THE ROLE OF PEPTIDERGIC NEURONS IN THE REGULATION OF SATIETY IN *DROSOPHILA*

Thesis by

Anne Christina Hergarden

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ABSTRACT

Understanding the neural mechanisms that motivate us to eat is important because of the increasing rates of obesity and the consequential increasing rates of diabetes and cardiovascular disease in our society. The aim of this dissertation is to gain insight into the neuromodulators and neural mechanisms that regulate satiety. To do this, we turned to *Drosophila melanogaster*, which has been a powerful model organism to study the molecular mechanisms underlying innate animal behaviors and which exhibits many conserved elements of feeding regulation and energy homeostasis found in mammals. A common theme in animal behavior is that food deprivation modifies behavioral responses, e.g., the likelihood that an animal will accept a low-nutrient food. I manipulated the parameters of a feeding assay to screen for animals that lacked several starvation-induced feeding behaviors: increased foraging for food, increased acceptance of low-nutrient food, and increased ingestion of low-quality food. Using this feeding assay, I identified a neuronal circuit manipulation that inhibits several starvation-induced behaviors. Activation of a subset of Allatostatin-A-expressing neurons, using a novel transgenic tool that we generated, inhibits starvation-induced changes in both the acceptance and the ingestion of low-quality foods. In contrast, this circuit manipulation did not affect starvation-induced metabolic changes or foraging behavior. This suggests that we tapped into a mechanism that regulates a specific subset of starvation-induced changes in feeding behavior that is independent from general starvation-induced behavioral responses and energy metabolism. Studies in blowflies have revealed that the primary mechanism that promotes satiety is inhibitory proprioceptive feedback from the gut, but whether such a mechanism operates in *Drosophila* is unclear. While Allatostatin A has been implicated as a satiety factor and as a myoinhibitor in several other insects, it has no known function in *Drosophila*. A mechanism that promotes satiety but that does not alter energy metabolism has not previously been identified in *Drosophila*. I have used this circuit manipulation to better understand how a state of satiety is achieved in *Drosophila*, by integrating the knowledge acquired from studies in other insects with the knowledge acquired from molecular genetic manipulations in *Drosophila.*

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ABREVIATIONS AND DEFINITIONS

Neuoropeptides and neuropeptide receptors

AKH Adipokinetic hormone promotes fat mobilization in nutrient-deprived flies. AKH mutants do not display starvation-induced feeding or starvation-induced hyperactivity.

AKHR AKH receptor mutants do not display starvation-induced feeding but display normal starvation-induced hyperactivity.

AstA Allatostatin A has been implicated as a satiety factor and myoinhibitor in other insects.

Crz Corazonin neurons have been implicated in the regulation of stress responses and promote similar metabolic effects as AKH.

DAR-1 and -2 *Drosophila* AstA receptors

DILP *Drosophila* insulin-like peptide promotes the deposition of fat stores when hemolymph sugar levels are high. DILP is also required for normal growth and development and some studies have implicated a role for DILP in the regulation of feeding.

Hugin neurons promote feeding on novel foods in unstarved flies.

InR DILP receptor

JH Juvenile Hormone promotes the diversion of fat stores towards egg production in mated females. AstA inhibits JH synthesis in some insects, not including flies.

Leucokinin plays a role in the regulation of meal size.

NPF Neuropeptide F promotes starvation-induced feeding and foraging.

NPFR NPF receptor

Transgenic tools

Gal4/UAS This is a binary expression system used to spatially and temporally restrict transgene expression in *Drosophila.*

UAS-mcd8::GFP Overexpresses a membrane-tethered green fluorescent protein.

UAS-Kir2.1 Overexpresses an inwardly-rectifying potassium channel which reduces neuronal excitability.

UAS-NaChBac Overexpresses a voltage-gated bacterial sodium channel which increases neuronal excitability.

UAS-Ricin Overexpresses a biotoxin that causes cell-autonomous cell death.

UAS-TNT Overexpresses tetanus toxin which blocks synaptic transmission.

UAS-TRPA1 Overexpresses a temperature-sensitive cation channel which increases neuronal excitability at 28°C but not at 22°C.

Other

CAFE assay Capillary Feeding Assay

CNS Central nervous system

DDC Dopa decarboxylase is an enzyme required for the synthesis of serotonin and dopamine.

MAN The median abdominal nerve transmits proprioceptive feedback from the crop, and is required to inhibit feeding in starved blowflies.

RN The recurrent nerve innervates the aorta, foregut, and crop. Proprioceptive feedback from the foregut, transmitted via the RN, inhibits feeding in blowflies.

SOG The subesophageal ganglion receives primary gustatory input.

SGS The stomatogastric nervous system regulates the motor patterns of feeding behavior and transmits peripheral sensory information from the gut. The SGS is analogous to the mammalian autonomic nervous system.

TH Tyrosine hydroxylase is required for dopamine synthesis.

VNC Ventral nerve cord

Chapter 1

INTRODUCTION

1. The scientific relevance of studying satiety

Globally there are more than 1 billion overweight adults, 1/3 of which are obese, and rates of obesity are increasing worldwide [1]. Obesity is one of the leading causes of preventable deaths in the United States, responsible for 1 in 10 deaths [2]; It increases the risk factor for medical complications such as diabetes, cardiovascular disease, and some forms of cancer [3]. In the U.S., 9.1 % of total medical expenditures in 1998 were due to medical complications caused by being overweight [4]. Because of the many health and economic costs of obesity, it is important to better understand the regulation of energy homeostasis and feeding behavior.

The sheer number and diversity of life forms can be attributed to developing proficiency at: converting a variety of compounds into utilizable energy; storing excess energy for use in times of scarcity; and acquiring ways to adapt to harsh environments [5, 6]. In animals, regulatory mechanisms promote feeding on high calorie foods, promote energy storage, and minimize energy expenditure. The ever-increasing incidence of obesity in Westernized culture is fueled by an excess of high fat, high sugar foods in a technologically advanced urban world, where physical activity is a choice [6, 7]. It is medically and economically relevant to understand the neural and molecular underpinnings of hunger and satiety, how food is attributed a hedonic value, how food intake is regulated, and how the body maintains homeostasis.

In mammals, the central nervous system (CNS) integrates internal and external cues, regulates feeding behavior, and coordinates the activities of multiple peripheral organs to maintain homeostasis [5, 8]. Complex feedback loops between peripheral organs and central regulatory centers interact to maintain stable blood glucose levels and to induce feeding and foraging to replenish energy stores [5, 8]. How this barrage of information is

tracked and integrated to regulate feeding and energy homeostasis is not very well understood, and is an important and difficult challenge to scientists today [5, 8–10].

Drosophila melanogaster (fruit flies) provide an excellent model system in which to study this complex problem because many elements of feeding regulation and homeostasis are conserved between *Drosophila* and mammals [7, 11–13]. Over 100 years of *Drosophila* genetic research has resulted in the characterization of many genes, signaling pathways, physiology, and behaviors [14]. Extensive libraries of mutants and transgenics have been created, as well as tools for manipulating genes and neuronal circuits [15]. Historically, a limitation of *Drosophila* as a model system has been determining neuronal connectivity in the central nervous system [15]. The brain, which is the size of a poppy seed, is a dense ball of interconnected axons and dendrites (neuropile) surrounded by a shell of neuronal cell bodies [15, 16]. Although neuromodulatory neurons have been described in terms of molecular signaling and neuroanatomy, connectivity has been difficult to determine because the same neuronal processes can contain both inputs and outputs [15–17]. How "the as yet impenetrable interneuron jungle" [18] of the brain regulates behavior has been a long-standing challenge [15, 16]. Recently, transgenic tools have been developed that allow us to establish connectivity, to visualize real-time neuronal activity, and to inducibly activate and silence neuronal circuits [15, 16]**.** This affords us the opportunity to leverage the knowledge of *Drosophila* genes, signaling pathways, physiology, and behavior to tackle the questions of how the CNS coordinates homeostasis and feeding behavior.

In *Drosophila*, little is known about how the CNS regulates feeding behavior in response to homeostatic perturbations [9, 11, 17]. Although energy homeostasis and feeding behavior are tightly regulated [11, 19], the majority of studies have focused on energy metabolism while mostly overlooking the regulation of feeding behavior [10, 17]. In addition, most of these studies have focused on the feeding behavior of larvae, which feed continuously and are in a life stage of rapid growth [7, 12]. In contrast, adult *Drosophila* have exited the growth phase, are discontinuous feeders, and face more complex life decisions than larvae [7, 12]. Understanding the regulation of feeding

behavior in adult *Drosophila* would provide a more accurate model of how mammalian feeding is regulated [7, 12]. Studying insect feeding behavior could also contribute to improved methods of pest control, which could be used to target insect populations that transmit diseases and that damage agricultural crops.

2. The regulation of satiety: Lessons from the blowfly

The majority of current knowledge about the neural regulation of insect feeding comes from in-depth investigations of the feeding behavior of the blowfly, or *Phormia regina*, and, according to studies conducted in other insects, this knowledge can be generalized to other insects [17, 18, 20]. The mechanisms that regulate feeding behavior in starved blowflies are different from those that regulate feeding under ad libitum[1] feeding conditions [19, 21]. I will focus mainly on how feeding is regulated under starvation conditions in order to better understand how a state of satiety is achieved. First, I will summarize the studies that characterized the neural regulation of feeding in starved blowflies; these studies are described in detail in [20].

In the blowfly, satiety is measured in terms of sugar responsiveness [21, 22]. A starved fly will respond to and accept a lower concentration of a sugar solution than will a fed fly [21, 22]. This can be quantified by stimulating the taste sensillae on the foreleg of a fly with stepwise increasing concentrations of a sugar solution until the fly responds by extending its proboscis[2], or mouthparts. The lowest concentration to which a fly responds is designated the acceptance threshold[3], which, depending on the degree of starvation, can vary over a hundred fold range of concentrations for some sugars. This method measures the relative degree of starvation either between flies or within a single fly over time. Thus a low or reduced acceptance threshold represents hungry or more nutrient-deprived flies

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[1] Ad libitum means "at one's discretion" in Latin.

[2] The fly proboscis is a retractrable straw-like appendage through which flies feed.

[3] "Although threshold lies somewhere between the concentration that elicits extension and the one in the series immediately below it, the higher of the two is arbitrarily designated as threshold. Since the aim of practically all experiments was to obtain data for comparative analysis, this fiction was acceptable" [20].

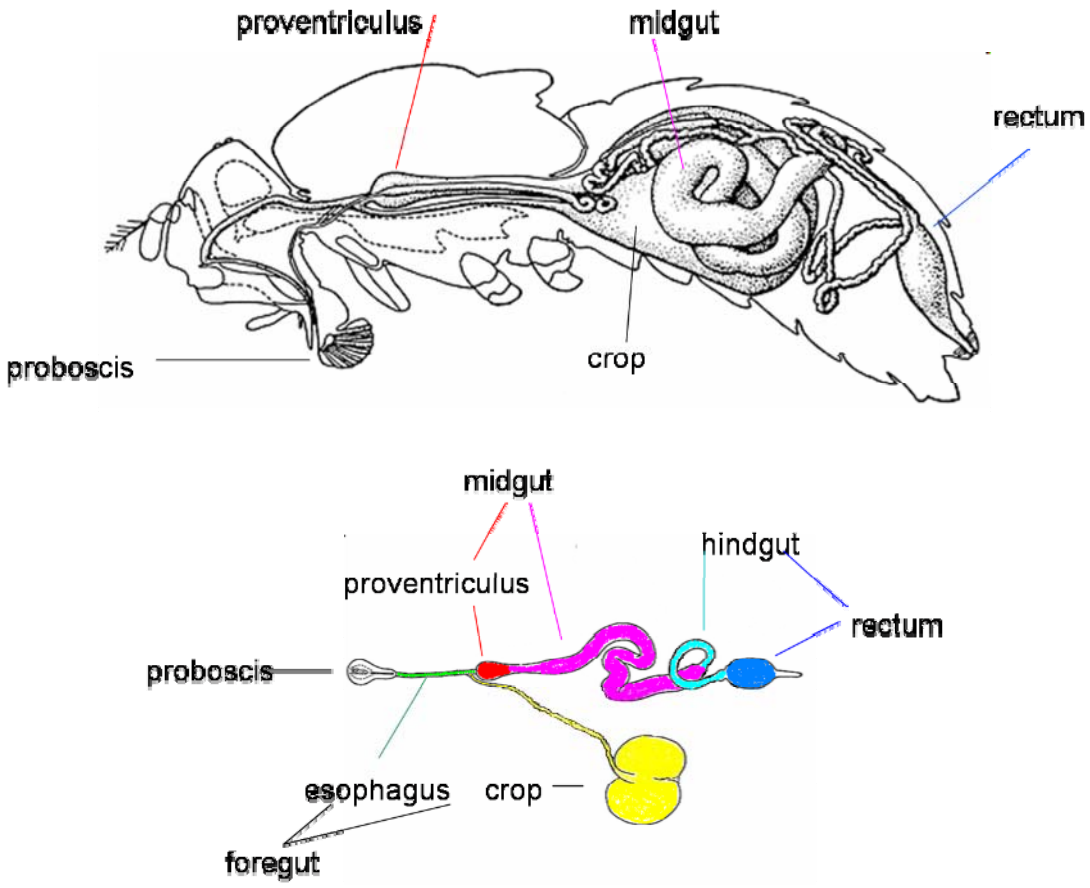
and a high acceptance threshold represents a state of satiety.

There are several physiological mechanisms that regulate satiety in the starved blowfly. The first and most potent inhibitor of feeding is crop distention [23]. The crop is an expandable sac that arises from an invagination of the foregut ([17], see Figure 1). When a starved fly feeds, food fills the midgut first, where the majority of digestion and nutrient absorption occurs [10], and then fills the crop, which is mostly used for food storage [17]. When food was prevented from entering the midgut, by tying off or severing the midgut, the volume of food intake of starved flies was comparable to sham operated starved flies, as was the increase in acceptance threshold [20]. Since little or no digestion occurs in the crop, this demonstrates that a state of satiety can be induced without food entering the midgut or ensuing nutrient absorption. Consistent with the hypothesis that nutrient absorption is not mediating these effects, injection of a sugar solution into the hemolymph of a starved fly was not sufficient to decrease the acceptance threshold [20]. Thus, a state of satiety can be achieved by the act of feeding, regardless of hemolymph sugar levels.

Anatomical and electrophysiological studies elucidated the mechanism by which crop distention regulates satiety. Studies demonstrated that severing the median abdominal nerve (MAN) results in hyperphagia, defined as at least a twofold increase in food intake relative to sham-operated controls [24]. The MAN contains projections from a nerve net surrounding the crop [25]. Electrophysiological recordings from this nerve revealed neurons that increased their firing rate upon crop distention, indicating that proprioceptors are monitoring crop distention. Therefore it is proposed that proprioceptive feedback from the crop is necessary and sufficient to promote satiety [26].

Figure 1. Anatomy of the digestive tract in *Drosophila*

A sagittal view of the digestive tract illustrated within the body of *Drosophila* (reprinted with permission from [27]. Shown below, is an illustration of the digestive tract indicating the foregut (green), crop (yellow), midgut (red and pink), and hindgut (blue). The crop duct branches off of the foregut. The proventriculus (red) contains a valve that separates midgut from foregut contents.



A second mechanism that contributes to the promotion of satiety in starved blowflies is foregut activity. When food was prevented from entering the crop, by lesioning or tying off the crop duct, sugar acceptance threshold rose for a few hours [20]. This suggests that the crop is not the only source of inhibitory feedback. To determine whether food within the midgut is sufficient to promote satiety, food was injected into the midgut of

a starved fly [20]. Surprisingly, 80% of these flies exhibited no change in sugar acceptance threshold compared to pre-injection. These flies were dissected to ensure that no food had passed into the foregut, which is separated from the midgut by the cardiac valve. Since tying off the crop duct before feeding was sufficient to suppress the acceptance threshold, but injecting food into the midgut was not, this suggests that food passing through the foregut was responsible for the satiety effect seen in cropless flies.

Additional lesioning studies revealed the mechanism underlying this satiety effect. When experimenters severed the recurrent nerve (RN), flies became hyperphagic [24]. The RN is part of the stomatogastric nervous system (SGS) in insects, which is analogous to the mammalian autonomic nervous system, and it innervates the aorta, foregut, crop, and hindgut [11]. To control for the possibility that severing the RN was impairing efferent motor control of the gut, several studies demonstrated that the motor patterning of food movement through the gut of these hyperphagic flies was normal [20]. First, food was able to enter the midgut and crop, and, as food was digested and the midgut emptied, the transfer of food from the crop to the midgut was normal[4] in hyperphagic flies. Further evidence that the SGS is not required for the motor patterning of food digestion is the fact that a digestive tract removed from the fly (dissected out and placed in saline) will continue the pattern of midgut emptying and food transfer until the crop is empty. These results support the hypothesis that the presence or movement of food through the foregut promotes a satiety effect, and that severing the RN does not sever motor neurons that are required to move food through the gut.

Electrophysiological recordings from the RN identified neurons whose firing rate was inversely correlated with the rate of crop emptying [26]. Although chemoreceptors exist in the foregut, they do not send their projections through the RN [23]. This led to the conclusion that proprioceptors are monitoring foregut contractions and are necessary and sufficient to produce a satiety effect. It is quite surprising that neither the presence of food in the midgut nor hemolymph sugar levels is sufficient to promote a satiety effect under

[4] A two-way crop valve opens and a "slug" (bolus) of food is transferred by reverse peristalsis up the crop duct and into the foregut. The cardiac valve, which separates the foregut from the midgut, opens and the slug is transferred into the midgut.

starvation conditions. This will be further discussed in a later section.

Hyperphagia resulted when either the MAN or the RN was severed, but the feeding behavior as a result of these lesions was different [23]. Severing the MAN resulted in flies taking one long continuous meal, whereas severing the RN resulted in flies taking repeated near-normal sized meals. These results suggest that crop distention (detected by the MAN) regulates meal cessation, while foregut contractions (detected by the RN) regulate meal initiation. Dramatically, when both nerves were severed, flies continued to feed until they burst.

RN and MAN lesioning in starved flies also results in polydypsia (water hyperphagia) [28]. Flies, like mammals, independently regulate water intake and food intake [19, 28]. To show that the primary effect of these lesions was not solely to promote drinking, the tastant stimulus presented to lesioned flies was varied [28]. When lesioned flies were feeding from a sucrose solution and the stimulus was switched to water, flies ceased drinking. Additional evidence was provided by switching the solution to ever increasing concentrations of solutions. When lesioned flies stopped drinking water, 100 mM sucrose was presented. This elicited feeding until the fly could not generate enough force to fill the crop any further despite continued efforts to feed. Thereupon, a 1 M sucrose solution was presented and this resulted in more vigorous pumping (sucking) until the fly burst.

There is evidence for additional mechanisms that promote satiety from ventral nerve cord (VNC) lesioning studies. Severing the cervical connective, which connects the brain to the VNC resulted in hyperphagia, and the effect of this lesion was stronger than that of severing the MAN, suggesting that the results are not merely due to a loss of the MAN (which projects to the VNC) [24]. The VNC is composed of several fused ganglia that control motor patterns, receive sensory feedback, and send and receive feedback to the brain [20]. This inhibitory feedback could be due to either loss of proprioceptive feedback from the abdomen or from locomotor centers in the VNC.

Hyperphagia as a result of lesioning the VNC may be due to loss of proprioceptive feedback from the abdomen. Experiments have demonstrated that abdomen distention is

necessary and sufficient to regulate drinking (water) [28]. "Bleeding" the fly, by nicking the cuticle and squeezing out hemolymph[5], stimulated drinking, whereas when fluid was injected into the abdomen, inhibited drinking. Surprisingly, injection of either hypertonic or hypotonic solutions was sufficient to inhibit drinking, which suggests that changes in hemolymph osmolarity are insufficient to promote drinking. Instead, these results suggest that abdomen distention regulates drinking.

Lesioning the VNC may also remove inhibitory feedback from locomotor centers. Hungry flies stop walking when they encounter a food source and during feeding, which suggests that feeding and locomotion are mutually exclusive events [29]. Indeed, it has been demonstrated that insects that are induced to fly exhibit a surprisingly high acceptance threshold despite the energy drain imposed by flight [20]. Therefore, locomotor centers in the VNC may be exerting an inhibitory influence on feeding behavior.

All of the previously described experiments were conducted on flies that were starved 24–48 hours. The lesioning studies in starved flies are provocative, and demonstrate that an empty gut and crop triggers a strong drive to feed that overrides any effects of hemolymph sugar levels or humoral factors. The crop is practically empty after 24 hours of starvation, only 5% full compared to the total capacity of the crop, and is empty after 48 hours of starvation [23]. In nature, the crop provides a safety net against starvation. Sometime between 24 and 48 hours of starvation, these flies will need to revert to internal energy reserves for energy.

These experiments demonstrate that in starved blowflies, feedback from the RN, the MAN, and the VNC is necessary to promote satiety, or reverse the drive to feed. Recording from the RN and the MAN electrophysiologically demonstrated that these nerves carry information about gut distention. This suggests that proprioceptors monitor crop volume and foregut activity, that sensory neurons relay this information via the RN and MAN, and that this proprioceptive information is necessary to return to a state of satiety. These findings are compelling, but it must be noted that these lesions disrupt

[5] Hemolymph is insect "blood".

feedback from a number of organs and dirsupt tissue integrity. The extent of the damage of this surgery is demonstrated by the high levels of morbidity: nerve-transected flies die within 1–3 days after surgery [20].

