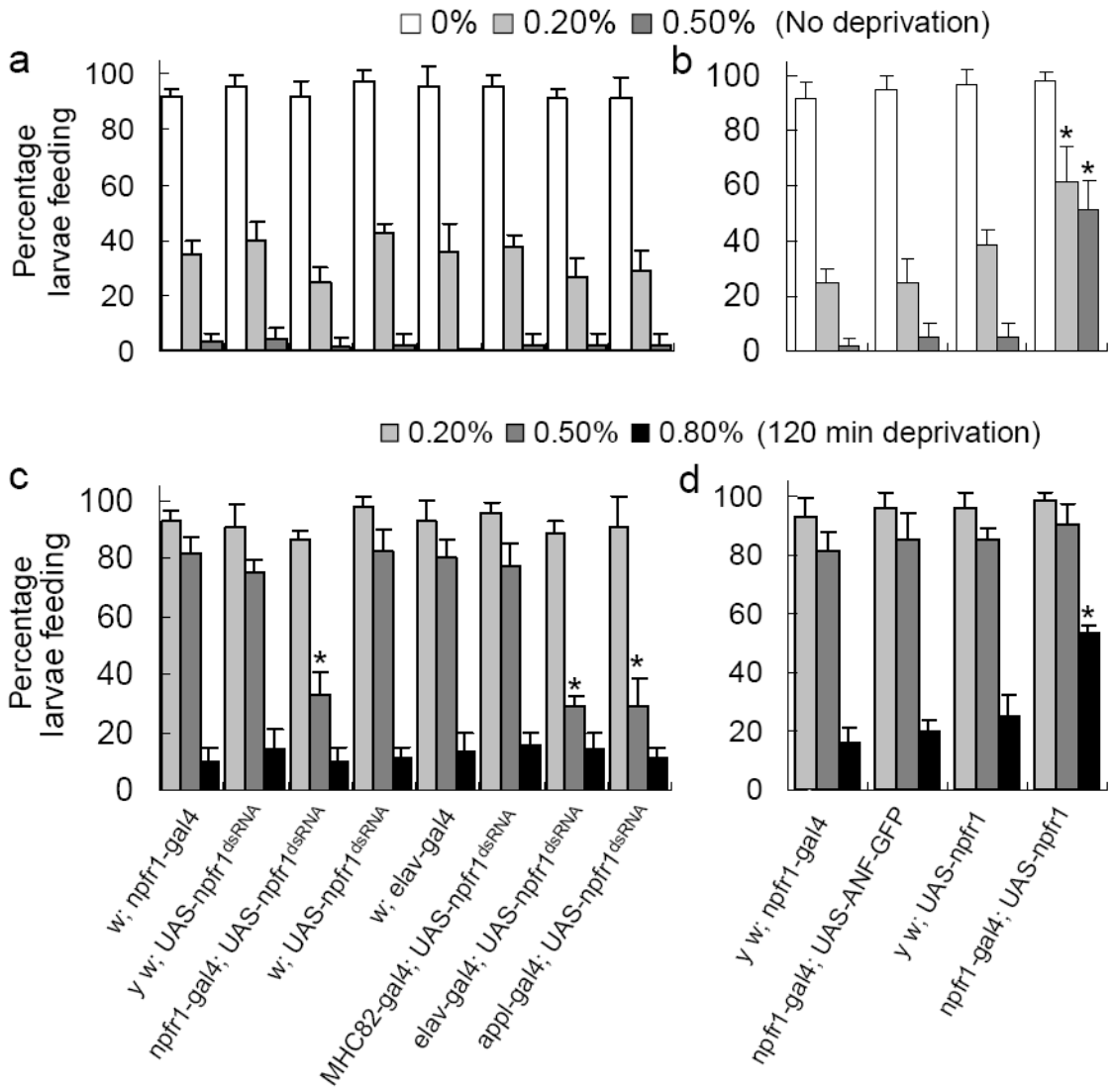

0% 0.20% 0.50% (No deprivation)
a
b
Percentage larvae feeding
100
80
60
40
20
0
*
*
0.20% 0.50% 0.80% (120 min deprivation)
c
d
Percentage larvae feeding
100
80
60
40
20
0
*
*
*
*
w; npfr1-gal4
y w; UAS-npfr1dsRNA
npfr1-gal4; UAS-npfr1dsRNA
w; UAS-npfr1dsRNA
w; elav-gal4
MHC82-gal4; UAS-npfr1dsRNA
elav-gal4; UAS-npfr1dsRNA
appl-gal4; UAS-npfr1dsRNA
y w; npfr1-gal4
npfr1-gal4; UAS-ANF-GFP
y w; UAS-npfr1
npfr1-gal4; UAS-npfr1

Figure 4.3 Up-regulation of dInR signaling in NPFR1 cells attenuates hunger-driven feeding of noxious food