In contrast to the drastic effects of nerve transection on feeding behavior in starved flies, ad libitum fed flies starved 8 hours or less only exhibit a mild decrease in acceptance threshold upon RN transection and acceptance threshold is unaffected by MAN transection [21]. Interestingly, ad libitum fed blowflies that are supplied with carbohydrates but not protein in their diet, do become hyperphagic when the RN or MAN are severed [30]. This suggests that the regulation of satiety in blowflies is affected by beginning nutritional state. Perhaps nutrient-deprived flies are more reliant on peripheral feedback to determine acceptance threshold, and that peripheral feedback is a requirement to return to steady state. Nutrient deprivation may trigger a switch in behavioral responses, towards a more aggressive drive to feed, and that proprioceptive detection of food in the gut is required in order to turn off this drive to feed.

Thus far, I have focused on what is known about peripheral mechanisms that promote satiety in blowflies. Such a thorough examination of the regulation of satiety has not been reported for any other insects, though similar mechanisms of inhibitory feedback have been demonstrated in other insects [17, 18, 20]. In *Drosophila*, very little is known about the regulation of satiety [17]. Lesioning experiments that would demonstrate inhibitory feedback from the crop or foregut have not been reported in *Drosophila melanogaster*, despite the comment that "Bodenstein perfected the technique" of RN severing in *Drosophila*, though this reference might have been to a larger species of *Drosophila* [20]. Due to their small size, lesioning experiments in *Drosophila melanogaster* would be difficult, but analogous experiments could be performed using transgenetic tools that silence or ablate neurons.

How does the feeding behavior of blowflies compare to what is known about *Drosophila* feeding behavior? Similar to blowflies, *Drosophila* increase their acceptance of low reward food cues with increasing starvation, and the volume of food consumed also increases as a function of starvation and the stimulatory value of the food offered [19, 21].

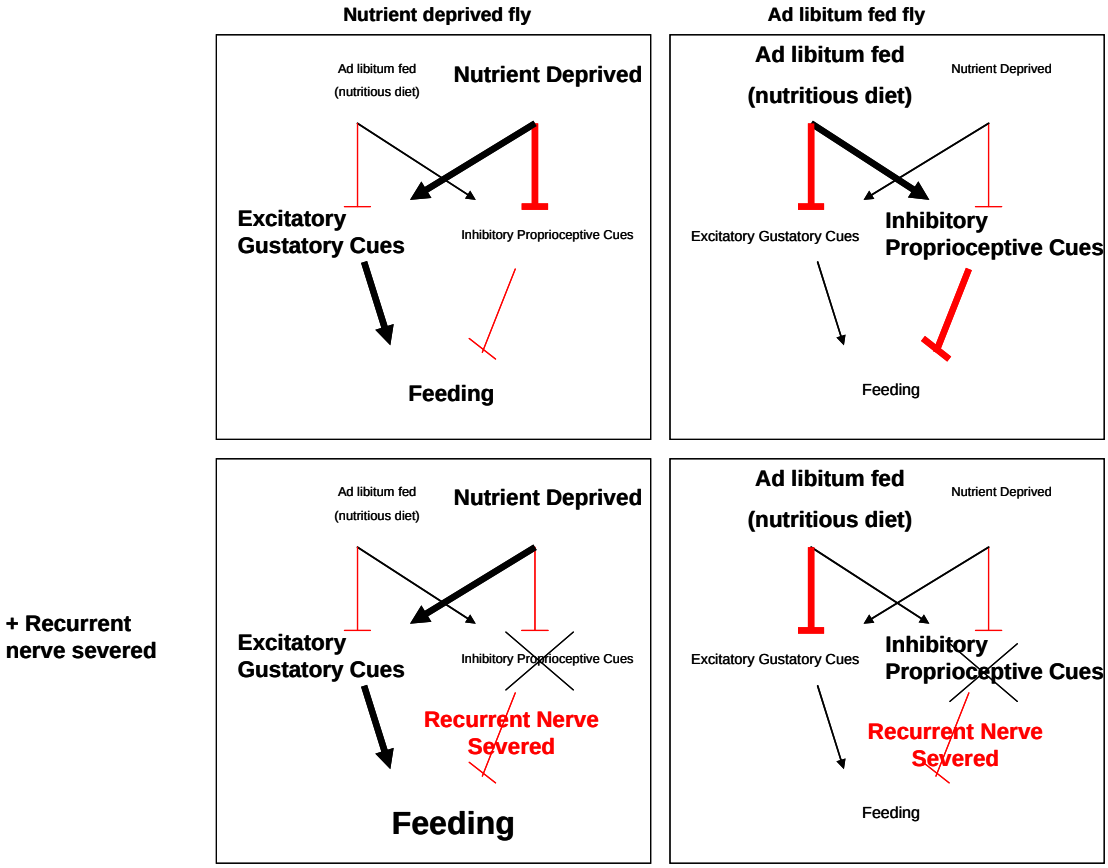
Dethier and colleagues [20] propose that feeding is regulated by gustatory cues, that satiety is regulated by proprioceptive feedback, and that the decision to feed involves a push-pull relationship between the stimulatory value of gustatory cues and the inhibitory feedback from gut distention. The feeding behavior of starved *Drosophila* agrees with this model, but similar to ad libitum fed blowflies, a different model is needed to describe the feeding behavior of unstarved *Drosophila*.

Nutritional state determines how food intake is regulated. Fed on a high concentration of sucrose (50 mM or higher), ad libitum fed *Drosophila* have little or no food in the crop, whereas fed on lower concentrations (10–25 mM), ad libitum fed flies store a significantly larger volume of food in the crop [19]. Here, more stimulating gustatory cues are not promoting increased food intake, and a simple model in which the decision to feed involves only inhibitory proprioceptive feedback and excitatory gustatory cues does not apply.

The neural mechanisms that regulate feeding behavior are dependent on nutritional state. A model that incorporates the influence of nutritional state on insect feeding behavior, and that is consistent with behavioral studies as well as lesioning studies, has not been reported. A model that is consistent with behavioral studies in blowflies and that incorporates the influence of nutritional state on feeding behavior is summarized in Figure 2. In nutrient-deprived flies, nutrient deprivation overrides inhibitory proprioceptive feedback, and promotes the acceptance of food. Ad libitum fed flies, fed a nutrient balanced diet, will not utilize the crop for food storage, and will only accept a highly stimulating food source. This suggests that inhibitory feedback from the gut is high in fed *Drosophila*. Since severing the RN and MAN in fed blowflies had little or no effect on food acceptance, suggests that these models need to be revised to agree with both blowfly and *Drosophila* feeding behavior. This model can account for this discrepancy, if gustatory cues and proprioceptive cues are simulateously regulated in opposite directions, depending on nutritional state. Excitatory gustatory cues and inhibitory proprioceptive cues are sensed immediately, whereas nutritional state only gradually changes. A nutrient-deprived fly (either starved or protein-deprived), has a strong drive to accept food, but when the RN is

severed, immediate inhibitory feedback that normally occurs after feeding is lost. This helps to explain the hyperphagia observed in starved nerve-severed flies. On the other hand, studies found that ad libitum fed flies only had a mild phenotype from RN transection. This could be explained if there was an independent mechanism providing an ongoing inhibition of food acceptance in a "sated" fly, which would minimize the feeding effects of removing inhibitory proprioceptive feedback.

Figure 2. A model that could explain the differential regulation of feeding in unstarved and nutrient-deprived flies



3. Neuromodulators that promote satiety in other insects

Many experiments were conducted to determine whether elements of hemolymph composition can regulate feeding behavior in blowflies, and some of these experiments led to some surprising conclusions. As mentioned previously, injecting a sugar solution into

the hemolymph of a starved fly was unable to decrease the acceptance threshold at any time in the following 24 hours [20]. Regardless of whether a hypo- or hyper-osmotic solution was injected, the acceptance threshold of the fly did not increase. Further attempts were directed at identifying soluble factors that could promote satiety. When a starved fly is given a transfusion of hemolymph from a fly that had taken a meal two hours previously, the acceptance threshold of 80% of the transfused flies did not increase within the following two hours from the starting threshold [20]. Parabiosis is a more direct method to determine whether a humoral satiety cue exists in flies. Parabiosis involves surgically connecting a pair of flies so that the hemolymph is shared. A section of cuticle is removed from each fly, and paraffin is used to seal one fly onto the back of the other. (The flies "took turns riding piggyback"). At the beginning of the experiment, both flies had been starved for 48 hours. Afterwards, only one of the two flies was fed to repletion, while the other was left unfed. The sugar acceptance threshold of the unfed fly was monitored, and no change in threshold was observed. This experiment was continued for three days, in which the fed fly was always fed to satiation, and the acceptance threshold of the unfed fly was monitored. After three days of experiments, it became clear that there was not a humoral factor present in the hemolymph that could promote satiety in an unfed fly.

Since these parabiosis studies concluded that no soluble factor (including sugars) could promote satiety, it would have been informative to characterize the volume of food intake of the fed fly. If the fed fly of the parabiosed pair of flies consumed the same volume of food compared to an unparabiosed fly, then this result would have provided further evidence that sugar levels or a humoral factor in the hemolymph is not sufficient to influence feeding behavior, since the hemolymph is shared in parabiosed flies and any nutrients absorbed by the fed fly would be diluted and any humoral factors released in response to feeding would be diluted. Alternatively, if the fed fly of the parabiosed pair of flies consumed an increased volume of food compared to a single unparabiosed fly, then this result would suggest that a humoral factor could promote feeding (but not promote satiety in an unfed fly).

Parabiosis studies suggest that a humoral factor in the hemolymph is not sufficient

to act as a satiety cue in starved flies. It is quite surprising that blood sugar levels or by-products of sugar conversion do not suppress hunger. A confound of transfusion and parabiosis experiments is that these experiments involve an unnatural series of events: The nervous system never encounters this combinatorial event—nutrition without ingestion. Nevertheless these findings, in combination with experiments discussed in the last section, suggests that at least for starved blowflies satiety is achieved solely through inhibitory proprioceptive feedback from the foregut, crop, and abdomen, and possibly also from inhibitory feedback from locomotor centers in the VNC.

In regards to the neural regulation of feeding behavior in unstarved flies, experiments characterizing the feeding behavior of blowflies starved less than 8 hours do not support the conclusion that peripheral inhibitory feedback is the only mechanism that can promote satiety. Several studies demonstrate that blowflies starved for 8 hours or less were unaffected by MAN lesioning and were only mildly affected by RN lesioning. These findings suggest that results from studies in starved blowflies involving nerve lesions or the direct manipulation of hemolymph content generate artificial scenarios (food intake without gut distention or replenished energy stores without food intake) ought to be interpreted conservatively.

How nutritional state alters the mechanisms by which feeding behavior is regulated is unknown. Gut proprioception may be involved in signaling nutritional state. This is because crop emptying rate is dependent on hemolymph osmolarity [20]. Differences in crop emptying rate would produce distinctive patterns of proprioceptive feedback, which could provide a readout of nutritional state.

In contrast to the findings that a humoral factor could not promote satiety in transfusion and parabiosis experiments, many studies have demonstrated that injected neuromodulators are able to promote satiety in blowflies and other insects. Injection of biogenic amines, including serotonin and dopamine, and neuropeptides, including sulfakinin, leucomyosuppressin, insulin-like peptides, and allatostatins have been demonstrated to decrease feeding behavior in insects.

Sulfakinin has been implicated as a “satiety factor” in several insects, including

blowflies, *Tabanus nigrovittatus* (salt marsh horse fly), *Blattella germanica* (cockroach), and *Schistocerca gregaria* (desert locust) [31]. In the blowfly, sulfakinin injection reduced carbohydrate but not protein intake of females (but not males) without reducing the percent of flies feeding [31]. In the locust, injection of sulfakinin reduced food intake without affecting the sensitivity of taste receptors [32]. In *B. germanica*, sulfakinin injection resulted in decreased food intake and sulfakinin was found to be myostimulatory [33]. It has also been shown to reduce feeding, stimulate gut contractions, and stimulate alpha-amylase secretion in the desert locust and blowflies [31, 32]. Interestingly, sulfakinin is a structural orthologue of mammalian Cholecystokinin, which is also known to decrease feeding, stimulate gut contractions, and stimulate secretion in mammals [31].

Despite being conserved as regulator of feeding behavior in other insects, in *Drosophila* sulfakinin is neither myostimulatory nor does it affect feeding behavior [34]. A long history of genetic studies in *Drosophila* have only identified few neuromodulators that promote satiety [10]. Neuromodulators that have been implicated in promoting *Drosophila* feeding include an insulin-like peptide, serotonin, and hugin.

Both *Drosophila* insulin-like peptide (DILP) and its receptor (InR) are required for normal larval feeding behavior [35–37]. Mutations in these genes caused a reduction in feeding, growth, and development, while overexpression caused an increase in feeding, growth, and development. This shows that the function of insulin has been conserved as a regulator of growth and development in animals [12]. There is also evidence for a conserved role for DILP neurons in the monitoring of internal energy stores. Transcripts of DILP are reduced during starvation and hemolymph DILP levels rise after feeding. In addition, changes in glucose or trehalose levels induce calcium release in adult DILP neurons [11].

Several lines of evidence suggest that DILP signaling is also involved in the regulation of feeding behavior independent of its metabolic/developmental effects. One study observed that the feeding deficits as a result of knocking down the DILP receptor (InR) expression preceded the developmental deficits. Furthermore, growth deficits of InR

null mutants were rescued by overexpression of InR in serotonergic neurons.[6] This suggests that the growth phenotype of InR mutants results from the feeding phenotype. Since InR and serotonin have many target tissues, it remains to be determined where these two signaling pathways overlap.

In both invertebrates and mammals, serotonin signaling regulates both metabolism and feeding behavior [38]. In the blowfly, serotonin injection inhibited both sugar and protein intake [39]. Injection also resulted in an increased acceptance threshold as well as weight loss. Similar effects on feeding due to serotonin injection have been observed in other insects [40]. Serotonin can stimulate foregut and crop contractions in other invertebrates [41, 42]. A functional role for serotonin in insect feeding behavior is supported by several immunohistological studies. In *Neobelliera bullata* (flesh fly), immunoreactivity of serotonin in the subesophageal ganglion (SOG), which receives primary gustatory input, decreased post-feeding [40]. This suggests that serotonin was released in this region as a result of feeding. Similar conclusions were drawn from studies in *Rhodnius prolixus* (triatomid bug), in which serotonin release from neurosecretory cells was observed in response to feeding [43]. Further supporting a neuromodulatory role for serotonin in feeding, is the presence of serotonin in the insect stomatogastric nervous system.

Consistent with earlier suggestions that DILP signaling in serotonergic neurons promotes feeding behavior in *Drosophila*, are studies by Neckameyer and colleagues that demonstrated that serotonin is involved in the regulation of feeding in *Drosophila* as well [44]. Adult flies with a null mutation in Tph2, which is one of the enzymes required for serotonin synthesis, exhibited decreased feeding behavior [44]. Unstarved adult Tph2 mutants consumed less over a 24 hour time period, exhibited decreased activity levels, and a decreased heart rate. Since there is a paralogue of Tph in *Drosophila*, and since Tph is

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[6] Overexpressing InR in DDC neurons rescued the InR -/- growth phenotype, whereas overexpressing InR in TH neurons did not. Dopa decarboxylase (DDC) is an enzyme that is required for the synthesis of both serotonin and dopamine. Tyrosine hydroxylase (TH) is required for dopamine synthesis. The expression of DDC and TH was found to only minimally overlap. Because neurons that coexpressed both TH and DDC were not proximal to DILP neurons, this led to the conclusion that DDC driven rescue of InR-/- occurred in serotonergic neurons and not dopaminergic neurons.

required for serotonin and dopamine synthesis, the authors demonstrated that Tph2 is exclusively expressed in serotonergic neurons in the adult, and not in dopaminergic neurons. These results support a conserved role for serotonin in regulating feeding behavior.

Conversely, Neckameyer observed that knockdown of Tph1 in the fat body resulted in increased mouthhook contractions and hyperactivity in larvae [44]. Feeding studies could not be done in Tph1 null mutants due to severe developmental deficits. Authors showed that dopamine but not serotonin is expressed in the fat body, and therefore concluded that the loss of serotonin was not responsible for the observed increased feeding behavior. Despite a previous study that demonstrated dopamine to modulate food intake and acceptance threshold [39], and numerous studies that have implicated dopamine in arousal [45, 46], the authors concluded that the feeding and hyperactivity phenotypes of fat body Tph1 knockdown larvae was due to toxic levels of tryptophan, the precursor to dopamine, and not due to serotonin or dopamine levels [44].

In *Drosophila*, two additional neuromodulators have been indirectly shown to modulate satiety-like behavior. Flies with null mutations in leucokinin or its receptor exhibited increased meal sizes compared to controls, though a gain of function phenotype for leucokinin was not reported. Leucokinin mutants consume larger than normal meals but compensate for the excess intake by decreasing meal frequency [47]. Given the expression of leucokinin in muscle tissue in the proventriculus it is likely that the leucokinin feeding phenotype is due to a malfunction of the cardiac valve, which separates the foregut from the midgut. A family member of leucokinin has similarly been implicated in modulating the activity of the cardiac valve. Injection of leucomyosuppressin into *Blattella germanica* resulted in a reduced feeding phenotype and an accumulation of food in the foregut [33].

Another study has provided indirect evidence of another potential satiety factor in *Drosophila.* Silencing of hugin neurons resulted in a contextual feeding phenotype: on being transferred from regular fly food to yeast paste, silencing of hugin neurons in adult flies resulted in a much shorter latency to feed than in control flies [48]. Transferred from yeast to yeast, or yeast to regular fly food, or regular food to regular food did not result in

differences in latency to feed, and in all conditions total food intake was no different from controls. This contextual phenotype was observed in only unstarved flies, and silencing of hugin neurons did not alter the rate of food intake in starved flies. Starvation-induced feeding was also normal. Since starvation reduces the latency to accept novel foods, and since hugin is downregulated in response to starvation, these results suggest that hugin neurons play a role in promoting the acceptance of novel foods. Another interpretation could be that hugin neurons are involved in the regulation of protein feeding, since many standard fly foods provide low levels of protein and increased acceptance of yeast paste could reflect increased protein hunger. This would explain why the other feeding conditions did not result in differences in latency to feed. Indeed, authors also reported that hugin expression was downregulated in wild-type flies fed only sugar (protein-deprived) [48]. The expression pattern of hugin further supports a role for hugin neurons in feeding behavior, as hugin is expressed in neurons that project to pharyngeal muscles and to regions of the brain where primary gustatory neurons and DILP cell bodies are located [17]. Overexpressing hugin within hugin neurons did not alter rates of food intake. This could indicate that additional neuromodulators expressed in hugin neurons are responsible for the effects observed upon silencing these neurons. Another possibility is that hugin-overexpressing flies were not tested under the proper feeding conditions (testing protein versus carbohydrate feeding). It would be interesting to determine whether hugin is involved in regulating protein metabolism. Ubiquitous overexpression of hugin resulted in growth deficits and lethality, which suggests a role for hugin development. Further studies are needed to establish the function of hugin-expressing neurons in the regulation of feeding behavior.

4. Allatostatin A

In insects, the neuropeptide Allatostatin A (AstA) is a potential satiety factor. In the cockroach, injection of AstA reduced food intake by 60% [49, 50]. Expression of AstA was shown to be anti-correlated with the feeding behavior of females. During the 7-day gonadotrophic cycle, or female reproductive cycle, food consumption was highest in the

middle of the cycle, and AstA transcript levels were highest at the beginning and the end of the cycle, consistent with a role for AstA to inhibit feeding. This observation was inconsistent with an independent study that demonstrated that peptide levels increased steadily during the gonadotrophic cycle. Additional effects of AstA injection in these studies included the inhibition of hindgut (but not foregut) contractions and an increase in the secretion of alpha-amylase, an enzyme that digests starch, in the midgut [50].

The function of AstA in adult cockroach, crickets, and termites is to inhibit Juvenile Hormone (JH) synthesis [51]. JH regulates the metabolic switch that occurs in females post-mating, by diverting diverting fat stores towards egg production. Mated females also exhibit increased feeding behavior compared to unmated females. In conditions of starvation and short photoperiod/low temperatures, AstA levels increase, inhibiting JH synthesis which, in turn, inhibits egg production and leads to the build-up of fat stores. Thus, reproductive state and environmental conditions regulate AstA levels, and AstA inhibits the metabolic switch that occurs as a result of mating in some insects.

Another study implicated a role for AstA in the regulation of feeding, but results were difficult to interpret. In *Gryllus bimaculatus*, females were injected with AstA (RNAi) at emergence and tested 2 days later. Knockdown of AstA was confirmed by Q-RT-PCR [49]. Food intake of injected virgin females was 38% reduced after 30 minutes but 79% increased after 60 minutes of feeding [49, 52]. Since AstA inhibits JH synthesis in this species, and AstA levels are high in unmated females, knockdown of AstA in virgin females would be predicted to result in the disinhibiton of JH, which would lead to a diversion of fat stores to egg production and to increased feeding. This study next reported that injected mated females, which should have low levels of AstA and high levels of JH, exhibited 30% decreased alpha-amylase activity after one day, yet after two days, enzyme activity was increased by 300%. Mated females would likely have low levels of AstA and therefore knockdown of AstA would have little effect on JH synthesis. Perhaps the logic of the authors was to tease apart a dual role for AstA in the cascade of effects due to JH synthesis from a role for AstA in the gut to promote alpha-amylase release.

AstA has also been implicated in the regulation of feeding behavior in insects in

which AstA does not inhibit JH synthesis, suggesting that the effects of AstA on feeding is not necessarily an indirect effect of inhibiting JH synthesis. Injection of AstA into the larvae of both *L. oleracea* and *S. littoralis* resulted in decreased feeding and growth, but only if the larval stage injected was in a feeding stage (injection during a non-feeding stage had no effect) [10].

Many neuropeptides that have been implicated in insect feeding behavior also have myoinhibitory or myostimulatory effects [10]. Perhaps their effects on feeding are by modulating proprioceptive feedback from the gut. It has been difficult demonstrate a causal role for central versus peripheral expression of neuropeptides because many are expressed both centrally and peripherally, and many are expressed at neurohemal release sites, or sites where neuromodulators can be directly released into the hemolymph [10, 53].

5. Does *Drosophila* have a brain-gut axis?

In mammals, the gastrointestinal tract is "the largest endocrine organ in the body" [54]. Mechano-chemo-, and noci-ceptive information is communicated to the CNS directly via the vagus nerve and by neuromodulators via the circulatory systems. The gut can produce over 100 bioactive peptides and some of these can modulate feeding and satiety; gut distention also affects feeding behavior in mammals.

The role of the adult stomatogastric nervous system (SGS) in the regulation of feeding behavior has been overlooked in *Drosophila* [10, 55]. The SGS innervates and regulates the aorta, foregut, crop, and proventriculus [11]. Little is known about the type of information afferent sensory cues transmit or how efferent cues control digestion and gut motility [55]. A number of neuropeptides that have been implicated in insect feeding behavior are either expressed in the gut or the SGS: sNPF and FMRFamide are expressed in neurons that project to the anterior midgut; FMRFamide and possibly dromyosuppressin are expressed in neurons that innervate the crop [56]; DILP3 is expressed in gut muscle in the proventriculus, foregut, and midgut [56]; leucokinin is expressed in muscle near the proventriculus [47]; and motorneurons expressing AstA, pigment dispersing factor (PDF), and proctolin innervate the posterior midgut and hindgut [51, 56]. In addition, receptors for

AstA, DILP, PDF, leucokinin, tachykinin, and diuretic hormone are expressed in the midgut and hindgut, and receptors for NPF and hugin are expressed in the crop according to microarray studies [65]. The function of neuropeptide expression in the gut and SGS has not been adequately addressed.

Neuroendocrine cells in the midgut of *Drosophila* express a variety of different neuropeptides, including NPF, AstA, AstB, AstC, tachykinin, diuretic hormone, FMRFamide, and possibly sulfakinin [56]. A number of these neuropeptides have been implicated in the regulation of feeding and homeostasis but there has been little attempt to address the potential contribution of gut neuroendocrine expression to feeding phenotypes. The functions of these midgut endocrine cells could be to sense gut content, to stimulate or produce digestive enzymes, or to modulate gut motility.

Understanding the function of these various neuropeptides in digestion and feeding behavior would be informative. If proprioceptive feedback is primarily responsible for modulating feeding behavior in *Drosophila*, then neuropeptides with myoinhibitory and myostimulatory properties could indirectly modify feeding behavior. The satiety effects observed in other insects upon AstA injection, could be due to myinhibitory properties of AstA. If feeding behavior is regulated by gut motility that is sensed by proprioceptive feedback, then directly altering gut motility would affect feeding behavior. The myoinhibitory effects of AstA have been demonstrated in cockroach (*Leucophaea maderae, Blattella germanica*), moth (*Manduca sexta*), lobster (*Homarus americanus, Homarus gammarus*), and crab (*Cancer borealis*). Interestingly, in the crayfish, AstA has myostimulatory properties [50, 57]. Alternatively the effects of AstA on feeding behavior could be to directly modulate the firing proprerties of proprioceptive neurons. AstA is co-expressed with serotonin in the SGS of *Crustacea*, in both lobsters and crabs [58, 59] Co-expression has been demonstrated in stretch receptors neurons that inhibit the pyloric and gastric mill rhythms in these organisms. In the crab, bath application of both AstA and serotonin inhibited contractions and co-application had a stronger effect than either alone [60]. AstA was also shown to be co-expressed with Acetylocholine in the SGS.

Based on the co-expression of serotonin and AstA in other species, these may be

coexpressed in *Drosophila* as well. This possibility is supported by evidence that both neuromodulators inhibit feeding in multiple organisms [10, 38]. The function of AstA in *Drosophila* is unknown [53]. Expression of AstA in feeding-related endocrine centers of *Drosophila* suggests that AstA might play a role in feeding behavior [61]. *Drosophila* eclose with food in the gut from their last meal as larvae [61]. It has been suggested that expression in the hindgut of pupae might serve to withhold the final meal of the larvae [61]. In addition, there is a conserved role for orthologues of AstA receptors in the feeding behavior of mammals and *Caenorhabditis elegans* [62, 63].

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Chapter 2

ANATOMICAL CHARACTERIZATION OF ASTA-GAL4 TRANSGENIC FLIES

1. Introduction

The neuropeptide Allatostatin A (AstA) has been implicated as a satiety factor and as a myoinhibitor in several other insects [1–3]. Currently, there is no known function for AstA or for AstA neurons in *Drosophila melanogaster*. Expression of AstA in feeding-related endocrine centers suggests that AstA may play a role in the regulation of *Drosophila* satiety as well [3]. In order to study the potential function of AstA neurons in *Drosophila*, we generated AstA-Gal4 transgenic flies.

The Gal4/UAS binary expression system takes advantage of the yeast transcription factor, Gal4, and its target, upstream activating sequence (UAS), to spatially and temporally restrict the expression of various transgenes using *Drosophila* enhancers [4]. Enhancer-Gal4 transgenic flies can be crossed to any of a large number of UAS-reporter transgenic flies, to express the reporter in a limited manner. Reporters include transgenes that encode fluorescent molecules which can be used to characterize the expression pattern of an enhancer-Gal4 line and transgenes which encode ion channels that can be used to manipulate neuronal activity [5]. UAS-reporter lines can also be used to either knockdown or overexpress specific genes.

In order to validate the specificity of these AstA-Gal4 transgenic lines, we used an antibody against AstA that had been used in previous studies to characterize the expression pattern of AstA in *Drosophila*. Several antibodies against AstA have been used to characterize the expression pattern of AstA in *Drosophila*. A monoclonal antibody raised against *Diploptera punctata* AstA was used to characterize larval, pupal, and adult expression patterns of *Drosophila* AstA [3, 6]. The specificity of this antibody was determined by preabsorbing it with the synthetic peptide against which the antibody was raised, and observing that immunoreactivity was abolished [3]. A second (polyclonal) antibody that was raised against *D. punctata* AstA, was reported to label the same cells as the monoclonal antibody [3]. Support for the specificity of this antibody to detect *Drosophila* AstA, is that immunolabeling reveals similar patterns of expression in both

Drosophila and *D. punctata.* The only difference was that there was no immunoreactivity in the *Drosophila* corpus allata, where Juvenile Hormone (JH) is synthesized. This supports the finding that AstA does not inhibit JH synthesis in *Drosophila*, which is a function of AstA in several other insects (see Introduction). In this section, we demonstrate that the AstA-Gal4 transgenic flies that we generated, specifically express Gal4 in a subset of neurons that endogenously express AstA.

2. Results

An AstA promoter-Gal4 transgenic was made by cloning 2.1 kb of 5' flanking DNA up to but not including the predicted transcriptional start site (Figure 1a). We obtained five independent insertion lines which we crossed to UAS-mcd8::GFP [7], a membrane-tethered fluorescent reporter, in order to compare and characterize the expression patterns of the independent insertion lines. Immunostaining with an antibody against green fluorescent protein (GFP) revealed no differences in expression patterns between the different independent insertion lines (data not shown). To address the specificity of the Gal4 expression pattern, we double labeled adult tissues with a monoclonal antibody raised against *Diploptera punctata* AstA that had previously been used to characterize AstA expression in *Drosophila* [3].

AstA-Gal4 labeled three pairs of neurons in the brain and ~ 30 neurons in each optic lobe. AllAstA-Gal4-expressing neurons co-localized with AstA antibody (Figures 1b–1g, cell bodies are circled in Figures 1b–d). We saw extensive innervation of the subesophageal ganglion (SOG), protocerebrum, and pars intercerebralis (PI) (Figure 2c–2e, brain neural architecture is diagrammed in Figure 2b). In addition, at higher magnification, we observed a descending axon that arborized in the dorsal-most neuromeres of the ventral nerve cord (VNC) (Figure 2a). We were unable to differentiate and trace the axons of the three pairs of brain AstA-Gal4 neurons due to the overlap of the axon tracts and the saturation of the GFP immunostain.

In the last abdominal neuromere of the VNC, Gal4 and AstA co-localized to three pairs of cell bodies (Supplementary Figure 1 shows a higher magnification image that illustrates the number of cell bodies), which send projections dorsally (Figures 2f–2k, indicated with an arrow in Figure 2f–h). These projections innervate the lower midgut, the

hindgut, and the rectum (Figures 3a–3f). Also co-labeled were single neurons in each of two nerves that exit the VNC laterally (Figures 2h–2j, cell bodies are circled).

In the gut, less than half of the AstA-immunoreactive neuroendocrine cells located in the lower midgut expressed Gal4 (Figures 3j–3l), a few of which were only weakly immunoreactive to AstA (In these figures, AstA-immunoreactive neuroendocrine cells are surrounded by squares, Gal4-immunoreactive neuroendocrine cells by circles. Notice the number of squares without circles (AstA+/Gal4-) and the circles without squares (weak AstA+/Gal4+).

Also co-labeled was tissue on both ends of the midgut, adjacent to the proventriculus and at the midgut/hindgut transition (Figures 3a–3c, 3g–3i, indicated using red arrows). In addition, there is speckled immunolabeling that is smaller than the size of neuroendocrine cells and does not appear to be associated with motorneuron projections (Figure 3j–3l). We see this type of labeling in the upper midgut as well (Figure 3g–i). This was not due to precipitates of the fluorescent antibodies, because immunostaining with only secondary fluorescent antibodies did not create this pattern of staining. Instead, we consistently see the highest density of speckling near the midgut transitions, and a gradual decrease in density further from the transitions (Figure 3a–c). The dense and speckled staining is likely staining muscle tissue, but in the lower midgut, it could also be staining varicosities of motor neurons. In the lower midgut, there are many more AstA+/Gal4- speckles than AstA+/Gal4+ speckles (Figure 3a–c, 3j–3l), whereas in the upper midgut, we see AstA+/Gal4-, AstA+/Gal4+ (indicated by arrows), and AstA-/Gal4+ (indicated inside circles) speckles (Figure 3g–i).

Similar to what we observed in adults, AstA-Gal4 is only expressed in a subset of AstA neurons in 3rd instar wandering larvae. Immunostaining the CNS of AstA-Gal4/ UAS-nuclear-lacZ larvae with anti-B-galactosidase, reveals expression of Gal4 in five cell bodies, which all co-express AstA, as assessed by fluorescent in situ hybridization (Figure 4a–4f). The total number and general location of AstA-expressing cell bodies, as assessed by in situ hybridization, was identical to the number and location of neurons previously reported using the *D. punctata* antibody (Figure 4a–4f, AstA+/Gal4- cell bodies are indicated using arrows and [3]). These results support the specificity of the AstA antibody

for *Drosophila* AstA, and the conclusion that AstA-Gal4 is only expressed in a subset of AstA-expressing neurons.

Previous reports did not observe AstA immunoreactivity in the foregut or in the muscle tissue at the midgut transitions [7, 8]. In order to determine whether the AstA-Gal4 expression that we observed in these regions was due to auto-fluorescence, we labeled AstA-Gal4,UAS-mcd8::GFP/UAS-Ricin tissues with an antibody against GFP. Ricin promotes cell autonomous death by inhibiting protein synthesis [9]. Expression of Ricin in AstA neurons abolishes GFP immunoreactivity in the gut, indicating that Ricin ablates AstA+/Gal4+ cells and that immunolabelling witnessed in the gut is specific (Figure 4h).

In the brain and VNC, GFP immunolabelling was also abolished in AstA-Gal4,UAS-mcd8::GFP/UAS-Ricin flies (data not shown). This manipulation allowed us to observe the expression pattern of AstA+/Gal4- neurons. Interestingly, the remaining five AstA+/Gal4- neurons in the brain extensively innervate the same regions as the AstA+/Gal4+ neurons: the protocerebrum, the pars intercerebralis, and the SOG (Figure 4g). In addition, expression in the central complex is also seen.

In order to localize potential post-synaptic terminals of AstA neurons, we used UAS-ANF::GFP, which expresses rat atrial natriuretic hormone (ANF) fused to GFP [10]. This neuropeptide is packaged and shuttled similar to *Drosophila* neuropeptides, thus allowing us to visual neuropeptide trafficking in AstA neurons. Labeling AstA-Gal4/UAS-ANF::GFP fly brains with an antibody against GFP, revealed that potential synaptic terminals occur in the protocerebrum, pars intercerebralis, and SOG (Figure 4i).

3. Discussion

We have demonstrated that the expression pattern of Gal4 in AstA-Gal4 transgenic flies faithfully reproduces the expression pattern of a subset of AstA-expressing neurons. All of the Gal4-positive neurons and neuroendocrine cells were also AstA-positive. There were five additional neurons in the brain, and 10 addition neurons in the VNC that were AstA-immunoreactive neurons but that did not express Gal4. We found that Gal4 co-localized with AstA in the following locations:

—3 of 8 AstA neurons in the brain (per hemisphere)

—3 of 13 AstA neurons in the VNC (per hemisphere)

—all ~30 AstA neurons in the optic lobe (per hemisphere)

—1 AstA neuron in each haltere nerve

—1 AstA neuron in each wing nerve

—lower midgut endocrine cells

—motorneurons innervating the lower midgut, hindgut, and rectum, whose cell bodies are located in VNC

—muscle at both midgut transitions

3.1 Projections of centrally expressed AstA neurons

Although we could not trace the projections of individual AstA neurons in the brain, we can infer the projection patterns of the three individual brain Gal4+/AstA+ neurons from previous anatomical studies. The projection patterns of individual AstA neurons in the *Drosophila* brain have been precisely characterized using camera lucida drawings [3]. Thanks to the sparse expression of AstA and the distinctive morphology and location of AstA cell bodies, we were able to infer the identity of Gal4+ neurons.

According to Yoon and Stay [3], the first pair of neurons, named ALT2, are located in the anterolateral tritocerebrum and these neurons project to the region of the pars intercerebralis. The second pair of cell bodies, named VMS, are located below the subesophageal ganglion, and project to the medial subesophageal ganglion. The third pair of cells, named PLT, is located in the posterior lateral tritocerebrum with varying positions medially. These neurons were only weakly immunoreactive to AstA, and the projections of these neurons were not immunoreactive.

We propose that the innervation of the protocerebrum and the descending neuron(s) in the cervical connective arise from the PLT cell bodies. We reach this conclusion by process of elimination, since AstA-Gal4 is only expressed in three neurons in the brain, two of which (ALT2 and VMS) were not reported to project to the protocerebrum and cervical connective. Support for this suggestion is that PLT neurons project to these regions in the larval brain. Yoon described larval PLT neurons as large and highly immunoreactive, and demonstrated that these neurons persist through pupation to adulthood. In larvae, the PLT neurons extensively arborize the CNS, with projections to both the larval lateral protocerebrum and down to the dorsal end of the ventral ganglion. In agreement with our observations in the adult, Yoon observed projections within the cervical connective that were immunoreactive, but could not determine the source of these projections.

Finally, all AstA neurons in the optic lobes coexpress Gal4. These neurons were reported to project to the base of the medulla, and likely play a role in visual processing.

3.2 Projections of VNC neurons and peripheral neurons

In the abdominal ganglion, Gal4 and AstA co-label three pairs of cell bodies and their projections to the gut, where they innervate the lower midgut, hindgut and rectum. Yoon named these neurons DLAa (dorsolateral abdominal) and reported these to be motor neurons that project through the median abdominal nerve to the lower midgut, hindgut and rectum. Also co-labeled is a single cell body in, or on, each of the nerves exiting the VNC towards the wing and the haltere. Based on projections from these neurons, which encircle these nerves, Yoon suggested that these projections may represent neurohemal release sites, secreting neuromodulators into the hemolymph or targeting neurons projecting through the nerve.

In agreement with previous findings, we saw AstA immunoreactivity in neuroendocrine cells in the lower midgut [3, 8]. Contrary to previous observations, we found AstA immunoreactivity in muscle tissue on both ends of the midgut, adjacent to the proventriculus and at the midgut/hindgut transition. Because AstA-Gal4 was co-expressed in these regions, and ablating AstA-Gal4 neurons eliminated GFP immunofluorescence, we believe that this additional Gal4 expression represents real expression of AstA and not ectopic expression of Gal4.

The discrepancy in upper midgut neuroendocrine expression, between our observations and previous studies, may be due to sexually dimorphic expression, or to differences in peptide expression due to genetic strain, nutritional regime, or nutritional state. We characterized expression in males, whereas previous studies characterized females. Further studies will be needed to clarify this discrepancy.

3.3 Why is AstA-Gal4 only expressed in a subset of AstA-immunoreactive neurons?

There are several potential explanations for the expression of AstA-Gal4 in only a subset of AstA-immunoreactive neurons. The most likely explanation is that we did not amplify the entire promoter/enhancer region of AstA, and the AstA-Gal4 transgene is lacking one or more enhancers for the missing neurons. It is unlikely that the site of transgene insertion is inhibiting Gal4 expression in some AstA+ cells, because all five independent insertions of AstA-Gal4 exhibited identical expression patterns.

One tempting hypothesis is that the enhancer region we used to generate the AstA-Gal4 transgenic construct captured one of the four putative isoforms of AstA that are splice variants of the prepropeptide. Three isoforms of AstA have been isolated from adult *Drosophila* by MALDI-TOF mass spectrophotometric analysis [11]. Only one isoform was isolated from the brain, from the cell bodies of the Pars intercerebralis/Pars lateralis, and from the SOG. Three isoforms were isolated from the VNC.

Another possibility is that the antibody used to identify the expression pattern of AstA is binding to another molecule, and therefore that the AstA+/Gal4- neurons do not actually express AstA. Evidence against this possibility is the total agreement between AstA transcript expression that we observed using in situ hybridization and reported antibody labeling in larval brains, and the degree of overlap between Gal4 and anti-AstA labeling. The available antibodies were raised against *Diploptera puntata* AstA, and validated by blocking the antibody with *D. punctata* AstA. We attempted to pre-absorb this antibody with *Drosophila* AstA, but in our hands, this did not eliminate immunostaining in *Drosophila.* However, we did not have a positive control to demonstrate that the synthesized peptide that we used was functional and that it had not

been degraded. In further support of the specificity of the antibody to AstA in *Drosophila*, is the similarity in the expression pattern of AstA between insects [3].

Finally, it is also possible that some AstA immunostaining represents peptide uptake, and not endogenous AstA expression. There have been examples in insects, where peptides but not transcripts were localized to tissues. Since AstA neurons project to several neurohemal release sites, this suggests that there is a function for circulating AstA.

3.4 AstA-Gal4-expressing neurons may play an adult-specific role in regulating feeding behavior.

Previous studies suggested that AstA may play a larger role in *Drosophila* adults than in larvae, based on the observation that there is an increase in the number AstA neurons and an increase in the amount of arborization in the brain of the adult compared to larvae [3]. Of the three AstA neurons that express Gal4 in the adult brain, two appear only in the adult. Whereas the third neuron, PLT, is large and highly immunoreactive in the larvae, this neuron is only weakly immunoreactive in the adult. Yoon and Stay [3] reported that the 30 neurons in the optic lobes only appear in the adult. In contrast, we saw co-expression of both AstA-Gal4 and AstA in the developing eye disc of wandering third instar larvae (data not shown) [3]. Furthermore, there is one additional neuron in the CNS and five in the VNC that are new to the adult but that do not express Gal4. Finally, there are tenfold more gut neuroendocrine cells in the adult than in the larvae, and AstA-Gal4 is expressed in less than half of the AstA-immunoreactive gut neuroendocrine cells in the adult. Therefore, AstA-Gal4 is expressed in a number of adult-specific AstA neurons.

AstA neurons innervate a number of feeding-related brain centers and tissues [3]. In addition to expression in gut motorneurons, gut neuronendocrine cells, and gut muscle, AstA neurons innervate the SOG, where primary gustatory information is relayed, and the pars intercerebralis, where *Drosophila* insulin-like peptide is synthesized. Isolation of AstA peptides from the adult brain suggests that AstA has a neuromodulatory function in the CNS [11].

AstA peptide expression at various potential neurohemal release sites supports a role for AstA as a humoral factor. A large potential target site is the neural sheath, which is highly immunoreactive to one of the AstA receptors, DAR-2 [12]. Three pairs of AstA

neurons in the VNC innervate the neural sheath surrounding the VNC, and two pairs of neurons appear to innervate the surface of the wing and haltere nerves. Support for this hypothesis, is the presence of AstA peptide in the hemolymph in *Diploptera* [3].