UAS-*dS6K*^{DN} and UAS-*dS6K*^{ACT} encode a dominant-negative and a constitutively active form of dS6K, respectively. UAS-*dInR*^{DN} and UAS-*dInR*^{ACT} each encode a dominant-negative and a constitutively active form of dInR. UAS-*dPI3K*^{DN} encodes a dominant-negative form of dPI3K. UAS-*dp110* and UAS-*dPTEN* encode a catalytic subunit of dPI3K and wild type dPTEN, respectively (Gao et al., 2000;Leevers et al., 1996). **a, c)** When fed *ad libitum*, > 40% of experimental larvae (*npfr1-gal4;UAS-dS6K*^{DN}, *npfr1-gal4;UAS-dInR*^{DN}, *npfr1-gal4;UAS-dPI3K*^{DN}, and *npfr1-gal4;UAS-dPTEN*) displayed feeding responses to 0.5% quinine food. In contrast, < 10% of paired controls (*y w;UAS-dS6K*^{DN}, *y w; UAS-dInR*^{DN}, *y w;UAS-dPI3K*^{DN} and *y w;UAS-dPTEN*) showed feeding responses to the same food. Under deprived conditions, the experimental larvae also displayed significantly higher feeding response to 0.8% quinine ($P <$ 0.01). **b, d)** Deprived larvae expressing dS6K^{ACT}, InR^{ACT} or Dp110 in NPFR1 cells (*npfr1-gal4;UAS-dS6K*^{ACT}, *npfr1-gal4;UAS-dInR*^{ACT} and *npfr1-gal4;UAS-dp110*) exhibited reduced feeding response to 0.5% quinine food relative to their paired controls (*y w*;*UAS-dS6K*^{ACT}, *y w;UAS-dInR*^{Act} and *y w;UAS-dp110*, $P < 0.001$). Moreover, *elav-gal4;UAS-dilp2* larvae that overexpress DILP2 in the nervous system displayed significantly attenuated feeding of 0.5% quinine food relative to controls (*elav-gal4;UAS-GFP* and *w;UAS-dilp2*, $P < 0.001$).

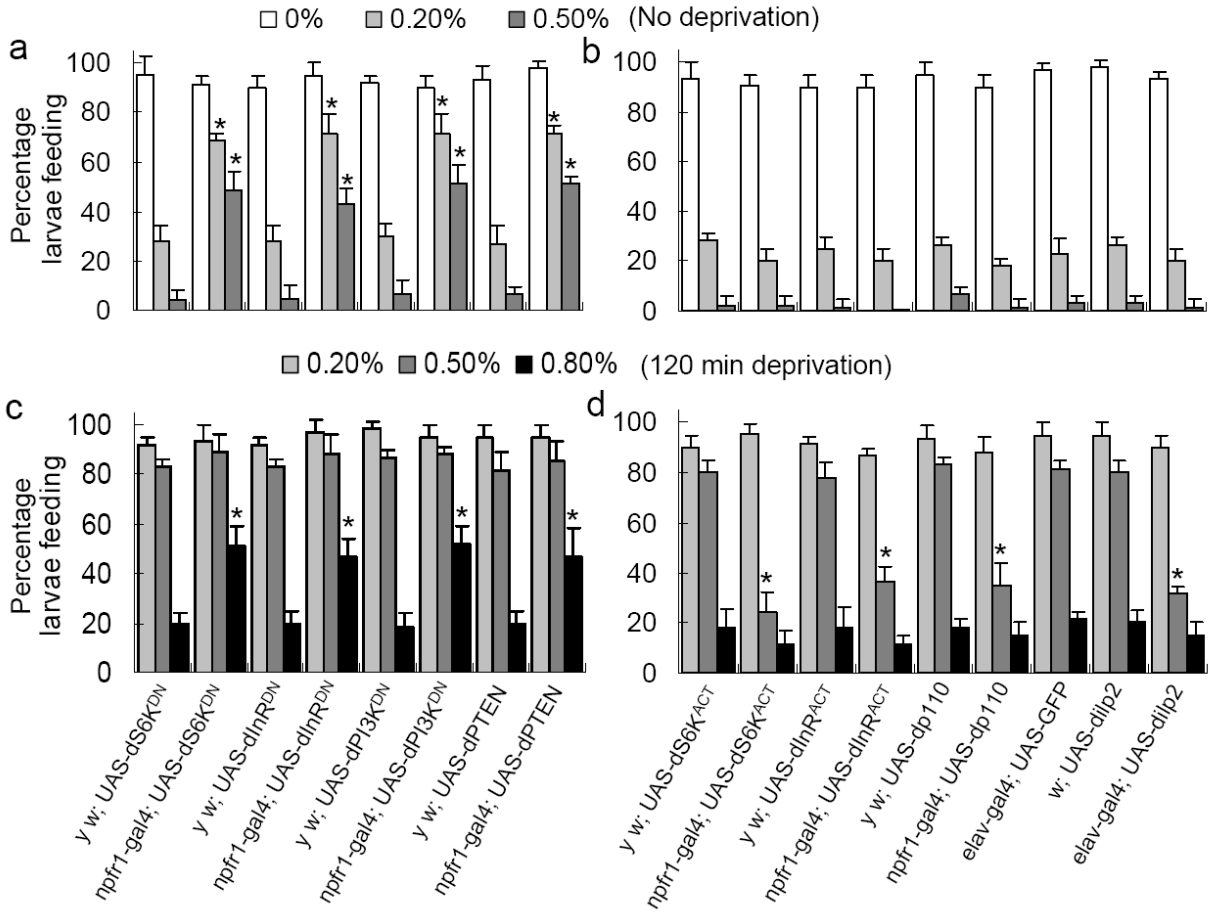

0% 0.20% 0.50% (No deprivation)
a
b
Percentage larvae feeding
100
80
60
40
20
0
0.20% 0.50% 0.80% (120 min deprivation)
c
d
y w; UAS-dS6K^DN
npfr1-gal4; UAS-dS6K^DN
y w; UAS-dInR^DN
npfr1-gal4; UAS-dInR^DN
y w; UAS-dPI3K^DN
npfr1-gal4; UAS-dPI3K^DN
y w; UAS-dPTEN
npfr1-gal4; UAS-dPTEN
y w; UAS-dS6K^ACT
npfr1-gal4; UAS-dS6K^ACT
y w; UAS-dInR^ACT
npfr1-gal4; UAS-dInR^ACT
y w; UAS-dp110
npfr1-gal4; UAS-dp110
elav-gal4; UAS-GFP
w; UAS-dilp2
elav-gal4; UAS-dilp2

Figure 4.4 Simultaneous modulation of *npfr1* and *dS6K* activities in NPFR1 cells suggests that dS6K negatively regulates NPFR1 signaling