4. Materials and methods

Fly husbandry

All flies were reared on standard media [13] at 25ºC, 70% relative humidity, and under a 12 h:12h light:dark regime unless otherwise indicated. Flies used for immunohistochemistry and behavior were 5–10 day old males. Fly lines used included UAS-mcd8::GFP (Bloomington stock #5137) [7], UAS-preproANF-EMD (Bloomington stock #7001) [10], UAS-Ricin [9], *w* (Exelixis background), *w* (Dr. Tim Tully lab isogenic strain), Canton-S flies obtained from Dr. Seymour Benzer.

Gal4 construct and transgenic fly lines

The primer sequences GATTCTAGACTGGATCTCAAGTGCACATTGCACTGCG, and GATGGATCCTCGGTTAAGTTTTGAATCCGCATCCGCTG were used to amplify a 2096 bp fragment of DNA upstream of but not including the putative transcriptional start site of *Drosophila* AstA (NCBI Reference Sequence NM_079765.2) from whole fly cDNA. The fragment was subcloned into the Xba I and BamH I restriction sites of pC3G4 vector (*Drosophila* Genomics Resource Center) [4]. Plasmid DNA was then collected using a Maxiprep kit (Qiagen) and microinjected into *w* (Exelixis background) embryos (microinjection was outsourced to BestGene, Inc.). We obtained a total of 5 stable independent transformant fly lines. We crossed these lines to UAS-mcd8::GFP to visualize their expression patterns. Co-labeling tissues with anti-GFP and anti-AstA confirmed that the AstA-enhancer-Gal4 lines faithfully reproduce the expression pattern of a subset of endogenously-expressed AstA. Larval double in situ/immunohistochemistry yielded similar results.

Immunohistochemistry

Male adult tissues were dissected in phosphate-buffered saline (PBS) and fixed in 2% paraformaldehyde at room temperature for one hour. After washing with 0.5% Triton X-100 in PBS (PBT), tissues were blocked in 10% normalized goat serum in PBT and then

incubated with primary antibodies in blocking solution for 2 days at 4ºC and then with secondary antibodies overnight at 4ºC. Tissues were then mounted in Vectashield (Vecta Labs) and visualized using a Leica confocal microscope. Primary antibodies used include nc82 (mouse monoclonal IgG1 used at 1:20, from Developmental Studies Hybridoma Bank (DSHB)), 5F10 (mouse monoclonal anti-*Diploptera punctata* Ast7 IgG1, used at 1:2, from DSHB, [3]), 3C11 (mouse monoclonal anti-synapsin IgG2b, used at 1:200, from DSHB), rabbit anti-green fluorescent protein (used at 1:300, Molecular Probes). Secondary antibodies used were Alexa Fluor 488-goat anti-rabbit, 583-goat anti-mouse IgG1, or 633-goat anti-mouse IgG2b or IgG1.

In situ hybridization

Double fluorescent in situ hybridization and immunohistochemistry protocol was modified from [14]. Briefly, tissues were dissected in chilled phosphate buffered saline (PBS) and fixed for one hour in 3.7% formaldehyde at room temperature. After washing in PBS, the samples were transferred stepwise into 100% methanol and stored overnight at -20°C. The next day, the samples were transferred stepwise back into PBS and then refixed for 15 minutes in 3.7% formaldehyde. After washing in PBS again, the samples were hybridized overnight at 58°C with a digoxygenin-labeled riboprobe. The next day the samples were washed in PBS plus 0.5% Triton-X 100 (PBT) and incubated overnight at 4°C with POD-coupled sheep anti-digoxigenin (Roche 1207733, at 1:20), anti-β-galactosidase (mouse IgG2a, Promega, at 1:200) and nc82 (1:20). After washing in PBT, the samples were incubated in 1:150 fluorescein-tyramide for 10 minutes (Tyramide Signal Amplification kit, Perkin Elmer NEL704A001KT, Cy3) and then washed again. Samples were then incubated overnight with secondary antibodies (goat anti-mouse IgG2a, Alexafluor 488 at 1:250, Molecular Probes a21131, and goat anti-mouse IgG1-Cy5 at 1:250, Southern Biotechnology Associates 1070-15) and mounted in Vectashield (Vector Labs).

5. Figure legends

Figure 1. Generation and characterization of AstA-Gal4 transgenic flies

(**a**) At the top is the schematic representation of the predicted gene structure of *Drosophila* Allatostatin A (AstA), where blocks represent exons and highlighted in orange is the coding region. Below is a schematic of the AstA-Gal4 construct. The blue block indicates the 2.1 kb region of upstream DNA that was amplified to make the AstA-Gal4 trasngenic construct. (**b**–**d**) AstA-Gal4 and AstA are co-expressed in three pairs of neurons in the brain (**e**–**g**) and in ~30 neurons in the optic lobes. Co-expression was determined by double immunofluorescent labeling of adult male AstA-Gal4/UAS-mcd8::GFP brains with antibodies against GFP (in green, **b** and **e**) and *Diploptera* AstA (in red, **c** and **f**, [3]). A merged image (**d** and **g)** of the two also includes anti-synapsin (in blue, labels synapses). The three co-immunostained cell bodies are circled in (**b**–**d**), and labeled according to the nomenclature of Yoon and Stay, 1995 [3]. UAS-mcd8::GFP is a reporter line that targets green fluorescence protein (GFP) to the cell membrane [7].

Figure 2. Expression of AstA and AstA-Gal4 in the adult brain and ventral nerve cord

Immunofluorescent detection of AstA (**d, g,** and **j**) and of UAS-mcd8::GFP driven by AstA-Gal4 (**a, c, f**, and **i**). Merged images are shown in (**e, h**, and **k**). (**a**) Immunofluorescent detection of UAS-mcd8::GFP in the cervical connective. An arrow points to the pair of medial dorsal cell bodies (VMS) in the SOG. (**b**) Adult brain image with illustrations of the location of the pars intercerebralis, protocerebrum, and subesophageal ganglion (SOG). (**c–e**) Arborization of neurons expressing AstA-Gal4 in the protocerebrum, pars intercerebralis, and SOG. (**f–k**) AstA-Gal4 and AstA are co-expressed in three pairs of neurons in the abdominal ganglion, marked by an arrow in (**h**), with axons projecting through the median abdominal nerve. See Supplementary Figure 1 for a higher resolution image. Also co-labeled are cell bodies in/on the nerves projecting to the wing and haltere (**i–k**); cell bodies are circled. The GFP immunofluorescence seen in (**i**) that is illustrated by an arrow, labels the wing nerve (not the VNC).

Figure 3. Expression of AstA and AstA-Gal4 in the adult midgut, hindgut, and rectum
Gut expression of the AstA-promoter-Gal4 enhancer trap line. Double immunofluorescent labeling of the lower midgut (**a–c, j–l**), the upper midgut (**g–i**), and the rectum (**d–f**) of adult male AstA-Gal4/UAS-mcd8::GFP flies with antibodies against GFP (**a, d, g,** and **j**) and AstA (**b, e, h,** and **k**). Projections from the abdominal VNC terminate in the lower midgut and the hindgut (**a–c**), and the rectum (**d–f**). Labeling of the muscle is marked by a red arrow, just anterior to the midgut-to-hindgut transition, which is marked by a blue arrow. In the upper midgut (**g–i**) muscle tissue is also labeled, indicated by a red arrow, just below the foregut-to-midgut transition. Speckled labeling of the foregut is indicated by circles, in cases where gfp+, AstA- labeling is seen, and white arrows in cases where gfp+,AstA+ labeling is seen. Neuroendocrine cells of the lower midgut (**j–l**), are highlighted by circles where GFP+ labeling is seen, and squares, where AstA+ labeling is seen. Merged images are shown in (**c, f, i, l**) and all images are positioned so that anterior is upper left.

Figure 4. Co-expression of AstA and AstA-Gal4 in the CNS of third instar larvae, ablating AstA-Gal4 cells highlights AstA+/Gal4- expression, potential AstA release sites in the brain
(**a–f**) Immunofluorescent detection of AstA and lacZ in the CNS of AstA-Gal4/UAS-(nuclear)::lacZ wandering third instar larvae by whole-mount AstA in situ hybridization (**b** and **e**), and immunolabeling of reporter expression using anti-β-Galactosidase (**a** and **d**). Arrows in (**a–c**) indicate three AstA+, Gal4- cell bodies. A higher magnification view of the larval brain (**d–f**) identifies at least four additional AstA+ Gal4- cell bodies, indicated by arrows. Merged images shown in (**c**) and (**f**). Immunofluorescent detection of GFP in the brain of AstA-Gal4,UAS-mcd8::GFP/UAS-Ricin flies confirmed the ablation of AstA-Gal4-expression in the brain (data not shown). (**g**) Immunofluorescent detection of *Diploptera* AstA in the adult brain of AstA-Gal4/UAS-Ricin flies reveals the extent of arborization of the non-Gal4-expressing but AstA-expressing neurons in the brain. (**h**) Immunofluorescent detection of GFP in the gut of AstA-Gal4,UAS-mcd8::GFP/UAS-Ricin flies confirmed the ablation of AstA-expressing muscle, neuroendocrine cells, and

projections in the midgut. (**i**) A reporter that highlights neuropeptide trafficking, UAS-ANF::GFP, was used to predict potential sites of neuropeptide release. Immunofluorescent detection of GFP in the CNS of AstA-Gal4/UAS-ANF::GFP. Note the density of GFP expression in the protocerebrum, pars intercerebralis, and SOG.

Supplementary Figure 1. High-resolution images of the abdominal ganglion

High-resolution images of the abdominal ganglion illustrating that AstA and AstA-Gal4 are co-expressed in three pairs of cells. Immunofluorescent detection of AstA (**e, f**, and **g**) and of UAS-mcd8::GFP driven by AstA-Gal4 (**b, c**, and **d**). (**a**) Merged image of the abdominal ganglion with arrows pointing to the relative location of three separate cell bodies. (**b** and **e**), (**c** and **f)**, and (**d** and **g**) are images of different planes/slices that illustrate that indeed three distinct cell bodies are co-expressing AstA and GFP.

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Figure 1

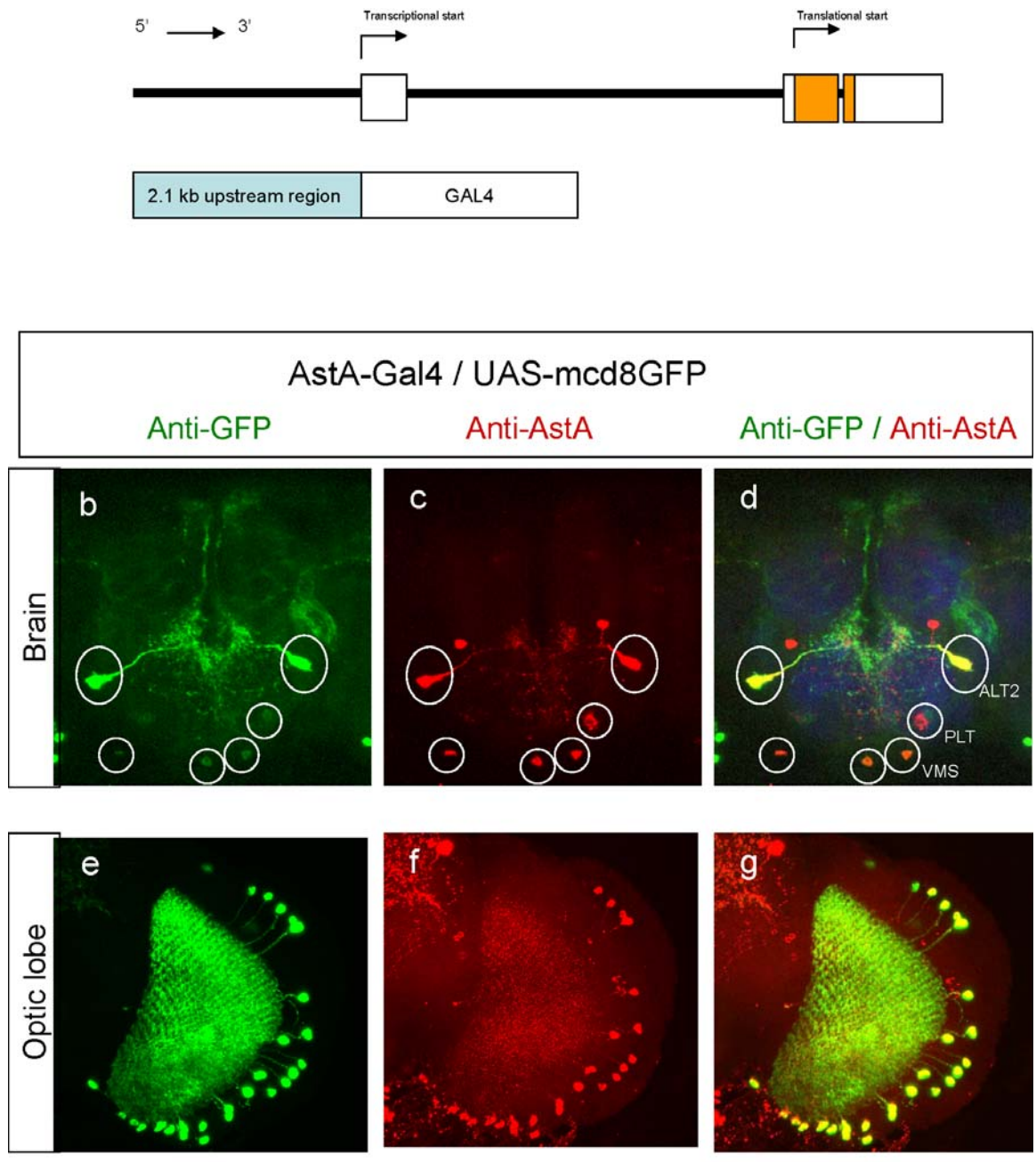

a.
5'
3'
Transcriptional start
Translational start
2.1 kb upstream region
GAL4
AstA-Gal4 / UAS-mcd8GFP
Anti-GFP
Anti-AstA
Anti-GFP / Anti-AstA
Brain
b
c
d
ALT2
PLT
VMS
Optic lobe
e
f
g

Figure 2 AstA-Gal4 / UAS-mcd8GFP

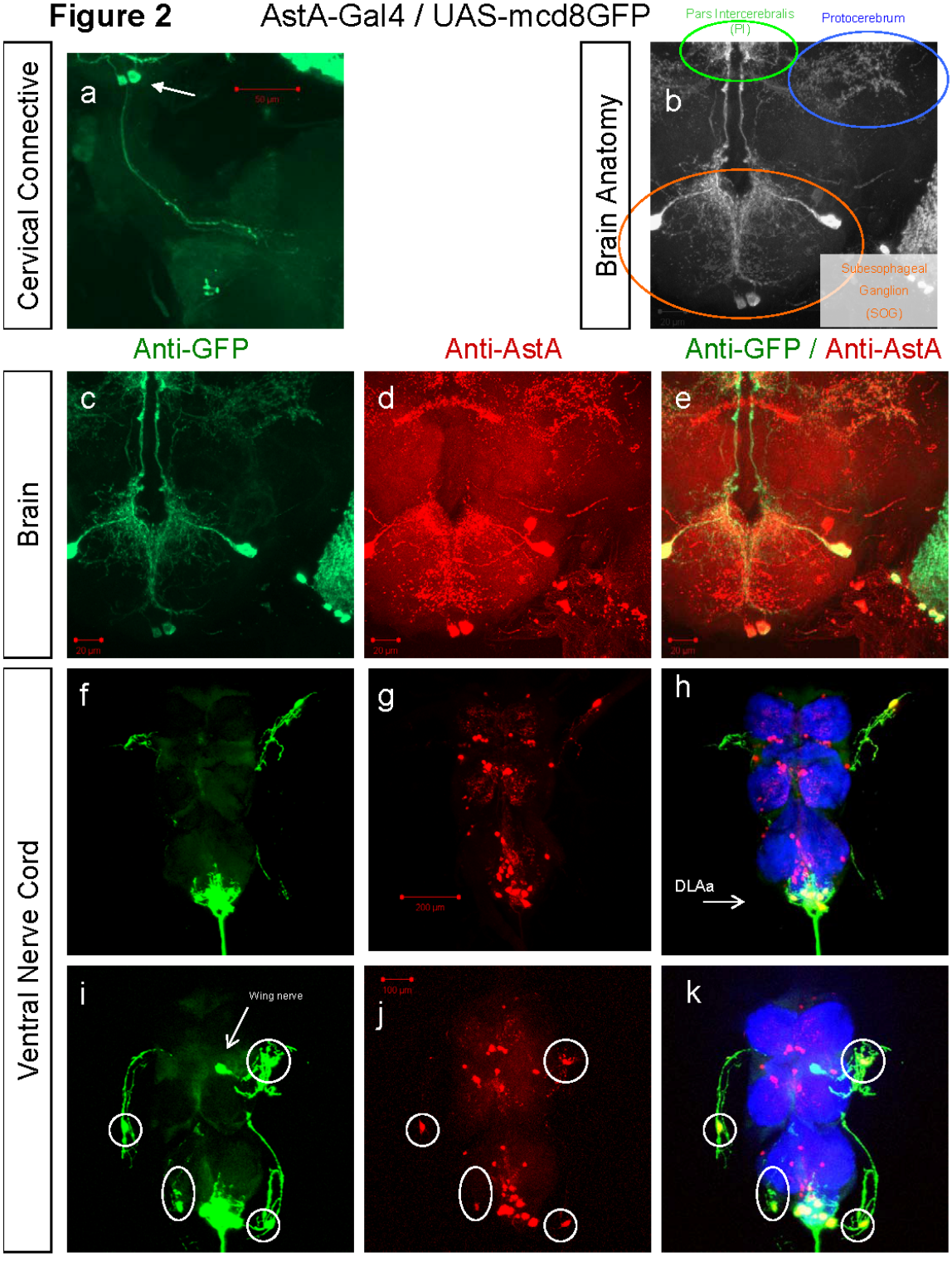
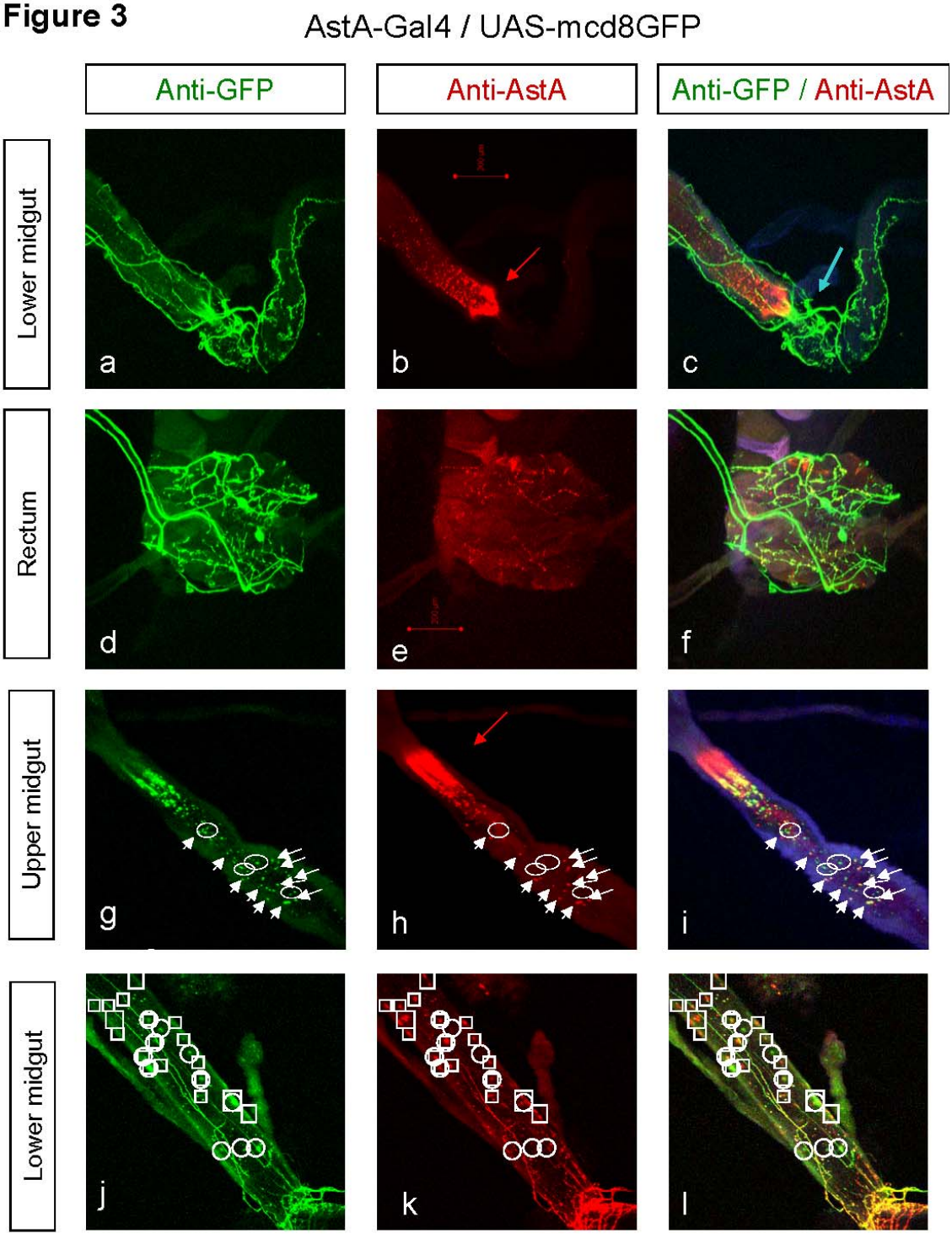


Figure 3


AstA-Gal4 / UAS-mcd8GFP
Anti-GFP
Anti-AstA
Anti-GFP / Anti-AstA
Lower midgut
Rectum
Upper midgut
Lower midgut
a
b
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d
e
f
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i
j
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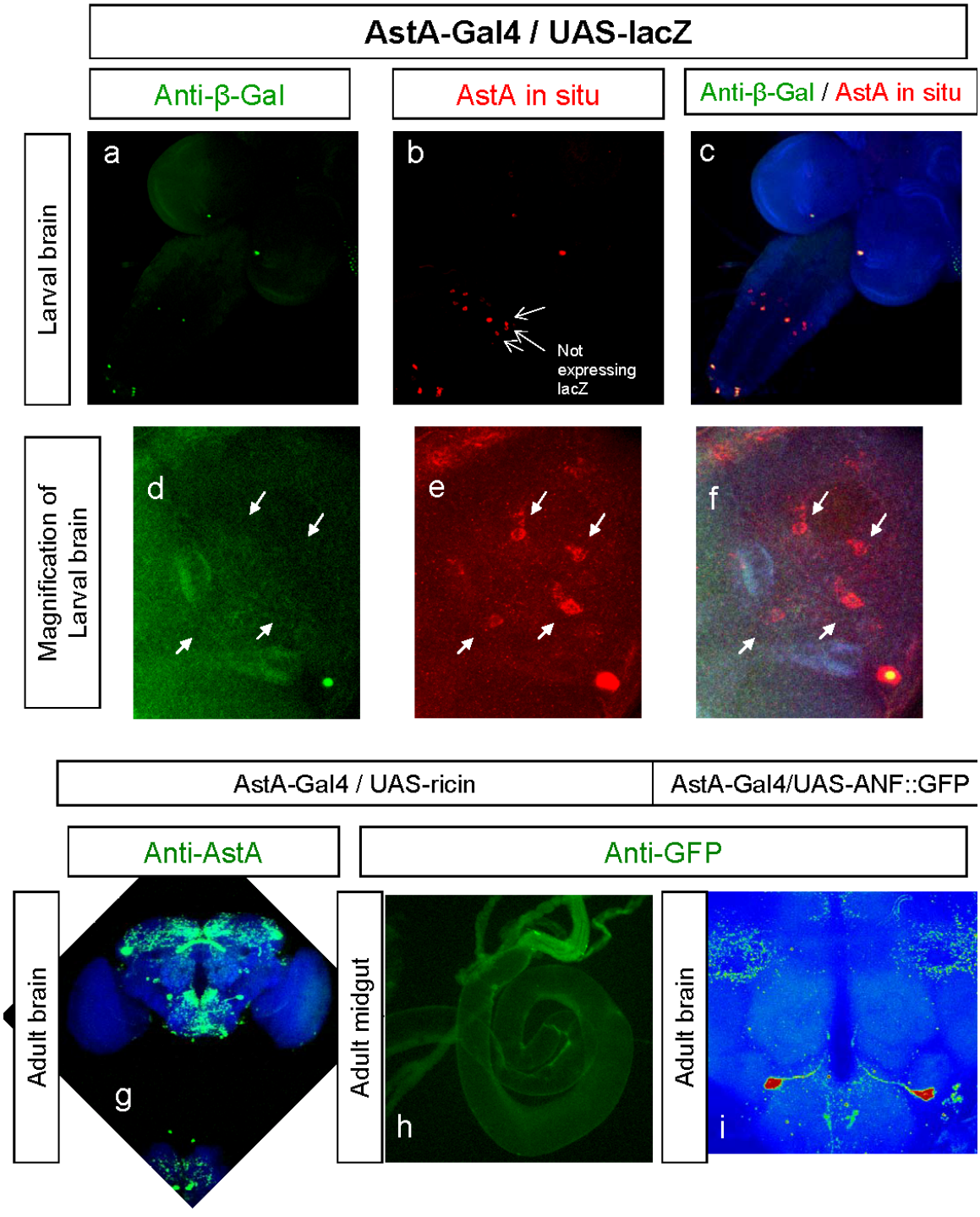
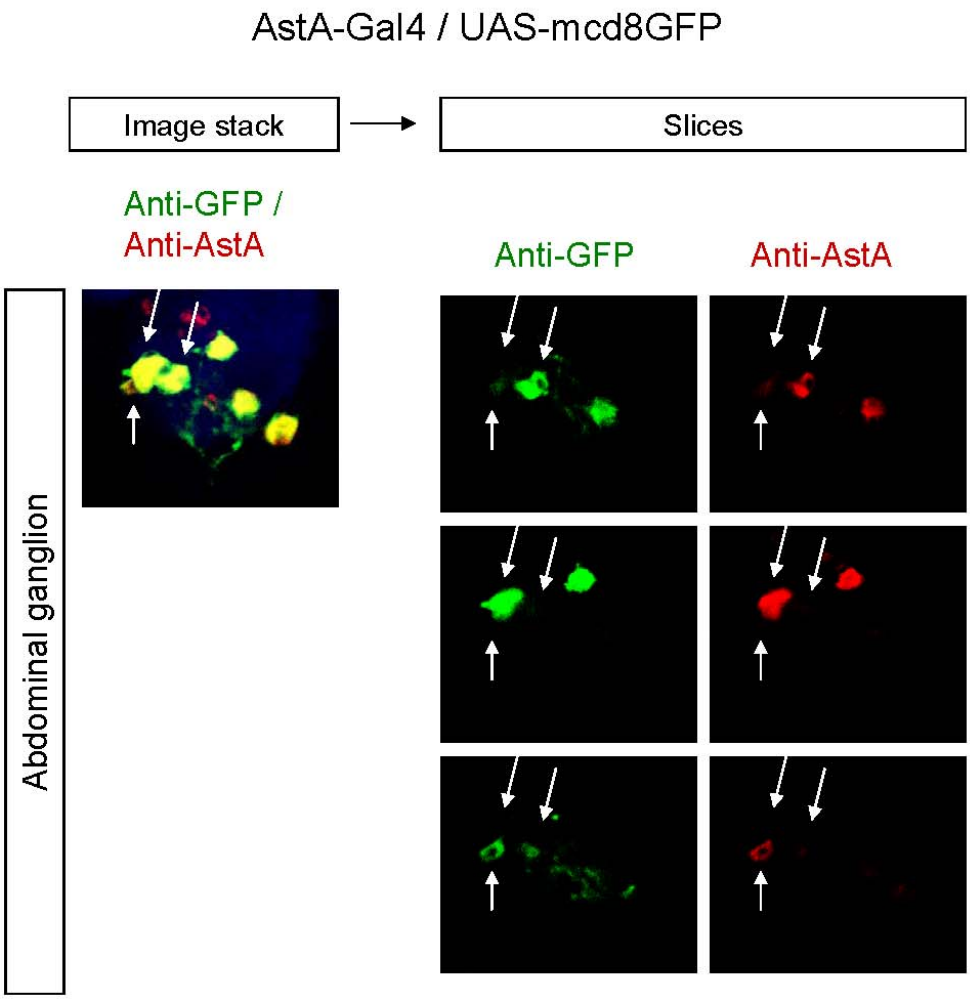
l

Figure 4


AstA-Gal4 / UAS-lacZ
Anti-β-Gal
AstA in situ
Anti-β-Gal / AstA in situ
Larval brain
a
b
c
Not expressing lacZ
Magnification of Larval brain
d
e
f
AstA-Gal4 / UAS-ricin
AstA-Gal4/UAS-ANF::GFP
Anti-AstA
Anti-GFP
Adult brain
g
Adult midgut
h
Adult brain
i

Supplementary Figure 1



Chapter 3

ACTIVATION OF ASTA NEURONS RESULTS IN SIGNIFICANTLY REDUCED STARVATION-INDUCED FEEDING BEHAVIOR

1. Introduction

The neuropeptide Allatostatin A (AstA) has been implicated as a satiety factor and as a myoinhibitor in several other insects [1–3]. Currently, there is no known function for AstA or for AstA neurons in *Drosophila* [1–3]. Expression of AstA in feeding-related endocrine centers suggests that AstA may play a role in the regulation of *Drosophila* satiety as well [2]. To test whether AstA regulates *Drosophila* satiety, we modified an existing feeding assay in order to obtain large differences in food intake between ad libitum fed flies and starved flies. This new assay interrogates physiologically-relevant behavioral changes that occur upon food deprivation, which includes increased food search behavior (foraging) and increased sensitivity to food cues (increased acceptance of low reward foods) [4]. Using this assay, we demonstrate that activation of AstA neurons results in significantly reduced starvation-induced food intake.

2. Results

2.1 Description of a feeding assay that measures starvation-induced feeding behaviors

The use of dyes to quantify feeding behavior has been well established [5]. This method of quantifying feeding makes it possible to do large scale experiments that examine both the feeding habits of populations of flies as well as the variability of the feeding habits within a population of flies. Dye is added to the food source in order to measure food intake. The volume of dye-laced food consumed by a population of flies can be quantified by measuring the absorbance of homogenized flies at the wavelength of the blue dye. The volume of food intake is interpolated from absorbance measurements of a dilution series of the dye. To gain more information about the feeding behavior of individual flies within a population of flies we visually inspected individual flies for the presence or absence of blue dye in the abdomen of each fly (before homogenizing). Detecting very small meal sizes

by eye under a dissecting microscope is unambiguous and is a much more sensitive method than using colorimetric quantification[1] (see Supplementary Figure 1a–1c). Scoring by eye also allows us to monitor for an abnormal distribution of meal sizes within a population of flies[2].

We modified feeding assay parameters in order to measure differences in starvation-induced feeding behaviors, which include an increase in food search behavior, food intake, and an increased sensitivity to food cues [4, 5]. Therefore, in a feeding assay, we wanted to use a concentration of sucrose that was not very stimulating to unstarved flies but that, as a result of starvation, became a stimulating concentration, as measured by food intake. We found that by manipulating the location and the size of the food patch, we could also manipulate the fraction of flies feeding, suggesting that we could use these parameters to incorporate food search behavior into our assay. We determined that by providing sucrose in a small raised dish placed inside a standard fly vial resulted in 100% of starved flies feeding, but only a fraction of unstarved flies feeding. The final parameters that we used in our feeding assay produced clearly distinguishable differences in both the number of flies feeding and in amount consumed between starved and unstarved animals (see Supplementary Figure 1d).

Unless otherwise noted, the following conditions were used in all feeding experiments: Twenty male flies were deprived of food but not water for 24 hours and then allowed one hour to feed on 10 mM sucrose, which was located in a small raised dish[3]. These assay conditions resulted in nearly 100% of starved flies feeding ~120 nL each (Figures 1a and 1b). In contrast, not only did a much smaller percentage of unstarved flies feed under these conditions, but they also consumed around sixfold less sucrose than starved flies (data not shown). These results are highly reproducible and confirm that our feeding assay measures changes in starvation-induced feeding behaviors.

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[1] By eye, a meal size of 20 nL or less per fly can be unambiguously detected (see Supplementary Figure 1a–c). By colorimetric quantification, the limit of detection is 400 nL per 20 flies.

[2] Using only colorimetric quantification to measure the food intake of a population of flies assumes that meal size is homogeneous across a population of flies and does not discriminate between a case in which 2 flies consumed 200 nL from 20 flies that consumed 20 nL each.

[3] Sucrose was dissolved in 0.5% agar and supplied in the screwtop lid of a 1.5 mL microcentrifuge tube, which was placed inside of a standard fly vial in which flies were starved.

2.2 Hyperexcitation of AstA neurons results in significantly reduced starvation-induced feeding.

Since the neural regulation of feeding is essential to the survival of an organism, it would be evolutionarily beneficial to have redundancy or compensatory mechanisms in place if elements of the circuit are lost (due to mutation or cell death). Consequently, instead of silencing or ablating AstA neurons, we initially chose to use a gain of function manipulation in order to probe the function of AstA neurons. We used UAS-NaChBac[4] [6], which would express a leaky sodium channel in AstA neurons, in order to increase the neuronal excitability of AstA neurons. We tested flies in the feeding assay described in the last section, in order to determine whether AstA neurons play a role in the regulation of feeding behavior.

Constitutive activation of AstA neurons resulted in significantly fewer flies feeding relative to controls (Figure 1a). As measured by the volume of food intake, control flies consumed on average ~120 nL per fly, while AstA/NaChBac[5] flies consumed only ~15 nL per fly, an eightfold reduction in total feeding (Figure 1b). This reduction in feeding behavior was observed using either of two independent insertions of AstA-Gal4 (labeled AstA3 and AstA5). The reduction of volume consumed is not due to most flies taking smaller meals, but to fewer flies feeding. Visual inspection of individuals showed that nearly 100% of control flies fed, but fewer than 20% of AstA/NaChBac flies fed. To ensure that we were accurately scoring non-feeding flies, which might have been mis-scored if they only consumed minute portions of food that were visually undetectable through the cuticle, we routinely dissected flies to directly visualize the contents of the gut and crop, and found few cases where dye was present in the gut but was undetectable through the cuticle.

To confirm that the feeding impairment phenotype observed by constitutively expressing NaChBac in AstA neurons was indeed due to hyper-activating these neurons, we asked whether the feeding phenotype could be rescued by simultaneously co-expressing an inwardly rectifying potassium channel using UAS-Kir2.1, which would decrease

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[4] A voltage-gated bacterial sodium channel

[5] AstA-Gal4/UAS-NaChBac. To simplify text, we will henceforth not include "Gal4" and "UAS" when referring to genotypes.

neuronal excitability [7]. As previously observed, significantly fewer AstA5,NaChBac/+ flies fed, but the fraction of AstA5,NaChBac/Kir2.1 flies that fed was indistinguishable from the fraction of control genotypes that fed (Figure 1c). This rescue by constitutive hyperpolarization shows that the feeding phenotype of AstA/NaChBac flies is due to the hyperactivation of AstA neurons.

Constitutively activating neurons could result in developmental defects or other defects that could accumulate over time. Therefore, we used UAS-TRPA1 [8], a warmth-activated cation channel, to acutely activate AstA neurons. Flies were raised at 22°C, a temperature at which TRPA1 is not activated, and shifted to the permissive temperature, 28°C, an hour before and during the feeding assay. When tested at 28°C, significantly fewer AstA/TRPA1 flies fed compared to genetic controls tested at the same temperature (Figure 1d). When flies were raised and tested at 22°C, feeding of AstA/TRPA1 flies did not differ from controls. There was no significant difference in the food intake of control flies as a consequence of the temperature shift. Therefore, acute hyperactivation of AstA neurons phenocopied the feeding phenotype observed due to chronic hyperactivation. The magnitude of this effect was less than that obtained using NaChBac which could be due to differences in the potency of the two effectors. These results indicate that the feeding phenotype of AstA/NaChBac flies was not due to developmental defects, or other accumulated effects from chronic excitation, but instead was a result of acutely activating AstA neurons.

2.3 The feeding behavior of AstA/NaChBac flies is not an indirect effect of assay parameters.

Several control experiments were conducted to ensure that the reduced feeding observed among AstA/NaChBac flies was not due to the presence of dye or agar in food, social interactions, locomotor or foraging deficits, novelty, or visual deficits. In an alternate feeding assay called CAFE (for CApillary FEeder) [9], flies were allowed one hour to feed on 100 mM sucrose supplied in capillary tubes. As flies drink the sucrose solution from the capillairies, the meniscus drops, and the amount consumed was determined by measuring the change in the height of the meniscus from the beginning to

the end of the experiment. As observed in our standard feeding assay, AstA/NaChBac flies consumed significantly less than control genotypes (Figure 2a). Since feeding on a novel food source represents a risk-prone behavior, we repeated our standard feeding assay using standard fly food. Again, AstA/NaChBac flies consumed significantly less than control genotypes (Figure 2b). Taken together, these results suggest that the reduced feeding behavior of AstA/NaChBac flies is not specific to a low concentration of sucrose and is not an indirect effect of agar or dye, since the CAFE assay eliminates the need to use food coloring or agar. In other control experiments, reduced feeding behavior of AstA/NaChBac flies persisted when provided an excess of food, when tested at various times during the day, and when tested in the dark (data not shown).

2.4 Silencing or ablating Gal4-expressing AstA neurons does not alter feeding behavior.

Since activating AstA neurons suppresses feeding behavior, we tested whether the opposite was true: does silencing or ablating AstA neurons enhance feeding behavior? Kir2.1 overexpression reduces neuronal excitability [7], and its rescue of the feeding phenotype of AstA/NaChBac flies suggests that this is true of AstA neurons. We measured the feeding behavior of AstA/Kir2.1 flies in a variety of feeding assays, varying assay conditions such as the degree of starvation, food quality, food accessibility, and time to feed. We did not detect any differences in the feeding behavior of AstA/Kir2.1 flies compared to control genotypes when tested unstarved or starved, on high- or low-quality food sources, or when we tested the rate of feeding (data not shown). We also tested the feeding behavior of flies in which we ablated Gal4-expressing AstA neurons by overexpressing a cell-death-promoting gene using UAS-Ricin [10]. We confirmed the ablation of Gal4-expressing AstA neurons by co-expressing UAS-mcd8::GFP and UAS-Ricin and co-labeling tissue with antibodies against AstA and GFP (See Chapter 2, Figure 4). We did not detect any differences in the feeding behavior of either starved or unstarved AstA/Ricin flies compared to control flies (data not shown).

2.5 Activation of NPF neurons rescues the decreased feeding behavior of AstA/NaChBac flies.

To address the possibility that activation of AstA neurons is artificially or non-specifically inhibiting feeding behavior, we next asked whether activation of AstA neurons exhibited functional interactions with a known modulator of normal feeding behavior. Neuropeptide F (NPF) is a neuropeptide that mediates many of the behavioral responses to starvation; Overexpression of NPF increases foraging behavior and increases the acceptance of less rewarding food sources in starved flies [11]. Since NPF overexpression and activation of AstA neurons yield opposing behavioral responses in starved flies, we decided to investigate potential epistatic interactions between these two pathways. If activation of AstA neurons were artificially or non-specifically inhibiting feeding behavior, we would expect that simultaneous activation of NPF neurons would fail to override this inhibition. On the other hand, if simultaneous activation of NPF neurons overrides the inhibition of feeding caused by activation of AstA neurons, it would suggest that the mechanism by which AstA neuron activation impairs feeding occurs through a mechanism that impacts, directly or indirectly, pathways that normally control food intake.

The majority of studies characterizing the role of NPF signaling in feeding behavior have focused on larvae, but several studies have demonstrated a role for NPF signaling in adult feeding as well. Although increased feeding behavior as a result of activation of NPF neurons has not been directly demonstrated, one study has demonstrated that activation of NPF neurons is necessary and sufficient to produce the motivational state required for food-based associative learning [17]. When odor cues are paired with sucrose, starved flies will exhibit a preference for the sucrose-paired odorant in subsequent trials, but unstarved flies fail to exhibit this preference. It was demonstrated that activation of NPF neurons, using UAS-TRPA1, can elicit a preference for the sucrose-paired odorant in unstarved flies, suggesting that activation of NPF neurons promotes a hunger-state in unstarved flies.

In order to determine whether hyperactivation of NPF neurons would further enhance the hunger-state of starved flies, we first examined the feeding behavior of starved NPF/NaChBac flies. Our feeding assay parameters were not designed to identify an increase in the fraction of flies feeding because we optimized parameters to detect the opposite. By changing the food source in our feeding assay to 100 mM sucrose and 500

mM NaCl (a highly stimulating concentration of sugar combined with an aversive concentration of salt), a significantly larger fraction of NPF/NaChBac flies fed compared to control genotypes under these conditions (see Supplementary Figure 1e). In addition, even though we did not see a significant increase in the fraction of flies feeding using our original assay parameters (10 mM sucrose) due to a ceiling effect, NPF/NaChBac flies exhibited significantly increased food intake compared to control genotypes under these conditions (see Supplementary Figure 1f). Therefore, constitutively activating NPF neurons in further enhances the hunger-state of starved flies, and can affect two aspects of feeding behavior: The initiation of feeding (fraction of flies feeding) and the termination of feeding (total volume consumed).

We next tested what the effects of co-activation of NPF and AstA neurons was on the feeding behavior of starved flies in our original assay, containing 10 mM sucrose. Activation of NPF neurons partially rescued the reduced feeding behavior of AstA/NaChBac flies, as measured by fraction of flies feeding (Figure 2c, $p<0.0001$). Activation of NPF neurons overrides the reduced feeding caused by activation of AstA neurons, and this suggests that activation of AstA neurons does not impair the motor control of feeding or cause a physical inability to feed.

3. Discussion

3.1 A behavioral assay that measures starvation-induced changes in feeding behavior

We developed a robust, high-throughput feeding assay that requires characteristic starvation-induced behaviors, including foraging and the acceptance of a low reward food source. Many studies have identified neuropeptides and signaling pathways that are involved in the promotion of feeding behavior, but very little is known about the regulation or promotion of satiety in *Drosophila* [1]. This is likely because the majority of studies have focused on larvae, which feed almost constantly, and not adults, which are discontinuous feeders. In contrast to larvae, adults have a vast repertoire of behaviors and face a multitude of challenges. The regulation of feeding in adults could provide insight into the regulation of satiety. Our results demonstrate that we have developed a behavioral feeding assay that can identify flies with deficits in starvation-induced feeding behaviors.