Larvae from each group were tested individually for feeding response to quinine-containing foods ($n > 40$ for each of three separate trials). **a, c**) Nondeprived larvae coexpressing $dS6K^{DN}$ and $npfr1^{dsRNA}$ or expressing $npfr1^{dsRNA}$ alone in the NPFR1 cells exhibited strong aversive response to 0.2% and 0.5% quinine-containing foods, similar to control larvae (*y w;UAS-*$npfr1^{dsRNA}$, *y w;UAS-*$dS6K^{DN}$ and *UAS-*$npfr1^{dsRNA}$*;UAS-*$dS6K^{DN}$, $P > 0.17$). In contrast, larvae with reduced dS6K activity only (*npfr1-gal4;UAS-*$dS6K^{DN}$) caused significant feeding response to the same diet ($P < 0.001$). After 120-min deprivation, larvae coexpressing $dS6K^{DN}$ and $npfr1^{dsRNA}$ also showed deficits in feeding of 0.5% quinine food similar to *npfr1-gal4;UAS-*$npfr1^{dsRNA}$ larvae ($P > 0.25$). **b, d**) Under nondeprived conditions, virtually all of the control groups (*y w;UAS-npfr1, y w;UAS-*$dS6K^{ACT}$ and *UAS-npfr1;UAS-*$dS6K^{ACT}$) as well as *npfr1-gal4;UAS-*$dS6K^{ACT}$ larvae declined the 0.5% quinine food ($P > 0.15$). In contrast, larvae coexpressing $dS6K^{ACT}$ and *npfr1 cDNA* or expressing *UAS-npfr1*l alone in NPFR1 cells displayed significant feeding response to 0.5% quinine food ($P < 0.001$). Deprived larvae coexpressing $dS6K^{ACT}$ and *npfr1 cDNA* showed enhanced response to the 0.8% quinine diet similar to larvae overexpressing *npfr1* alone. These results suggested that NPFR1 activity exerts a dominant effect over that of dS6K.

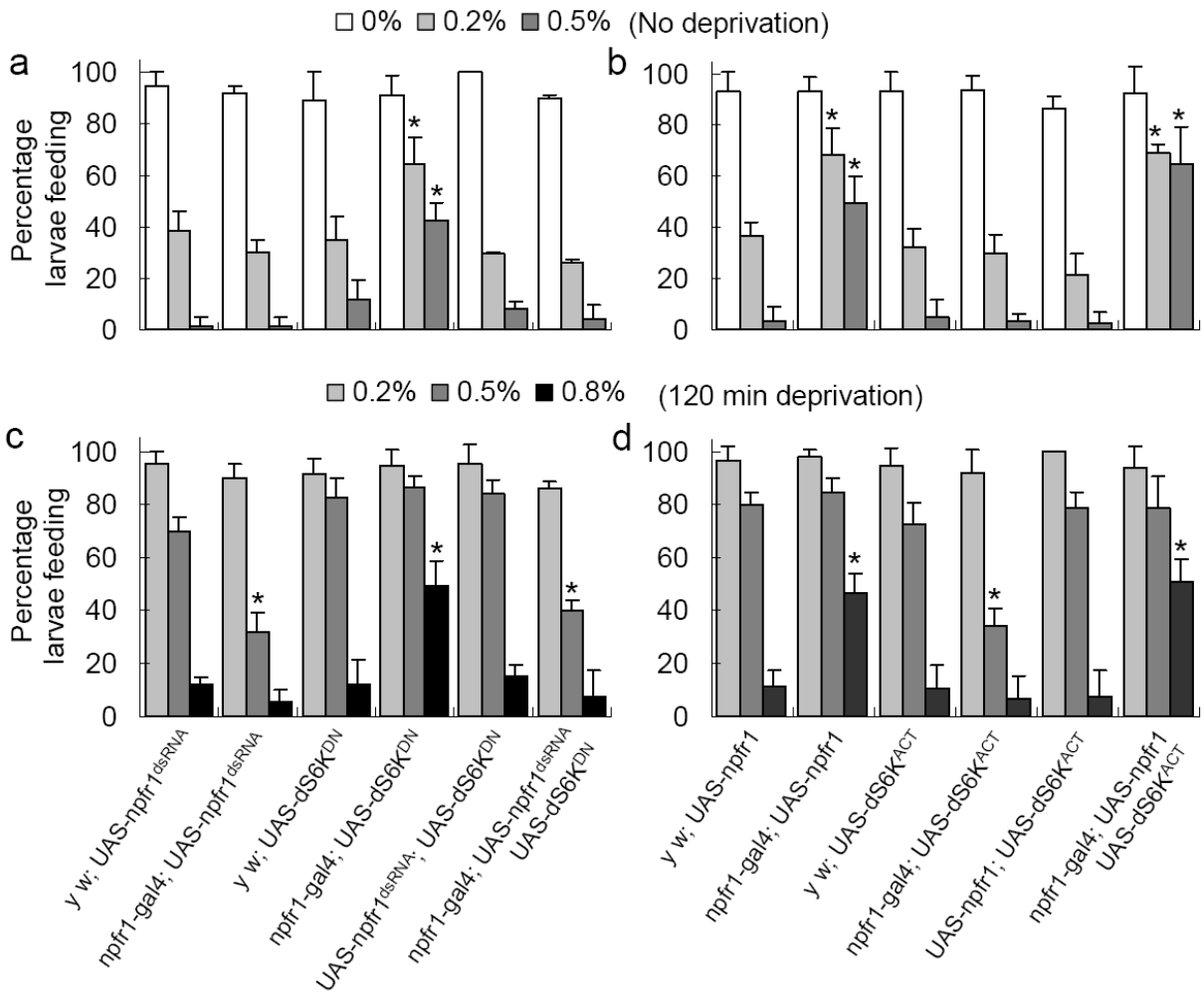

□ 0% ■ 0.2% ■ 0.5% (No deprivation)
a
b
Percentage larvae feeding
100
80
60
40
20
0
*
■ 0.2% ■ 0.5% ■ 0.8% (120 min deprivation)
c
d
y w; UAS-npfr1dsRNA
npfr1-gal4; UAS-npfr1dsRNA
y w; UAS-dS6KDN
npfr1-gal4; UAS-dS6KDN
UAS-npfr1dsRNA; UAS-dS6KDN
npfr1-gal4; UAS-npfr1dsRNA UAS-dS6KDN
y w; UAS-npfr1
npfr1-gal4; UAS-npfr1
y w; UAS-dS6KACT
npfr1-gal4; UAS-dS6KACT
UAS-npfr1; UAS-dS6KACT
npfr1-gal4; UAS-npfr1 UAS-dS6KACT

Figure 4.5 Representative images of feeding and non-feeding larvae in quinine assays
The larval gut is folded in a stereotypical pattern. To objectively measure larval feeding activity, we defined feeding larvae as those containing green food in > 50% linear length of the gut (**a, b**), and non-feeding larvae as those with little or no green food (< 10% linear length; **c, d**). Larvae largely behaved in a bimodal fashion under our assay conditions: they either fed persistently or declined the food (see Figure 4.6). **e**) Nondeprived Canton S larvae were initially transferred into 0.5% quinine-containing green yeast paste and their feeding response were tested as described before. Additionally, aliquots of quinine-free red yeast paste (5ml total) were spotted onto the agar plate around quinine food at about 5cm in distance. The percentage of feeding larvae in each diet was scored after 20min. The majority of larvae (> 80%) were able to locate the new quinine-free food source. $n = 60$ from three separate trials. **f**) A representative image of a larva feeding in quinine-free diet.

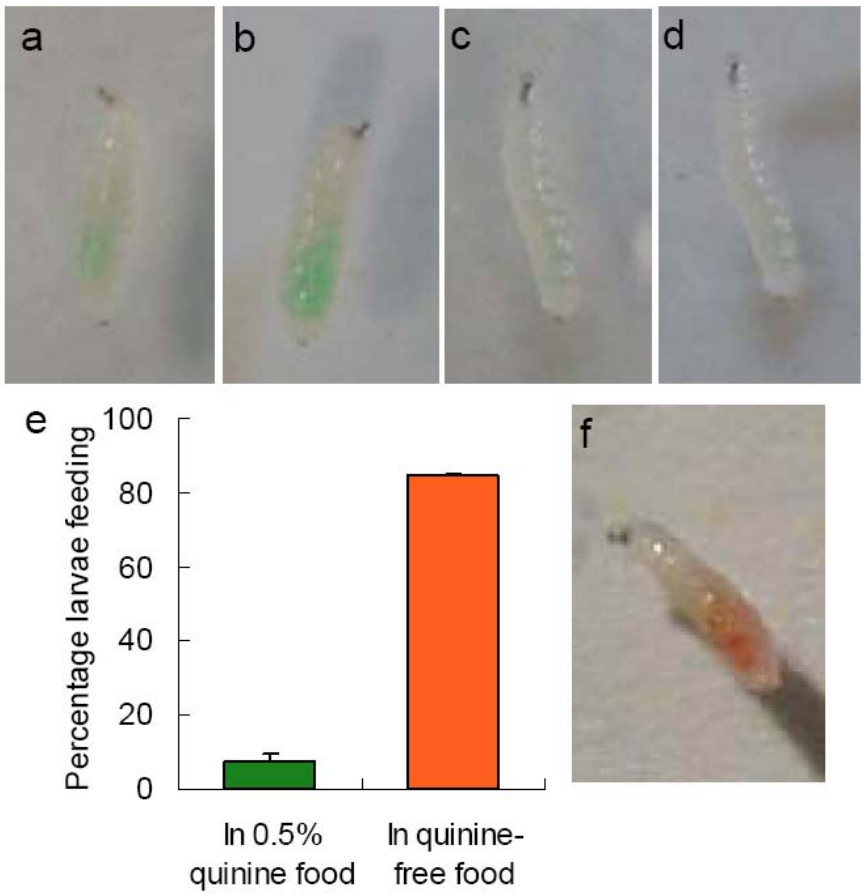

a
b
c
d
e
Percentage larvae feeding
100
80
60
40
20
0
In 0.5% quinine food
In quinine-free food
f

Figure 4.6 Dissection of the midgut of feeding and non-feeding larvae
The representative images of the dissected gut (**a, c, e, g**) from the corresponding larva (**b, d, f, h**). The numbers in parentheses indicate the percentage of the total gut length filled with green food. Typically, larvae can be divided into two groups: those that fed persistently, and had green coloring in > 50% of the gut length (**a-d**); those that sensed/tasted and declined feeding, and had little or no green-dyed food in the gut (< 10% of the midgut length, **e-h**).