3.2 Activation of AstA neurons results in significantly reduced starvation-induced feeding.

Using this feeding assay, we demonstrated that activation of AstA neurons results in significantly reduced starvation-induced feeding relative to controls. Both constitutive activation and acute activation of AstA neurons resulted in significantly reduced feeding. This reduced feeding behavior is caused by manipulating the firing properties of AstA neurons, because co-expression of UAS-Kir2.1 and UAS-NaChBac rescued the reduced feeding phenotype.

3.3 The reduced feeding behavior of AstA/NaChBac flies is not due to indirect effects.

We have ruled out potential defects that could result in decreased feeding behavior that are not directly related to feeding behavior. AstA/NaChBac flies fed significantly less in an established feeding assay, called the CAFE assay [9], which suggests that the reduced feeding behavior is reproducible using an established feeding assay, using a highly stimulating sucrose concentration, and is not due to an aversion to either agar or dye in the food. Since the failure to find or access the food could be a result of impaired locomotor

activity or foraging, sensory deficits, or social interactions, we demonstrated that AstA/NaChBac flies fed significantly less when an excess of food was supplied. These results imply that the decreased feeding of AstA/NaChBac flies was not a result of reduced encounter with the food source. The decreased feeding behavior is not due to a disinclination of AstA/NaChBac flies to accept a novel food source. The acceptance of a novel food source is considered a risk-prone behavior, and is modulated by hugin-expressing peptidergic neurons [12]. Using standard fly food in the feeding assay did not rescue the decreased feeding behavior of AstA/NaChBac flies, eliminating the possibility that the feeding behavior is a result of an aversion to feeding on a novel food source. In addition, since standard fly food contains a mixture of nutrients, including protein and fat, the decreased feeding behavior of AstA/NaChBac flies is not specific to sucrose. These control experiments convinced us that AstA/NaChBac flies exhibit significantly decreased feeding relative to controls and that this is not an indirect effect on some other aspect of fly behavior. It also validates that the behavioral feeding assay that we developed is a useful method to screen for flies with satiety-like feeding behavior.

3.4 Silencing or ablating Gal4-expressing AstA neurons has no effect on feeding.

We were unable to detect any differences in the feeding behavior of flies with either silenced or ablated AstA neurons compared to control flies. Silencing experiments are important because they can teach us about the function of a neuronal circuit in normal behavior. If activation and silencing of a neuronal circuit gives opposing effects, it can prove the necessity and sufficiency of a circuit. Silencing or ablating Gal4-expressing AstA neurons has no effect on feeding, suggesting that Gal4-expressing AstA neurons are not necessary for normal feeding behavior although these neurons are sufficient to modulate feeding behavior. There are three potential explanations for these results:

1) We have not yet tested flies under the proper conditions to uncover a role of AstA in the regulation of feeding. Activation of AstA neurons may be tapping into a mechanism that is only used under very specific circumstances, e.g., when avoiding a predator, encountering a mate, or undergoing diapause. Diapause is a state of dormancy employed by insects to survive harsh environmental conditions, such as low temperatures

and short length of day [13]. In flies, diapause results in reduced food intake, a slowed metabolism, an increase in fat stores, arrested development, and reproductive dormancy [14, 15]. Activation of AstA could trigger a state of diapause but silencing these neurons would not result in altered feeding behavior under normal lab conditions.

2) The function of AstA neurons could be redundant. In Chapter 2, we demonstrated that AstA-Gal4 is expressed in only a subset of AstA-expressing neurons. In the brain, AstA-Gal4 is expressed in only three of eight AstA neurons, and in the VNC, it is expressed in only three of 13 AstA neurons. Therefore, AstA+/Gal4- neurons may be compensating for silenced or ablated AstA+/Gal4+ neurons. Alternatively, constitutive loss of AstA neurons may lead to the recruitment of alternate circuits during development that fulfill the function of AstA neurons. This possibility could be addressed by testing the feeding behavior when acutely silencing AstA neurons.

3) Activation of AstA neurons results in a gain of function phenotype that does not represent the role of AstA neurons in satiety. For example, constitutive activation of AstA neurons could result in the secretion of abnormally high concentrations of a neuromodulator that regulates satiety, but systemic secretion of this neuromodulator does not occur as a result of the normal firing properties of AstA neurons.

3.5 Activation of NPF neurons rescues the decreased feeding behavior of AstA/NaChBac flies.

Simultaneous activation of NPF and AstA neurons resulted in a partial rescue of the decreased feeding observed in AstA/NaChBac flies. The foregoing results suggest that NPF neurons act downstream of (or in parallel to) AstA neurons in their control of feeding. If we had obtained no rescue (i.e., reduced feeding due to AstA neurons acting downstream of NPF neurons), then it would leave open the possibility that activation of AstA neurons impairs the motor control of feeding, or causes a physical inability to feed, e.g., constipation or motor impairment. In agreement with other control experiments mentioned in earlier sections, these results rule out many non-specific potential causes for the reduced feeding behavior of AstA/NaChBac flies, such as motor deficits and sensory deficits.

4. Materials and methods

Fly husbandry

All flies were reared on standard media [16] and maintained at 25ºC, 70% relative humidity under a 12h:12h light:dark regime unless otherwise indicated. For TRPA1 experiments, flies were raised at 22ºC. Flies used for immunohistochemistry and behavior were 5–10 day old males. Fly lines used included AstA-Gal4 (described in Chapter 2), UAS-NaChBac::eGFP (a generous gift from Dr. Bader Al-Anzi) [6], UAS-TRPA1 flies express two independent insertions of the transgene (the recombinant was a kind gift from Kenta Asahina) [8], UAS-eGFP::Kir2.1 [7], *w* (Exelixis background, used for UAS-responder x *w* crosses), *w* (isogenic strain from the Dr. Tim Tully lab, used for *w* x Gal4 crosses), NPF-Gal4 (Bloomington stock #25681)[11], and Canton-S flies (obtained from the Seymour Benzer lab). For experiments using flies expressing three transgenes, AstA5-Gal4 and UAS-NaChBac::eGFP were recombined onto the same chromosome.

Behavior

Feeding assays

Unless otherwise noted, the following conditions were used in all feeding assays. Twenty adult male flies were wet-starved for 24 hours in standard fly vials containing 1% agar. A small dish containing 10 mM sucrose was placed into fly vials for one hour. Sucrose was dissolved in 0.5% agar plus 0.5% FD&C Blue #1 and was provided within the center ring (28 mm^2 surface area) of a screwcap lid (belonging to 1.5 mL microcentrifuge tubes, Fisher 02-681-347). After each experiment, the fraction of flies feeding was visually scored as the presence or absence of blue dye in the abdomen using a dissecting microscope (for a more detailed explanation of this method of quantification, see Supplementary Figure 1a). After visual scoring, the volume of food intake was determined by colorimetric quantification. Each population of flies was beheaded, homogenized, centrifuged, and the absorbance of the supernatant was measured at the wavelength of the blue dye (625 nm). To control for any background absorbance from homogenized flies, a set of starved, unfed flies were simultaneously processed. Only males were used in feeding experiments because the regulation of feeding behavior in females is complicated by reproductive state, since feeding behavior and metabolism are altered by mating and egg production in females [17]. All experiments were conducted during midday, within a six-hour time window that did not

overlap with the morning and evening peaks of fly activity. Before experiments, flies were acclimated for one hour within the testing chamber at 25°C and 60% relative humidity. Flies were allowed to recover from CO_2 anesthesia (used to sort flies) for at least 24 hours before starvation was initiated.

The CAFE assay was previously described in [9]. Briefly, a 100 mM sucrose solution was provided in small glass capillary tubes from which 20 flies were allowed to feed for 1 hour. Amount consumed was Meniscus levels were measured before and after the experiment. Evaporation was controlled for by measuring meniscus levels from capillaries that were set up during the experiment, but without flies. The changes in meniscus distance in evaporation controls were subtracted from experimental measurements.

Data and statistics

GraphPad Prism software was used to generate graphs as well as for statistical analysis.

Bar graphs plot the mean and standard error. Unless otherwise noted, all data was analyzed using one-way ANOVA with Bonferroni correction for multiple comparisons.

5. Figure Legends

Figure 1. Hyperexcitation of AstA neurons results in significantly reduced feeding. (**a, c,** and **d**) The fraction of food-deprived flies that fed and (**b**) the total volume of 10 mM sucrose consumed. (**a** and **b**) Both the fraction of flies feeding and the total volume consumed was significantly reduced for AstA/NaChBac flies relative to genetic controls. ($p<0.0001$, N=4–20). The total volume of sucrose consumed for each population of flies from (**a**) is shown in (**b**). Unless otherwise noted, all feeding assays allowed twenty adult male flies one hour to feed from 10 mM sucrose after being starved for 24 hours with access to water. Sucrose was dissolved in 0.5% agar and 0.5% FD&C Blue #1 and was provided in the scewtop lid of a 1.5 mL microcentrifuge tube. In order to calculate the fraction of flies feeding, individual flies were scored by eye under a dissecting microscope for the presence or absence of blue dye in the abdomen. This method of scoring was unambiguous above a meal size of ~15 nL (see Supplementary Figure 1a). After visual scoring, the amount of sucrose consumed by each population of flies was determined in the following way: flies were beheaded, homogenized, and centrifuged, and the absorbance in the wavelength of the dye (625 nm) of the supernatant was measured. The total volume of sucrose consumed was interpolated from this absorbance measurement (minus the absorbance measurement of unfed flies processed in parallel) and the absorbance measurements of a dilution series of the dye. [5] used similar feeding conditions (males wet-starved 24 hours, and allowed one hour to feed on 10 mM sucrose containing 0.5% FD&C Blue #1) and reported an average food intake of 110 nL per fly, similar to the amount of intake of control genotypes. NaChBac expression increases the electrical excitability of neurons [6]. Crosses were UAS-NaChBac x *w*, UAS-NaChBac x AstA-Gal4, and *w* x AstA-Gal4. Two independent insertions of AstA-Gal4 were used (labelled AstA3 and AstA5). "Gal4" and "UAS" will be eliminated from future text when referring to genotypes to simplify figures and text. (**c**) The fraction of AstA5,NaChBac/+ flies that fed was significantly reduced compared to that of all control genotypes ($p<0.0001$), but the fraction of AstA5,NaChBac/Kir2.1 flies that fed was not significantly different from that of genetic controls ($p=0.2429$, N=3–7). Crosses were AstA5-Gal4,UAS-NaChBac x *w*, AstA5-Gal4,UAS-NaChBac x UAS-Kir2.1, and UAS-NaChBac x UAS-Kir2.1. (**d**) The fraction of AstA/TRPA1 flies that fed was significantly reduced when tested at a

temperature that activates TRPA1, 28°C, but not when tested at a temperature that does not activate TRPA1, 22°C (two-way ANOVA, $p<0.0001$, N=12–15, [8]). All flies were raised at 22°C, and either tested at 22°C or shifted to 28°C for one hour before and during the experiment. When raised and tested at 22°C, the fraction of AstA/TRPA1 flies that fed was not significantly different from that of genetic controls (one-way ANOVA, $p=0.1597$). For control genotypes, there was no interaction of temperature (two-way ANOVA, $p=0.7202$). Crosses were UAS-TRPA1 x *w*, UAS-TRPA1 x AstA5-Gal4, and *w* x AstA5-Gal4. Unless otherwise noted, the mean and standard error are plotted and one-way ANOVA with Bonferroni correction for multiple comparisons was used for statistical analysis. AstA/NaChBac flies are plotted in orange, +/NaChBac flies in blue, and AstA/+ flies in white or black. Asterisks indicate statistical significance of $p<0.05$ (*), $p<0.01$ (**), or $p<0.001$ (***). The letters that appear above graphs indicate the statistical significance between genotypes after Bonferroni correction.

Figure 2. AstA/NaChBac flies feed significantly less when tested in the CAFE assay or fed standard fly food. Activation of NPF neurons partially rescued the reduced feeding behavior or AstA/NaChBac flies.

(a) AstA/NaChBac flies consumed a significantly smaller volume of sucrose in the Capillary Feeding assay, or CAFE assay ($p=0.003$, N=2–6). Groups of 20 flies were given an hour to feed on a 100 mM sucrose solution supplied in capillary tubes. The amount consumed was measured by the distance that the meniscus moved, by marking the height of the meniscus before and after the experiment. Chambers were set up in parallel without flies to control for evaporation of the sucrose solution. (**b**) Average fraction of starved flies feeding on standard fly food. AstA/NaChBac flies fed significantly less than controls. (**c)** When AstA and NPF neurons were simultaneously activated (AstA,NaChBac/NPF flies, orange striped bars) the fraction of flies feeding was significantly increased compared to when only AstA neurons were activated (AstA,NaChBac/+, orange bars, unpaired t-test, $p<0.0001$, N=10–11). The fraction of AstA,NaChBac/NPF flies feeding was still significantly reduced compared to that of other genotypes tested (one-way ANOVA, $p<0.0001$, N=6–11). Feeding conditions described in Figure 1a were used.

Supplementary Figure 1. Small meal sizes are easily visualized using food coloring. Activation of NPF neurons results in significantly increased food intake and fraction of flies feeding.

(**a–c**) Photos that illustrate the ability to detect small amounts of dye-laced food in the abdomens of adult male flies by visual scoring under a dissecting microscope. Circled in (**a–c**) is the section of the abdomen scored for blue dye (used at the same concentration as in feeding assays). An images of an unfed fly (**a**), a fly that consumed 20 nL (**b**), and a fly that consumed 100 nL (**c**) of blue-laced dye. The midguts and crops (indicated by arrows) were dissected out these photographed flies and are shown in images to the right. The volume consumed was measured by processing the intact midguts and crops as described in Figure 1b, except that the intact dissected digestive tracts of single flies were homogenized in a smaller final volume. (**d**) Fraction of unstarved and starved flies feeding on 10 mM sucrose when required to "work" for the food. Supplying food in microcentrifuge tube lids is considered work, whereas the "no work" conditions supply a sucrose patch that spans the base of the fly vial, the surface area of which is 133 mm^2. The surface area supplied in the lid was 28 mm^2. (**e**) Constitutively activating NPF neurons resulted in significantly increased food intake compared to that of control genotypes ($p<0.0001$). Assay conditions were the same as those described in Figure 1a, and the method to quantify food intake is described in Figure 1b. (**f**) Constitutively activating NPF neurons resulted in significantly increased fraction of flies feeding compared to controls when the food source was 100 mM sucrose plus 500 mM sodium chloride ($p=0.038$, N=3–9). Changing the concentrations of tastants in our feeding assay allowed us to measure increases in fraction of flies feeding. All other assay conditions were the same as those described in Figure 1a.

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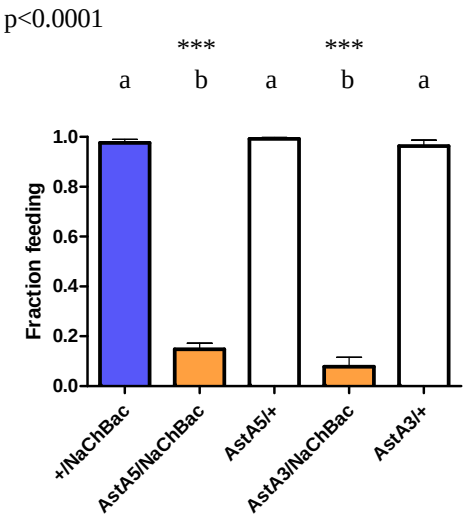
Figure 1

a.



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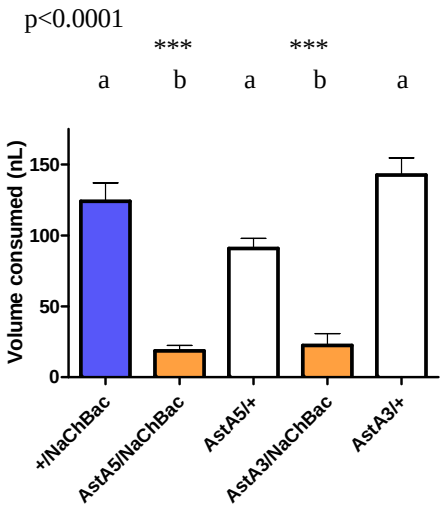

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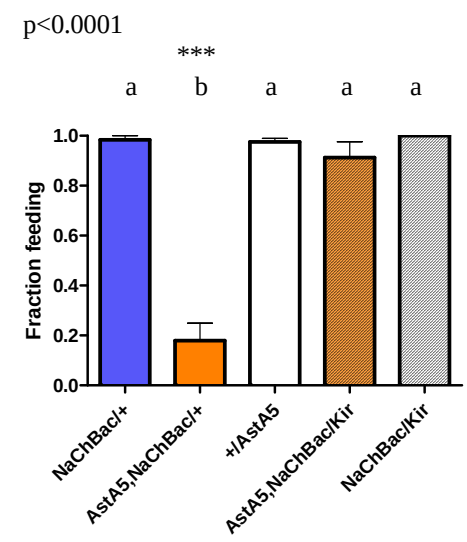


d.

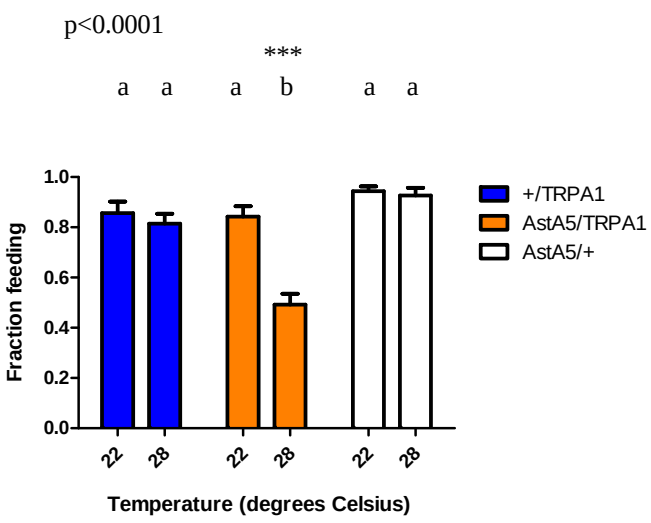
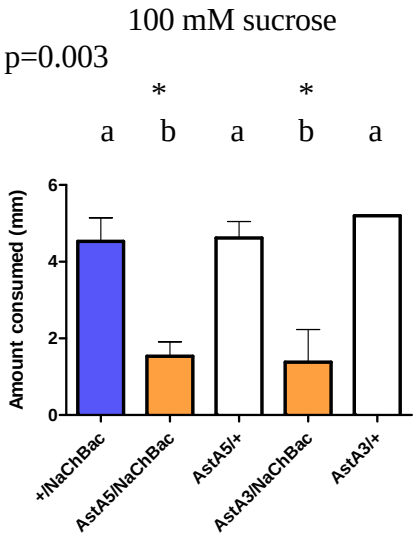
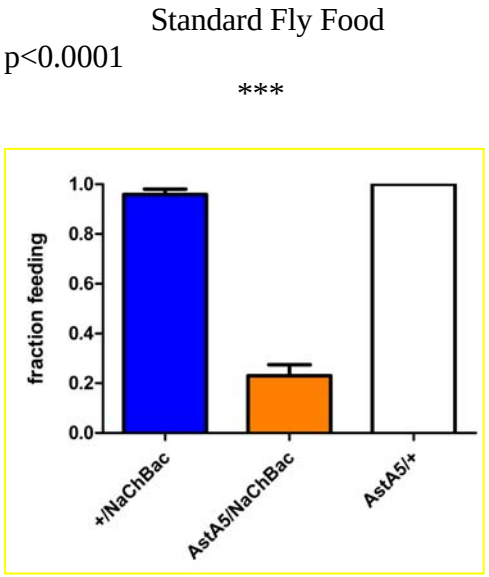


Figure 2

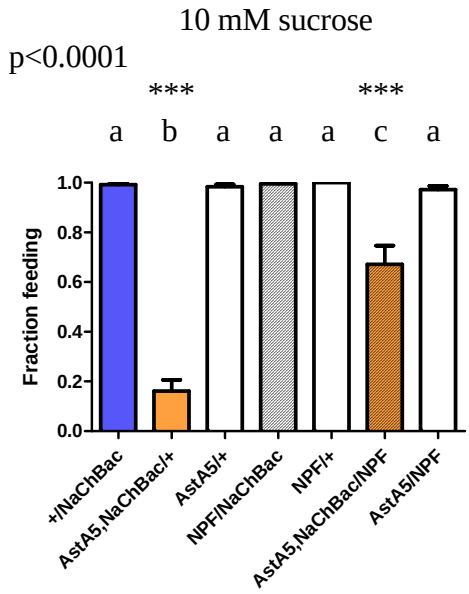
a.