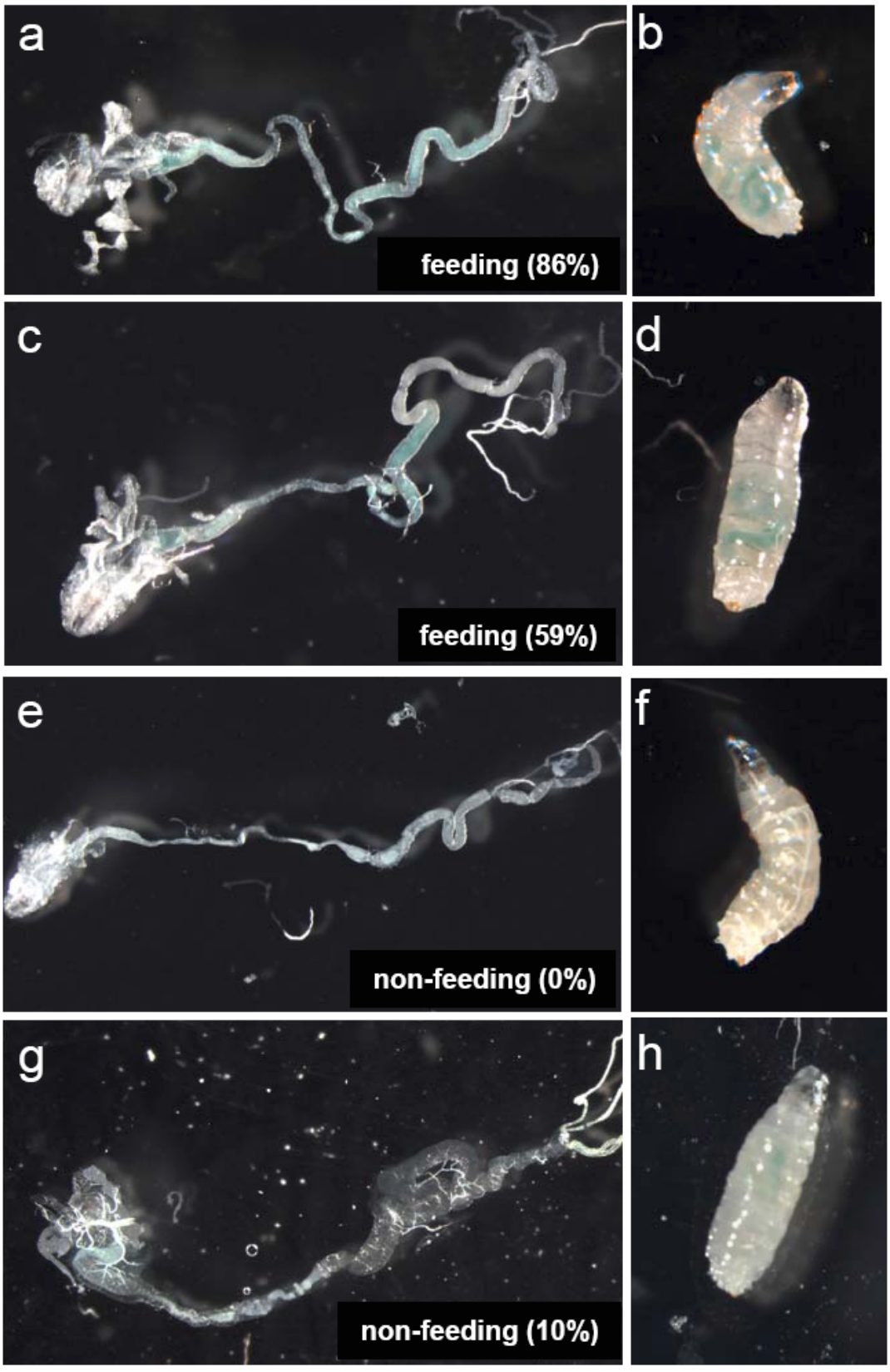

a
feeding (86%)
b
c
feeding (59%)
d
e
non-feeding (0%)
f
g
non-feeding (10%)
h

Figure 4.7 Comparison of the size of adult eyes
Seven-day old male flies fed *ad libitum* on a rich diet (yeast paste on apple-juice agar) were collected for each group ($n > 10$). **a**) The *GMR-gal4;UAS-dInRACT* (left) flies have larger eye size relative to the paired control (*w; UAS-dInRACT*). **b**) The *GMR-gal4;UAS-dInRDN* (right) flies have reduced eye size relative to the paired control (*w; UAS-dInRDN*). **c**) The eye sizes of experimental and control flies were quantified by NIH Imaging software ($P < 0.001$, ANOVA).

GMR-gal4; UAS-InR^{ACT} (left) **vs**
w; UAS-InR^{ACT} (right)

w; UAS-InR^{DN} (left) **vs**
GMR-gal4; UAS-InR^{DN} (right)