100 mM sucrose
p=0.003
*
*
a b a b a
Amount consumed (mm)
6
4
2
0
+/NaChBac
AstA5/NaChBac
AstA5/+
AstA3/NaChBac
AstA3/+

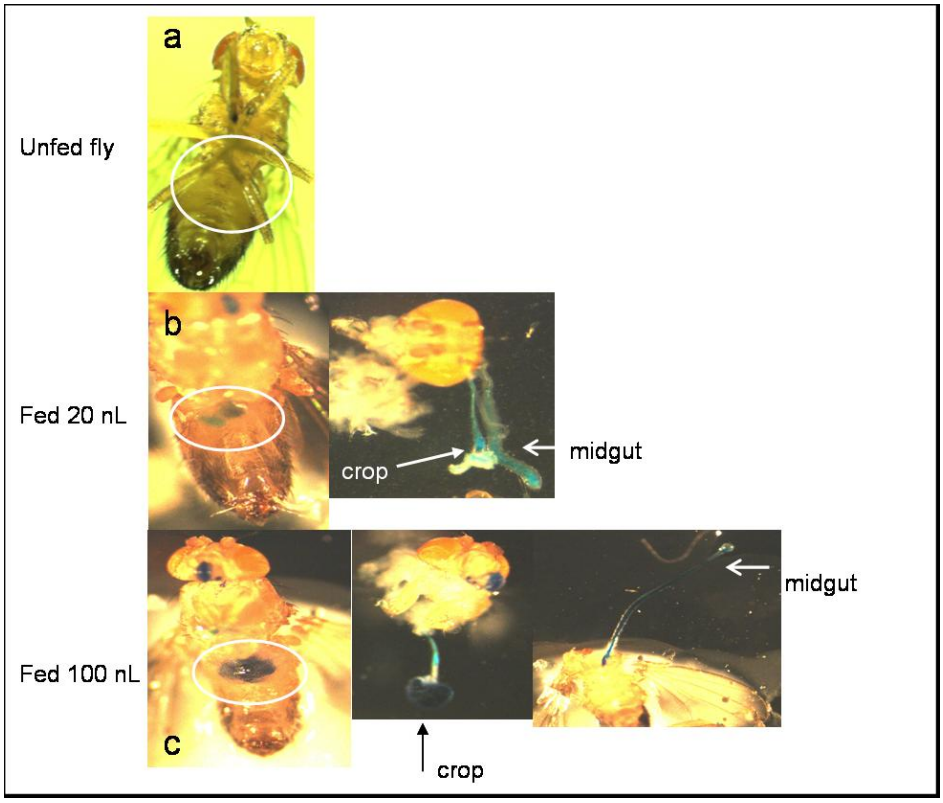
b.


Standard Fly Food
p<0.0001
***
fraction feeding
1.0
0.8
0.6
0.4
0.2
0.0
+/NaChBac
AstA5/NaChBac
AstA5/+

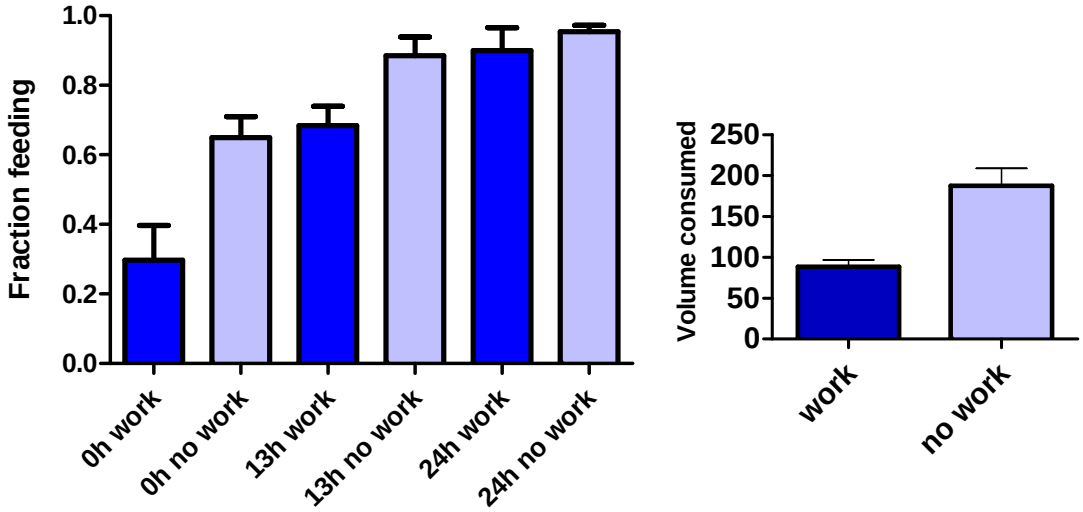
c.


10 mM sucrose
p<0.0001
***
***
a b a a a c a
Fraction feeding
1.0
0.8
0.6
0.4
0.2
0.0
+/NaChBac
AstA5,NaChBac/+
AstA5/+
NPF/NaChBac
NPF/+
AstA5,NaChBac/NPF
AstA5/NPF

Supplementary Figure 1

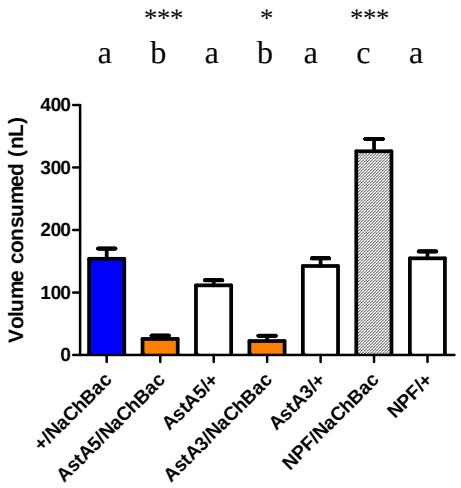


d. 10 mM sucrose



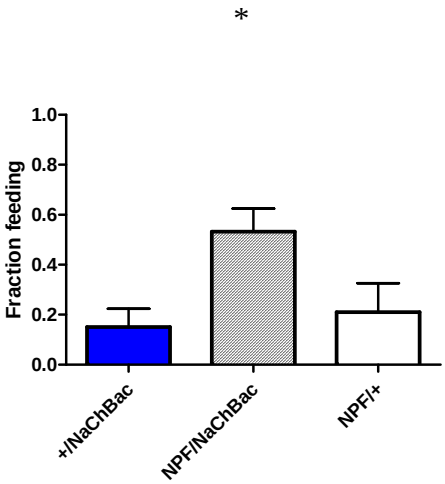
e.

10 mM sucrose



f.

100 mM sucrose + 500 mM NaCl



Chapter 4

MECHANISMS BY WHICH ACTIVATION OF ASTA NEURONS REDUCES STARVATION-INDUCED FEEDING BEHAVIOR

1. Introduction

In the previous chapter, I showed that activation of AstA neurons results in significantly reduced starvation-induced feeding. Here, I examined the behavioral and metabolic responses to starvation of flies with activated AstA neurons in order to further define the phenotype. First, I will discuss potential mechanisms that could explain the phenotype in relation to what is known about the regulation of feeding and metabolism in insects.

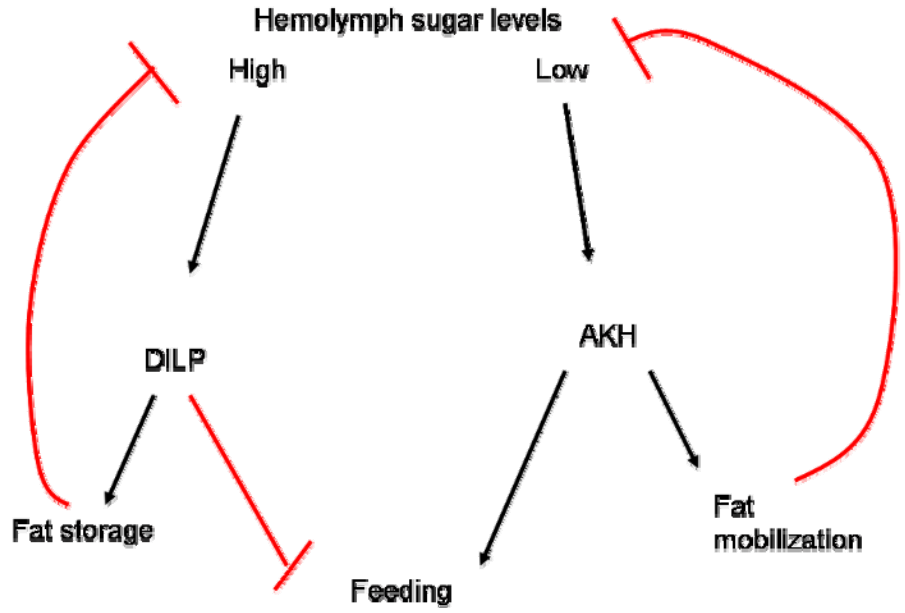
1.1 Nutritional state

The reduced food intake following starvation of AstA/NaChBac flies could be the result of higher energy reserves. *Drosophila* store energy in the form of triglycerides and glycerol, and utilize trehalose as an energy source [1]. If starvation-induced depletion of energy reserves plays a role in regulating feeding behavior, then a manipulation that prevents the depletion of energy reserves during food deprivation could reduce starvation-induced feeding. Depletion of energy stores during food deprivation could be prevented or decreased by a manipulation that causes decreased energy expenditure during starvation, decreased metabolism, or that endows flies with excess energy stores compared to controls.

In *Drosophila*, hemolymph sugar levels are monitored and regulated by two neuropeptides with opposing functions. Low hemolymph sugar levels prompt the release of adipokinetic hormone (AKH), which promotes fat mobilization [2]. Ablation of AKH neurons results in increased fat stores and increased resistance to starvation compared to controls, whereas overexpression of AKH has the reverse effect [3-5]. Conversely, high hemolymph sugar levels prompt the release of insulin-like peptide (DILP), which promotes fat storage [6-8] Ablation of DILP neurons results in increased hemolymph sugar levels and starvation sensitivity compared to controls, whereas overexpression of DILP has the reverse effects [6-8]. Manipulation of these metabolic regulators also alters feeding

behaviors: A null mutation in the AKH receptor or overexpression of DILP results in decreased food intake [5, 6]. Corazonin (Crz) neurons are involved in regulating stress responses and are upstream of AKH neurons; the metabolic effects of manipulating the neuronal activity of Crz neurons mimics those of manipulating AKH levels [9, 10].

Diagram 1. Neuropeptides regulate hemolymph sugar levels. This model simplifies the various interactions between nutritional state, neuromodulators, energy homeostasis, and behavior. How Crz neurons fit into this model is yet to be determined.



1.2 Excitatory and inhibitory sensory input

In the blowfly, proprioceptive feedback from the foregut, crop, or abdomen inhibits food intake, whereas the stimulating effect of tastants *promotes* food intake [11]. The stimulating effect of tastants increases with increasing sugar concentration and decreases with the addition of contaminating aversive tastants [12]. In *Drosophila*, the stimulating effect of tastants determines the volume of intake, but it has not been demonstrated that proprioceptive feedback inhibits food intake [13]. As mentioned previously, starvation in *Drosophila* increases responsiveness to food cues, but it is unclear if this is a result of tuning the sensitivity of primary gustatory neurons, a release of inhibition (disinhibition) from peripheral proprioceptors, or other internal changes.

If the activation of AstA neurons were drastically impairing gustatory acuity, flies in which NPF and AstA neurons were co-activated would not recognize the food offered, and would be unlikely to feed. But if gustatory acuity is slightly impaired, it is possible that NPF activation could overcome this impairment. Another possibility is that AstA neurons and NPF neurons are involved in tuning the sensitivity of gustatory neurons in opposite directions.

If AstA neurons were enhancing peripheral proprioceptive feedback, and if the regulation of feeding in *Drosophila* occurs in the same way as in blowflies, then we would not expect a rescue of feeding behavior in NPF,AstA/NaChBac flies. Blowfly studies demonstrated that in starved flies, proprioceptive feedback from the foregut, crop, and abdomen have a powerful inhibitory effect on feeding behavior. Given the robust feeding phenotype of AstA/NaChBac flies, we might not expect a central mechanism (NPF) that promotes starvation-induced feeding to override the effects of enhancing proprioceptive feedback. Based on blowfly studies, we would also expect inhibition of AstA neurons to result in hyperphagia, which did not occur (see Chapter 3).

1.3 Integration of nutritional state and gustatory/proprioceptive sensory cues

In blowflies, behavioral studies have demonstrated that gustatory discrimination and the decision to ingest are processed independently [14]. Previous studies have suggested that this might be the case in *Drosophila* as well [15]. AstA/NaChBac flies may be impaired in detecting nutritional state or in integrating this information with gustatory cues which would lead to impairments in feeding/ingestion “decision-making”.

In *Drosophila* gustatory sensory neurons, the regulation of feeding behavior, and the regulation of metabolism are well characterized. Very little is known about how gustatory input is integrated or how nutritional state alters feeding behavior. Integration of gustatory cues and nutritional state may occur in DILP neurons or the many types of neuropeptide-expressing neurons that send projections to both the SOG and to the vicinity of DILP neurons. DILP neurons are sensitive to hemolymph sugar levels, though direct connectivity has not been established. In addition, there is no overlap between primary gustatory neurons and motor neurons within the SOG, ruling out the possibility that feeding is a simple reflex circuit without any interneurons [16].

1.4 Regulation of motor patterns

In insects and mammals many complex behaviors are controlled motor pattern generators [17]. These motor patterns are fully functional independent of central nervous system input, but stimulus from central control centers is required to release the motor pattern, e.g. from descending inhibition. In *Drosophila*, motor neurons that control feeding arise from the SOG, which receives gustatory input, controls proboscis and head movements, and serves as a control center for motor patterns [18]. In blowflies, motor patterns control proboscis extension, sucking, transfer of food into the midgut and into the crop, transfer of food from the crop to the midgut, and excretion [14]. If AstA/NaChBac flies were impaired at initiating or executing motor patterns required in feeding, then we would not expect AstA,NPF/NaChBac flies to feed. However, this does not rule out the possibility that AstA and NPF modulate the initiation of motor patterns.

1.5 Additional factors that can alter feeding behavior

Other factors that influence feeding behavior include circadian rhythm, experience, social interactions, arousal, reproductive state, and environmental cues. In the previous chapter, we were able to rule out the possibility that circadian rhythm, experience, or social interactions are responsible for the feeding behavior of AstA/NaChBac flies. Remaining factors include arousal, reproductive state, and environmental cues.

It is possible that altered levels of arousal are contributing to the reduced feeding of AstA/NaChBac flies. In *Drosophila*, food deprivation prompts increased food search behavior, increased responsiveness to food cues, and a heightened central excitatory state [12, 19]. In insects, both stress and food deprivation results in the release of octopamine, which has functions analogous to that of adrenaline in vertebrates [20, 21]. In the blowfly, octopamine injection increases spontaneous locomotor activity, increases food intake, and decreases acceptance threshold [22]. Once food is located, locomotion is arrested and a period of quiescence follows feeding. In the blowfly, parabiotic studies have demonstrated that a humoral factor can induce the quiescence normally seen post-feeding [12]; an unfed fly will become quiescent after its pair is fed [14]. This humoral factor that mediates post-feeding quiescence has not been identified.

In males, reproductive state does not affect feeding [23]. Since all of our studies examined only male flies, it is unlikely that effects of reproductive state are altering AstA/NaChBac feeding behavior. Environmental cues such as low temperatures and short days can cause flies to enter a state of diapause, which is a state of dormancy employed by insects to survive harsh environmental conditions [24]. Female blowflies in diapause decrease their food intake because feeding would stimulate egg production, ending a state of diapause [25]. In *Drosophila* females, low temperatures can trigger arrested egg development, increased stress resistance, and increased fat reserves [24]. Null mutations in a gene called *couch potato* exhibit defects in diapause; they are hypoactive and exhibit only weak phototaxis and negative geotaxis [26]. If AstA/NaChBac flies were exhibiting a state of diapause, we would expect to observe changes in energy reserves, starvation resistance, phototaxis, geotaxis, and activity levels.

2. Results

2.1 Nutritional state

2.1.1 Starved AstA/NaChBac flies do not have excess energy stores.

One explanation for the decreased feeding behavior of starved AstA/NaChBac flies is that they have excess energy reserves compared to control flies. These excess energy reserves could be due to reduced motor activity in AstA/NaChBac flies. To examine differences in energy expenditure due to decreased locomotor activity during starvation, flies were monitored in group circadian monitors (Trikinetics) during starvation. The activity levels of AstA/NaChBac flies were not significantly different from controls during the first 24 hours of starvation (Supplementary Figure 1a). In addition, we looked at the activity levels of flies in which AstA neurons were acutely activated, using AstA/TRPA1 flies. We monitored activity levels during wet starvation for 22 hours at 22°C followed by an additional two hours at 28°C. The activity of AstA/TRPA1 flies was not significantly different from the activity levels of control flies (Supplementary Figure 1b). Thus, activation of AstA neurons does not result in decreased locomotor activity during starvation.

To further address the possibility that AstA/NaChBac flies have excess energy reserves or a slower metabolism, we measured starvation resistance, i.e., the length of time

that the flies can withstand starvation before they die. Activity was monitored during wet starvation in single-fly activity monitors (Trikinetics). The time of last beam crossing was considered time of death. The survival of AstA/NaChBac flies was not significantly different from the survival of +/NaChBac flies[1] (Figure 1a). Furthermore, activation of AstA neurons was unable to rescue the increased starvation sensitivity of Crz/NaChBac flies. As mentioned in the introduction of this chapter, activation of Crz neurons promotes fat mobilization and results in the depletion of fat stores causing increased starvation sensitivity. The survival of Crz/NaChBac flies and AstA,Crz/NaChBac flies were not significantly different from each other, but were significantly different from all other control genotypes (Figure 1a, see Supplementary Figure 1c for survival curves). AstA neuron activation neither prolonged survival upon starvation on its own, nor did it prolong the survival of starved Crz/NaChBac flies. Given that Crz/NaChBac flies have decreased energy reserves, these data indicate than activation of AstA neurons either decreases food intake by a mechanism that does not involve increasing energy reserves, or that any effect of AstA activation to increase such reserves can be overridden by simultaneous activation of Crz neurons.

If increased energy stores caused the reduced feeding of AstA/NaChBac flies, we would expect higher triglyceride, glucose, or protein levelss in these flies versus controls. AstA/NaChBac flies did not have excess energy reserves in the form of higher levels of triglycerides[2] or glucose[3] before starvation (Figure 1b, 1c). AstA/+ flies had higher triglyceride levels before starvation, which may be due to excess toxicity of the UAS-NaChBac transgene, but triglyceride levels were depleted to levels similar to

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[1] All Gal4/+ flies exhibited a slightly longer survival time than all Gal4/NaChBac and +/NaChBac flies, which was likely due to slight toxicity caused by UAS-NaChBac.

[2] There is an unresolved question regarding the accuracy of the method we used to quantify triglyceride levels. One study has shown that measurements using this method are inflated by fly eye pigment [27]; another demonstrates the assay's large dynamic range among flies with the same eye pigment. We did not observe changes in eye pigment levels in response to starvation regime, and thus we can confidently compare relative triglyceride levels within a single genotype before and after starvation. These measurements underly our statement that triglyceride levels of AstA/NaChBac flies deplete upon starvation. The inter-genotype measurements are motivated by finding a mechanism to explain reduced feeding. If increased triglyceride stores caused the reduced feeding, we would expect higher triglyceride measurements in the AstA/NaChBac flies than in the controls, a result that would need to be further investigated to assess the contribution of eye pigment to the increased triglyceride measurements. However, in our inter-genotype comparisons, AstA/NaChBac flies have triglyceride levels measured at or below those of the control genotypes. AstA/NaChBac flies thus do not have elevated triglycerides.

[3] Although *Drosophila* utilizes trehalose and not glucose as fuel, glucose levels are altered by the manipulation of DILP or AKH levels. Unfortunately, we were unsuccessful at measuring trehalose levels directly.

AstA/NaChBac and +/NaChBac flies after 24 hours of starvation. Most importantly, the energy stores of AstA/NaChBac flies after 24 hours of starvation were not significantly different from that of control flies (Figure 1b, 1c). Triglyceride and glucose levels were normalized to protein levels for these measurements, but raw triglyceride, glucose, and protein levels were also not significantly different from controls (data not shown).

We also addressed the possibility that excess energy reserves were being stored as undigested food in the gut and not reflected in our measurements of triglycerides, glucose, or protein. We let flies feed undisturbed on regular Caltech fly food containing blue dye for several days before starvation. After 24 hours of starvation, both AstA/NaChBac and control flies had negligible amounts of blue dye detectable in the abdomen (data not shown). This implies that AstA/NaChBac flies do not retain digested or undigested food after 24 hours starvation. This result eliminates the possibility that starved AstA/NaChBac flies are feeding less because of slower excretion.

Taken together, these results rule out the possibility that the decreased feeding behavior of AstA/NaChBac flies is a result of increased energy reserves. They also indicate that the decreased feeding behavior of AstA/NaChBac is not due to a state of diapause, because diapause leads to excess fat stores and increased resistance to starvation.

2.1.2 AstA/NaChBac flies fail to modulate their feeding behavior in response to a depletion of energy stores.

In order to clarify whether homeostatic regulation of energy levels and feeding behavior can be fully dissociated, we returned to the AstA,Crz/NaChBac flies. Due to their metabolic deficit, Crz/NaChBac flies have fewer energy stores than controls and also feed more than controls (Supplementary Figure 2 and [10, 28]). Given that AstA,Crz/NaChBac flies are starvation sensitive (Figure 1a), we will assume that, like Crz/NaChBac, these flies also have lower energy reserves.