c

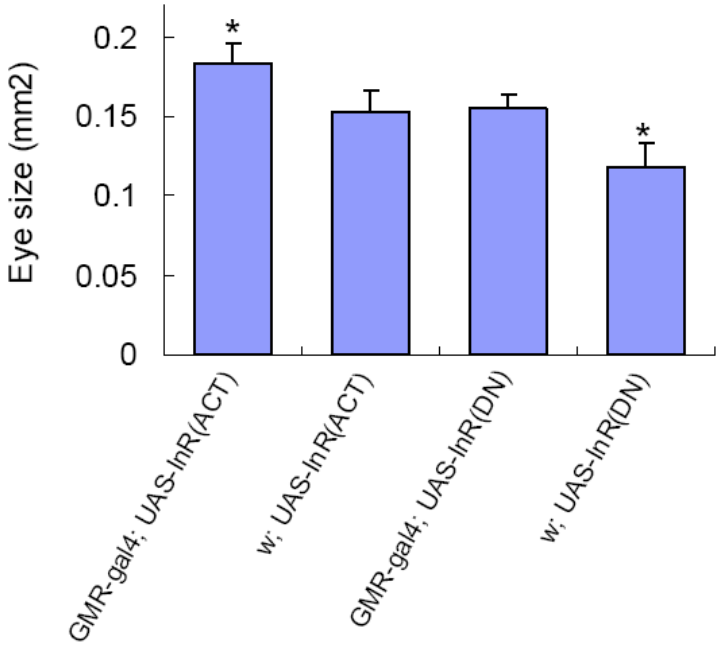
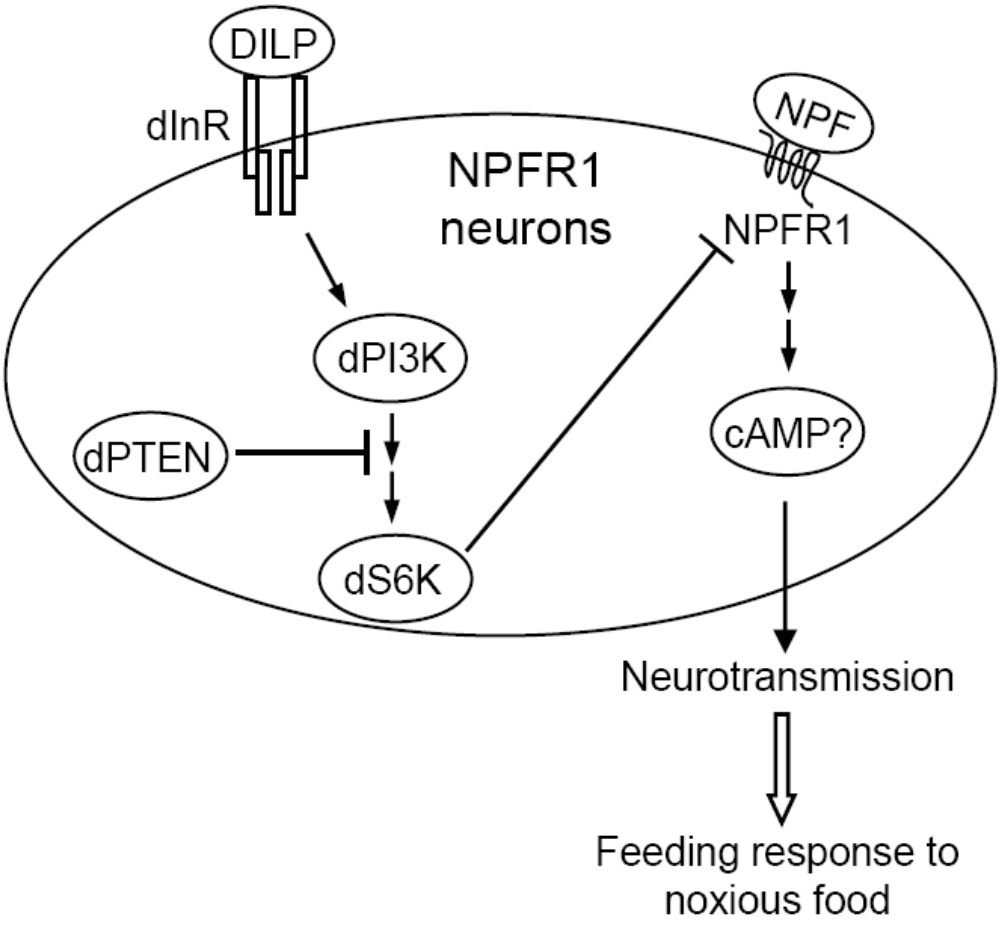


Figure 4.8 Model of the central regulation of risk-sensitive feeding response to noxious food
NPFR1 neurons integrate two distinct extracellular cues, DILPs and NPF. DILP/dInR signaling mediates the transduction of hunger stimuli to NPFR1 neurons through dS6K, which in turn negatively regulates the NPF/NPFR1 signaling pathway. Upon downregulation of dInR/dS6K signaling by hunger stimuli, the NPF/NPFR1 pathway acutely promote compromised intake of noxious food. It's has been previous shown that NPF inhibits forskolin-stimulated adenylyl cyclase activity in NPFR1 expressing cells (Garczynski et al., 2002).


DILP
dInR
NPF
NPFR1
neurons
NPFR1
dPI3K
dPTEN
cAMP?
dS6K
Neurotransmission
Feeding response to
noxious food

CHAPTER 5

CONCLUSIONS AND DISCUSSION

NPY-LIKE SYSTEM DEVELOPMENTALLY AND PHYSIOLOGICALLY MEDIATES FOOD-CONDITIONED BEHAVIOR

We demonstrated that the expression level of the *Drosophila npf* gene is developmentally regulated to switch on and off two opposite behaviors in third-instar larvae, feeding and food aversion. A high level of *npf* mRNA is detected in the brain of young larvae attracted to food, and loss of NPF signaling by either targeted cell ablation, temperature-controlled blockage of neurotransmission, or gene silencing through RNAi results in the phenotypes of premature insensitivity of food response, food-conditioned hypermobility, and precocious social burrowing. Conversely, *npf* expression is developmentally downregulated in the brain of older wandering larvae showing food-aversive behavior. Overexpression of *npf* broadly in the nervous system in a pattern encompassing peptidergic neurons leads to delayed transition to the nonfeeding phase and eliminates food aversion and social burrowing behaviors in older larvae.

More importantly, we provide strong evidence that the NPF signaling system is not essential for promoting baseline feeding, but rather for maintaining normal foraging activity under deprived or adverse conditions. These observations reflect a striking similarity between the *Drosophila* NPF and its mammalian counterpart, NPY. *Drosophila* larvae deficient in NPF signaling display normal growth and developmental profile when fed ad libitum. The intake rate of liquid food also remains identical between NPF signaling-deficient larvae and controls.

However, loss of NPF signaling causes drastically reduced response to less-accessible solid food, which is demonstrated by the fact that NPF-deficient larvae display much lower frequency in extracting food from solid agar in comparison with the control animals. In parallel, NPY knockout mice in a C57BL/6 background show up to 50% reduction of post-fast food intake (Segal-Lieberman et al., 2003). Among the six siblings (Y1 – Y6) of the NPY receptor family, Y1 receptor has been implicated in regulating fast-induced hyperphagia; Y1 homozygous mutant mice have slightly diminished daily food intake and NPY-induced food intake, while fast-induced feeding is significantly decreased (Pedrazzini et al., 1998). Our results suggest that NPFR1 is essential for motivational feeding under deprived condition and may serve a similar role as the mammalian Y1 receptor. Furthermore, phenotypes of increased anxiety, fear, and sensitivity to stress stimuli in NPY-deficit mice have been well documented (Silva et al., 2002) and are reminiscent of the sugar-induced hypermobility displayed by the NPF neuron-deficient larvae. In this dissertation, I have provided a large body of evidence to support the notion that NPY-like signaling system is not only structurally but also functionally conserved in *Drosophila* and mammals. Taken together with the fact that major components of the identified neuropeptide signaling pathways are structurally conserved between mammals and insects, the NPF system may provide a novel paradigm in studying the general mechanisms underlying the modulation of adaptive behavioral response by different neuropeptides.

Our experiments also suggest that the NPF system developmentally modulates the initiation of a cooperative burrowing behavior. Loss of NPF signaling in young feeding larvae is sufficient to trigger premature onset of social bordering and clumping phenotypes normally displayed by older wild-type larvae, while NPF-overexpressing larvae remain solitary throughout the third instar life and eventually form puparia on moist food-coated surface which is extremely

susceptible to the detrimental overgrowth of mold and bacteria. These results indicate that the developmental activation of social aggregation in wandering larvae initiates a cooperative burrowing behavior which allows larvae to efficiently penetrate food layers, thereby securing their migration from foraging precinct to desirable food-free loci to form puparia. Therefore, a critical biological significance underlying the developmental downregulation of NPF signaling in the larvae exiting the feeding stage has been revealed: it initiates food-aversive response and social burrowing activity, which greatly augments the odds of survival from pupae to adults.

DS6K DIFFERENTIALLY MEDIATES HUNGER-MOTIVATED FEEDING BEHAVIORS

Food deprivation elicits a broad array of adaptive responses at both the cellular and systemic levels. However, the molecular and neural mechanisms underlying the central integration of hunger stimuli and organization of behavioral adaptation still remain elusive. It has been reported here that dS6K, a previously known nutrient-sensing effector, exerts strong effects on hunger-motivated adaptive food response by integrating peripheral hunger signals into a neuronal circuit that consists of DILP/dInR and NPF/NPFR1 signaling pathways, along with a variety of corresponding intracellular components. More specifically, downregulation of dS6K activity in the DILP neurons of well-fed larvae leads to a significant increase of the ingestion rate of enriched foods (e.g., liquid yeast paste) as well as motivated intake of nonpreferred foods (e.g., a less-accessible solid food), while its upregulation attenuates such hunger-driven feeding responses in fasted animals. Gain-of-function analyses also imply that DILP2 and DILP4, but not DILP3, negatively regulate hunger-driven feeding response by acting as neurohormones on downstream targets including the NPFR1 neurons. On the other hand, larvae expressing a

dominant-negative form of dS6K in the NPFR1 neurons display hyperactive feeding of solid food, yet normal ingestion rates of the enriched liquid media. Conversely, deprived larvae with elevated dS6K activity in the NPFR1 neurons exhibit attenuated response to solid but not liquid food. Furthermore, functional disruption of key components of the dInR pathway in the NPFR1 neurons causes similar phenotypes as those displayed by larvae carrying loss-of-function mutation of dS6K in the same subset of cells. These findings indicate that the dS6K activity of the NPFR1 neurons is essential for the regulation of food preference, but does not contribute to the regulation of feeding rate.

The biological role of NPFR1 on motivational feeding has been assessed through up-/down-modulation of *npfr1* gene activity in *Drosophila* larvae under various conditions of food deprivation, availability, and quality. Under well-fed or mild-deprived (30 min) conditions, NPFR1 overexpression selectively promotes hunger-induced intake of nonpreferred solid food, while leaving ingestion rate intact. Yet the same larvae that are subject to prolonged deprivation for 120 min exhibit a normal response to food in comparison with the controls, which could be due to the possibility that the endogenous level of NPFR1 induced by severe hunger has approached a plateau. In contrast, targeted knock-down of *npfr1* activity in the nervous system of fasted larvae causes significant deficits in feeding of the solid but not liquid food, indicating that neural NPFR1 mediates hunger regulation of food preference.

Our results suggest the following model for illustrating the hunger regulation of adaptive feeding behaviors in *Drosophila*. Upon sensing the peripheral hunger stimuli, dS6K activity is downregulated in the DILP neurons, which leads to the suppression of DILP synthesis and/or release that negatively regulates a downstream NPF-dependent and another NPF-independent pathway. The former selectively mediates motivational feeding of nonpreferred foods (food

preference) by lowering the default threshold of food acceptance; the latter promotes a general increase in ingestion rate, thereby enabling more effective food consumption. A default pathway that mediates the feeding rate under optimal foraging conditions may also exist and is largely insensitive to the DILP and NPF signaling pathways, since overexpression of dS6K, DILPs, or NPFR1 in nondeprived larvae do not interfere with the uptake of rich liquid media. We hereby conclude that dS6K functions as a key regulator of diverse hunger-driven behaviors by signaling differentially through the insulin- and NPY-like systems under various nutritional states.

DROSOPHILA NPY- AND INSULIN-LIKE SYSTEMS COORDINATELY REGULATE RISK-SENSITIVE FORAGING BEHAVIOR

In terms of the compostion of food sources, animals can be divided into two categories, monophagy and omnivory. Because monophagous animals, such as vampire bats, can acquire a nutritional balanced diet by simply feeding on a single food or restricted range of foods, they have little demand to contact or ingest unfamiliar foods potentially containing toxins. In contrast, omnivores, including humans, rodents and fruitflies, must ingest a suitable mix of different foods in order to meet their nutritional requirements. Thus, in an attempt to find sources of adequate nutrients via broadly sampling among foods in the natural environment, omnivory has much higher frequency of being exposed to toxic agents than does monophagy. During the history of evolution, omnivores have developed a highly sophisticated mechanism that facilitates not only the selection of adequate diet but also the identification and rejection of toxic substances normally associated with aversive taste and/or smell. An Optimal Foraging Paradigm has been proposed by Charnov and colleagues in order to explain the strategies employed by animals to manage the risks associated with foraging behaviors (Charnov, 1976). Such risk-sensitive

preference of noxious/aversive-tasting foods is subject to the change of a range of physiological and environmental factors, among which the energetic status of the forager is very prominent. Generally speaking, animals on a positive energy budget (e.g. fed ad libitum) tend to be risk-averse by rejecting noxious foods; while animals on a negative energy budget (e.g. deprivation of food) are risk-prone in food acquisition. So far, the genetic and neural mechanisms underlying an animal's optimized strategy on risk-sensitive foraging are largely unknown.

Elucidating the underpinning mechanisms of adaptive response to noxious foods should be helpful for demystifying the complex risk management associated with foraging decisions. In this dissertation, we have been using *Drosophila* larvae as a model to analyze the aversive response to food adulterated with quinine, a bitter-tasting substance, under different energy states. Wild-type larvae fed ad libitum display strong feeding response to quinine-free diet; whereas larvae show compromised intake of quinine-adulterated media in proportion to the duration of deprivation. Our studies reveal that the NPFR1 neurons centrally mediate the response to noxious food by integrating two categories of extracellular cues, DILPs and NPF. Overexpression of NPFR1 is sufficient to bypass hunger stimuli and enables nondeprived larvae to accept noxious foods, whereas larvae with downregulated neural NPFR1 signaling display the opposite phenotype. A temperature-shift assay suggests that the NPF/NPFR1 signaling pathway is tonically required for suppressing aversion to noxious foods in deprived animals because blocking neural transmission for merely 15 min is sufficient to cause drastic reduction of noxious food uptake. Furthermore, it has been implicated that the dInR signaling pathway is responsible for transducing hunger stimuli to NPFR1 neurons through dS6K, which in turn negatively acts on the NPFR1 signaling pathway. For example, targeted disruption of dInR or one of the downstream components in the NPFR1 neurons leads to increased response to noxious foods by

nondeprived larvae. In contrast, fed larvae with elevated dInR signaling activity in NPFR1 neurons display attenuated feeding response to quinine-adulterated food similar as those shown by NPFR1-deficient larvae. Moreover, epistatic analyses through simultaneous up-/down-modulation of NPFR1 and dS6K in the same subset of NPFR1 cells indicate that NPFR1 exerts dominant effects over dS6K on the regulation of aversive response to noxious foods. Taken together, our results strongly support a novel conception that a neuronal circuit composed of the insulin- and NPY-like signaling systems is essential for the dynamic regulation of risk-sensitive foraging behaviors, which maintain a balance between the sufficiency of nutrient supply and the avoidance of ingestion-induced toxicosis.

OVERALL SIGNIFICANCE AND FUTURE DIRECTIONS

The overall significance of this dissertation is summarized as follows:

1) The research in this dissertation has provided substantial evidence to support a conception that the conserved NPY signaling system is not essential for the regulation of baseline feeding in both vertebrates and invertebrates, yet plays a pivotal role in maintaining motivated foraging activity (e.g. intake of less-nutritious foods or unpalatable foods) under deprived conditions. The stimulatory role of the NPY-like system in feeding behavior has been recapitulated and better characterized through conditional and inducible transgenic analyses in *Drosophila*. In conjunction with our results, a parallel study in transgenic rodent models by employing a similar strategy would be extremely helpful to override the contradictory statements from old literature and establish the endogenous NPY system as a stimulator of feeding and foraging activities when energy demands are increasing.

2) We have also provided strong evidence that the insulin-like peptides mediate hunger regulation of food preference by directly signaling to the downstream NPY receptor-like neurons. Furthermore, results of the epistatic analyses implicate that dS6K appears to bridge the insulin and NPY signaling pathways together and mediates diverse food response under different energy states. Our findings strongly support a notion that a conserved insulin-NPY interaction exists in the CNS and contributes to the regulation of energy balance. This conception has also been supported by limited evidence from rodent models (see details in Chapter One).

3) A model for the hunger regulation of adaptive feeding behaviors in *Drosophila* has been proposed accordingly. In this model, an NPF/DILP neuronal circuit has been shown to participate in the dynamic regulation of diverse hunger-driven behaviors, including food intake rate, food preference, and avoidance of noxious foods. In general, the *Drosophila* NPF/DILP neural network characterized in this dissertation provides an important entry point into understanding how genes, signaling pathways, and neural circuits are integrated in the dynamic regulation of feeding behaviors and energy homeostasis.

Hence, my research has laid the groundwork for future studies on the molecular and neural mechanism underlying the control of food intake. It still remains unknown as to what signaling molecules mediate the NPF-independent pathway for promoting general feeding rate downstream of the DILP/dInR pathway, and what is involved in the default feeding pathway that controls ad libtum feeding of high-quality foods. It would be rewarding to initiate a large scale genetic screen to look for the candidate genes that contribute to these two pathways. Moreover, it is necessary to determine the upstream sensory input and downstream cellular targets of the neural NPF/NPFR1 signaling pathway and the underlying mechanism of how they interact with each other. On the other hand, how the NPF signaling in the gut regulates feeding behavior has

not been addressed and the hypothesis that gut NPF may function as an inhibitory factor in appetite control just like its mammalian counterparts PYY needs to be tested carefully. We have shown for the first time the functional distinction among *dilp2-4* in control of food intake, but whether and how other *dilp* homologs contribute to the feeding behavioral are still unanswered.

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