Remarkably, despite sensitizing AstA/NaChBac flies to starvation by further depleting energy stores (by co-activation of Crz neurons), these flies also failed to show a starvation-induced increase in their feeding behavior. Flies were allowed to feed on 10 mM sucrose after 13 hours of wet starvation.[4] AstA,Crz/NaChBac flies behaved like

[4] Starvation time was shortened because most Crz/NaChBac flies do not survive 24 hours wet starvation.

AstA/NaChBac flies; the two were not significantly different from each other, but fed significantly less than all controls (Supplementary Figure 2c, $p<0.0001$). Thus, the suppression of food intake caused by activation of AstA neurons overrides the increased feeding behavior induced by the activation of Crz neurons.

2.2 Integration of nutritional state and sensory cues

2.2.1 AstA/NaChBac flies fail to exhibit starvation-induced changes in sucrose responsiveness

In wild-type flies, food deprivation results in an increased behavioral sensitivity to food cues. To test whether activation of AstA neurons influences this effect of starvation, we used proboscis extension assays to measure the sensitivity to food cues. In the proboscis extension reflex (PER) assay [29], the retracted proboscis is stimulated with stepwise increasing concentrations of a sucrose solution.[5] The probability of proboscis extension increases as the concentration of sucrose is increased, and the probability of proboscis extension is also increased as a result of starvation [30]. In other words, starved flies are more responsive to low concentrations of a sucrose solution than unstarved flies.

Using PER to test responsiveness, we observed that starved genetic control flies (+/NaChBac and AstA/+ flies), like wild-type flies, had a higher probability of proboscis extension at low sucrose concentrations than did unstarved genotype controls (Figure 2a, solid lines versus dashed lines, blue and black lines). Specifically, at 50 and 100 mM sucrose, the percentage of starved genotype control flies exhibiting a PER was significantly higher than that of unstarved genotype controls (Supplementary Figure 3a, 3b). In contrast, the PER of starved AstA/NaChBac flies was significantly lower than that of starved genotype controls at these sucrose concentrations (Figure 2a, orange solid line). Furthermore, the 50 and 100 mM PER responses of starved AstA/NaChBac flies did not differ significantly from that of any of the unstarved genotypes tested (Supplementary Figure 3a, 3b).

The foregoing experiments pooled the responses of all flies across all concentrations of sucrose. We also used these PER experiments to measure the PER

[5] A pipetman is used to produce a small droplet of the tastant. The droplet is briefly touched to the chemosensory hairs of the labellum (on the mouthparts) and quickly withdrawn before flies can drink.

threshold,[6] or the lowest concentration of sucrose that elicits proboscis extension in each fly. The PER threshold of unstarved AstA/NaChBac flies did not differ significantly from that of unstarved genetic controls ($p=0.4015$, Figure 2b), but the PER threshold of starved AstA/NaChBac flies was significantly higher than that of starved genetic controls (Figure 2c, $p<0.0001$). These experiments show that starved AstA/NaChBac flies behave as though unstarved with respect to sucrose responsiveness in the PER assay.

2.2.2 The PER phenotype of AstA/NaChBac flies is not responsible for the feeding phenotype.

The reduced feeding phenotype we observed in AstA/NaChBac flies could simply be a reflection of the impaired regulation of PER upon starvation. If the PER demonstrates the probability of whether flies will initiate feeding and if the reduced feeding behavior of AstA/NaChBac flies represents a decreased initiation of feeding behavior, then we would expect that the feeding behavior of AstA/NaChBac flies on a concentration of sucrose that elicits 100% PER would be similar to control flies. Alternatively, these two phenotypes may represent independent effects of activating AstA neurons. To resolve whether the feeding phenotype is purely due to the PER phenotype, we tested feeding behavior on 800 mM sucrose, a concentration that elicits 100% PER, even in AstA/NaChBac flies (Figure 2a). Thus, this concentration of sucrose overrides the PER deficit of AstA/NaChBac flies. Nevertheless, wet-starved AstA/NaChBac flies still exhibited significantly reduced feeding when allowed to feed on 800 mM sucrose over a 20 minute time period (Figure 2d, $p<0.0001$). These data suggest that the feeding deficit of AstA/NaChBac flies is not exclusively a consequence of their PER deficit (although it could still contribute).

An impairment in either starvation-induced increases in foraging activity or food intake could, in combination with the PER phenotype, account for the decreased feeding of starved AstA/NaChBac flies. Experiments discussed in Chapter 3 argue against the former possibility. To determine whether ingestion is impaired in these flies, we directly presented a sucrose solution that elicits 100% PER to mounted wet-starved flies. Under these "bottle-

[6] According to Dethier, "Although threshold lies somewhere between the concentration that elicits extension and the one in the series immediately below it, the higher of the two is arbitrarily designated as threshold. Since the aim of practically all experiments was to obtain data for comparative analysis, this fiction was acceptable" [14].

fed" conditions, locomotion, vision, olfaction, and gustatory acuity of foreleg chemoreceptors (which can detect the presence of food) are not required to feed. Single flies were fed to satiation with 800 mM sucrose containing blue dye, by allowing them to drink until they stopped extending their proboscis to the sucrose solution. The volume of intake of single flies was determined by colorimetric quantification as described in Chapter 3. The food intake of AstA/NaChBac flies was still significantly less than that of controls, even at this high sucrose concentration (Figure 2e, $p<0.0001$). Thus the reduced feeding of these flies is not due to deficits in foraging, vision, olfaction or gustatory acuity of foreleg chemoreceptors

We have demonstrated that AstA/NaChBac flies are impaired at modulating their feeding behavior in response to starvation, which in wild-type flies includes increased responsiveness to food cues and increased food intake as a consequence of starvation. We have also shown that the reduced feeding behavior of AstA/NaChBac flies is not due to excess energy stores. In the blowfly, proboscis extension and sucking are independently regulated and are controlled by a different set of muscles [14]. Our results imply that in *Drosophila*, starvation-induced changes in the sucrose-sensitivity of the PER and in the volume of food intake are independently regulated.

2.2.3 Sensory discrimination of starved AstA/NaChBac flies is normal at low sucrose concentrations.

The mechanism by which starvation alters responsiveness to food cues in flies has been contested. Some studies argue that primary gustatory neurons become more excitable [31, 32], while others argue that the firing properties of primary gustatory neurons are not altered by starvation [14]. Since we used a low concentration (10 mM) of sucrose in many of our feeding assays, and since there is a low probability of proboscis extension in this concentration range, we considered the possibility that a decreased ability of AstA/NaChBac flies to detect this low concentration of sucrose is the basis for its reduced feeding behavior.

In order to determine whether AstA/NaChBac flies have gustatory acuity deficits at low sucrose concentrations, we used a taste discrimination test as an indirect measure of gustatory acuity. Flies are capable of discriminating between 1 mM and 5 mM sucrose,

and will exhibit a preference for the higher concentration in a two-choice assay [33]. We therefore tested AstA/NaChBac flies for their ability to discriminate between these two very low concentrations of sucrose. We used red and blue food coloring to score for preference. Starved flies were allowed to feed for one hour, after which the abdomens of the flies were scored for the presence of red, blue, or purple dye. Half of the experiments were conducted using red 1 mM, blue 5 mM and the other half used the reverse combination to ensure that there was no bias introduced from either dyes. Nearly 100% of flies consumed only the higher concentration of sucrose (Figure 2f). Although a significantly smaller fraction of AstA/NaChBac flies fed ($p=0.0005$, 0.28 +/- 0.03% versus 0.71 +/- 0.04% and 0.74 +/- 0.10%), those that did were unimpaired at discriminating between the two concentrations ($p=0.3922$, Figure 2f). These data suggest that the gustatory sensitivity of starved AstA/NaChBac flies is normal with respect to sucrose, whereas their starvation-induced behavioral responses do not reflect those of a normal fly. However, electrophysiology or calcium imaging studies are required to formally exclude a mechanism by which the gustatory sensitivity of AstA/NaChBac flies is impaired in a manner that does not affect their ability to discriminate different concentrations of sucrose.

Our results demonstrate that starvation-induced changes in feeding behavior do not rely on neural mechanisms that alter gustatory sensitivity or discrimination in *Drosophila.* In blowflies, behavioral studies have demonstrated that gustatory discrimination and the decision to ingest are processed independently [14]. Previous studies have suggested that this might be the case in *Drosophila* as well [15], and our results confirm this hypothesis. The reduced feeding behavior of AstA/NaChBac flies is not a consequence of an impaired ability to detect sucrose.

2.3 AstA/NaChBac flies are able to generate the normal motor patterns that control digestion.

We have ruled out the possibility that AstA/NaChBac flies are unable to generate the motor patterns that control proboscis extension or food intake, or that they retain excess food that might prevent them from feeding. The possibility remains that motor patterns involved in food transfer into the gut are impaired. We tested this by dissecting out the gut and crop from flies immediately after feeding them dye-laced food. The entire length of

the midgut of AstA/NaChBac and control flies contained blue food similar to controls (data not shown). Depending on the volume of intake, the crop contained blue food coloring as well. Taken together, these results imply that AstA/NaChBac flies are able to execute the motor patterns involved in food intake and transfer of ingested food from the gut to the crop, and that excessive food retention is not impairing their physical capacity to feed.

2.4 The activation of AstA neurons does not impair all starvation-induced behavioral changes.

Starvation results in several types of behavioral responses: increased spontaneous locomotor activity, increased responsiveness to food cues, and a heightened central excitatory state [12, 34]. We were curious whether AstA/NaChBac flies were deficient in other starvation-induced behavioral responses.

2.4.1 Starvation-induced changes in spontaneous locomotor activity are normal in AstA/NaChBac flies.

Starvation elicits increased spontaneous locomotor activity [12, 35]. When animals reach a critical stage of starvation, it is more important to expend energy foraging for food than it is to conserve energy by not moving, in order to avoid death by starvation. Animals become hyperactive when this critical stage is reached. Using single-fly circadian monitors to track locomotor activity during wet starvation, we found that starvation-induced hyperactivity of AstA/NaChBac flies was normal (Figure 3). In Section 2.1, we demonstrated that triglyceride levels of AstA/NaChBac flies are depleted to the same levels as starvation-matched controls. These findings imply that starvation-induced changes in food responsiveness and food intake occurs downstream of, or in parallel to, starvation-induced changes in metabolism and spontaneous locomotor activity. The starvation-induced behavioral deficits of AstA/NaChBac flies are not due to a general inability to sense low hemolymph sugar levels (otherwise starvation-induced increases in locomotor activity should be reduced or eliminated). This in turn indicates that activation of AstA neurons does not impair all starvation-induced behavioral changes.

2.4.2 Activation of AstA neurons does not block memory performance.

In associative memory tasks using tastants as the unconditional stimulus, starvation is required for memory performance, as unstarved flies fail to learn [36]. Our colleagues (in Scott Waddell's lab) tested AstA/TRPA1 flies in food-associated learning and memory tasks, and found that memory performance was indistinguishable from controls (data not shown). This suggests that the mechanism by which AstA neurons are inhibiting feeding behavior is not by inhibiting (all) NPF signaling, which is required to disinhibit three pairs of dopamine neurons in the mushroom bodies in order for memory performance to occur [36]. Since activation of AstA neurons does not affect starvation-induced hyperactivity or memory performance, this demonstrates that this manipulation does not result in general starvation-induced behavioral responses.

2.5 AstA/NaChBac flies do not have general arousal deficits.

It has recently been demonstrated that there are at least two different forms of dopamine-mediated arousal and that these are controlled by discrete neural correlates [37]. These include sleep/wake arousal and startle-induced arousal.

2.5.1 Sleep/wake arousal deficits do not contribute to the feeding behavior of AstA/NaChBac flies.

Sleep/wake arousal and feeding behavior are linked. Total food intake is inversely proportional to the time spent sleeping [38]. The total time that AstA/NaChBac flies spent sleeping was indistinguishable from that of controls (data not shown). In addition, flies exhibit differences in feeding behavior at different times of day, and this is abolished by eliminating cycling of clock genes in the fat body, a tissue in which energy is stored [39]. In order to determine whether the feeding behavior of AstA/NaChBac flies was reflecting a difference in daytime arousal levels or reflecting a preference to feed at different times than control flies, we tested feeding at different times of day and night. AstA/NaChBac flies exhibited a decreased feeding phenotype independent of the time of day or night tested (see Chapter 3). These results imply that activation of AstA neurons is not altering starvation-induced responsiveness to food cues by altering circadian activity patterns.

2.5.2 Startle-induced arousal does not contribute to the feeding behavior of AstA/NaChBac flies.

It is possible that either stress levels or startle-induced arousal contribute to the decreased feeding behavior of AstA/NaChBac flies. In insects, both stress and food deprivation results in the release of octopamine [20, 21], which is thought to be the insect equivalent of adrenaline [20]. Octopamine injection increases spontaneous locomotor activity, increases food intake, and promotes trehalose synthesis [40]. If activation of AstA neurons is suppressing octopamine release, we would expect that these flies would also have altered locomotor activity. We have demonstrated that the locomotor activity of AstA/NaChBac flies is normal (Supplementary Figure 1a).

Repetitive mechanical stress induces a heightened state of arousal [37], and it is possible that either a heightened or a dampened sensitivity to mechanical stress could indirectly affect feeding behavior. The feeding behavior of AstA/NaChBac flies could reflect altered stress responses due to the mechanical stimulus that flies experience at the beginning of feeding experiments. To test whether AstA/NaChBac flies exhibit altered startle-induced locomotor activity, we monitored these flies in a circadian monitor. We administered a mechanical stimulus similar to that experienced by flies when transferred to a feeding assay, and that induced several hours of heightened activity levels in both AstA/NaChBac and control flies. The increased spontaneous locomotor activity of acutely stressed AstA/NaChBac flies was indistinguishable from that of controls (data not shown). This suggests that startle-induced arousal is not contributing to the decreased responsiveness to food cues observed in AstA/NaChBac flies.

2.5.3 The locomotor agility and performance of AstA/NaChBac flies are unimpaired.

Finally, we demonstrated that AstA/NaChBac flies performed no differently than control flies in other motivated behaviors that require locomotor agility, such as phototaxis and geotaxis. Phototaxis and geotaxis are the innate attraction towards light and against gravity, respectively. These behaviors can be used to motivate flies to pass through a maze that demands locomotor agility in order to navigate the maze successfully. Experiments discussed earlier suggest that the reduced feeding of starved AstA/NaChBac flies is

unlikely due to decreased locomotor or foraging activity, since these flies still exhibit a feeding phenotype when no work is required to access food. To further investigate this question, we tested the performance of AstA/NaChBac flies in both a phototaxis maze and a geotaxis maze. The performance of AstA NaChBac flies was both accurate and speedy in both assays, and indistinguishable from that of controls (see Supplementary Figures 3c, 3d, and 3e). These results confirm that AstA/NaChBac flies are unimpaired in their locomotor agility and that they do not exhibit a general deficit in motivated behaviors.

3. Brief summary

We have identified a group of neurons whose hyper-activation can specifically inhibit some, but not all, of the normal starvation-induced changes in the behavioral responses to food cues. Activation of these neurons has no effect on starvation-induced changes in metabolic homeostasis, food search behavior or the drive state ("motivation") necessary to perform a starvation-dependent appetitive conditioning task.

4. Materials and methods

Fly husbandry

All flies were reared on standard Caltech fly food [41] at 25ºC, 70% relative humidity, and under a 12 h:12 h light:dark regime unless otherwise indicated. For TRPA1 experiments, flies were raised at 22ºC. Flies used for immunohistochemistry and behavior were 5–10 day old males. Fly lines used: AstA-Gal4 (described in Chapter 2), UAS-NaChBac::eGFP (a generous gift from Dr. Bader Al-Anzi) [42], UAS-TRPA1 contained two copies of the responder (the recombinant was a kind gift from Kenta Asahina) [43], UAS-eGFP::Kir2.1 [44], *w* (Exelixis, used for UAS-responder x *w* crosses), *w* (Dr. Tim Tully lab isogenic strain, used for *w* x Gal4 crosses), NPF-Gal4 (Bloomington stock #25681) [45], Crz-Gal4 (T. Tayler, et al., unpublished), Canton-S flies obtained from Dr. Seymour Benzer, AKH-Gal4. Tim Tayler and I generated Crz-Gal4 transgenic flies independently. I have confirmed that the expression pattern of Crz-Gal4 in these flies is specifically expressed in Crz-expressing neurons in third instar larvae by double fluorescent in situ/ immunohistochemistry. The adult expression pattern of Crz-Gal4 transgenic flies has also been validated (T. Tayler, unpublished). The expression pattern of the Crz-Gal4 in adults is similar to the expression pattern of Crz-Gal4 transgenic flies described in [46].

Triglyceride, protein, and glucose measurements

Starved flies were transferred to vials containing 1% agar. Starved and unstarved samples of all genotypes were processed in parallel. 20 male flies, aged 6–9d old, were frozen and then homogenized in 500uL PBT (PBS +0.1% Triton X). Samples were sonicated then centrifuged at maximum speed for 15 minutes. 300 uL of supernatant was then diluted ten-fold for use in the assays. Triglyceride levels were measured using the Stanbio Triglyceride Kit, and the absorbance at 500 nm was measured. Protein levels were assayed using the BCA1 protein kit (Sigma), and the absorbance at 562 nm measured. Glucose levels were assayed using a kit as well (Sigma GAHK-20), and the absorbance at 340 nM was measured. All measurements were done using a spectrophotometer. Both the triglyceride and the glucose values were normalized to protein levels.

Behavior

Circadian activity and survival

Flies were monitored in single-fly locomotor activity monitors (Trikinetics, Waltham MA). For monitoring locomotor activity, flies were supplied with standard fly food media. For survival assays flies were supplied with 1% agar. Survival was determined by the time of last beam crossing per fly. Trikinetics data acquisition software was used to collect data in one-minute time bins. Trikinetics file scan software was used to bin data into 30-minute time bins for generating figures of locomotor activity.

Feeding assays

Unless otherwise noted, all feeding assays and quantification methods were done as described in Chapter 3. For the two-choice feeding assay [47], we optimized the final concentrations of red and blue food coloring so that mixtures in a 5:1 and a 1:5 ratio yielded an obvious purple mixture, distinct from the pure red or pure blue solution. Half of the experiments were conducted using red 1 mM sucrose and blue 5 mM sucrose and the other half used the reverse combination to ensure that there was no bias introduced from the dyes. Sucrose was dissolved in 0.5% agar, and 0.5 mL was pipeted into a standard fly vial that was positioned at a 45 degree angle. Once the agar had hardened, the vial was tilted 45 degrees in the opposite direction in order to pipet the second 0.5 mL sucrose solution. This created a V-shaped base of food on the bottom of the fly vial, except that the two sucrose patches did not touch. Flies were wet-starved for 24 hours, and then transferred to the two-choice sucrose vial for 1 hour. The abdomens of flies were then scored for the presence of red, blue, or purple dye under a dissecting microscope. Performance index (PI), which was calculated as [(# that eat 5 mM sucrose) + (0.5)*(# that eat both)] / (total # that eat).

“Bottle fed” or hand-fed flies, were first mounted on their backs using Elmers glue. A droplet of 800 mM sucrose solution containing 0.5% FD&C Blue #1 was produced using a 10 uL pipetman, and the droplet was touched to the proboscis of the fly. Flies were allowed to drink until they stopped extending their proboscis in response to the stimulus.

PER

The protocol referenced in [33] was roughly followed. Briefly, flies were mounted in 200 uL tips without anesthesia so that they were immobilized with only their heads exposed. Flies were given at least 30 minutes to acclimate before being presented with water, using a pipetteman, directly to the proboscis. If they extended their proboscis in response to water, those flies were eliminated from further data sets. Sucrose concentrations were then presented directly to the proboscis from lowest to highest concentration. The stimulus was promptly removed so that the flies were not able to ingest the solution. The lowest concentration of sucrose which elicited a proboscis extension was noted for each fly as well as the percent of total flies extending their proboscis at each concentration. Data represents the average of many experiments, each of which included 10–20 flies per experiment.

Geotaxis and phototaxis

The T-maze and the geotaxis maze were previously described [29]. For the phototaxis assay, flies were acclimated in the dark for one hour prior to testing. Groups of 20 flies were then transferred into the elevator of the T-maze, which was oriented so that one arm pointed towards a bright light source. The elevator was lowered to transfer flies into the two-pronged "maze" and 10 seconds later, the total number of flies in each arm of the maze was counted. Performance index was calculated as [(# of flies in the illuminated arm) – (# of flies in the un-illuminated arm)] / (total # of flies in both arms). For the geotaxis assay, flies were acclimated in the dark for one hour prior to testing. Twenty flies were transferred to the entrance of the geotaxis maze under red light conditions. The maze branches vertically a number of times, so that flies traveling through the maze have to choose to positively or negatively geotax at least eight times before they exit the maze. The innate negative geotaxis behavior of flies is so robust, that the majority of wild-type flies will exit the maze from the uppermost exits within an average of two minutes. Flies in the maze were watched and the time for the last fly to exit the maze was noted. Geotaxis scores reflect the average level at which flies exit the maze, where nine corresponds to the uppermost exit and 1 to the lowest exit. The geotaxis score represents the average level that a population of flies exited the maze, i.e. (# of flies at level n)*(n)/(total # of flies).

Data and statistics

GraphPad Prism software was used to generate graphs as well as for statistical analysis. Bar graphs represent mean and standard error. Unless otherwise noted, data was analyzed using one-way ANOVA with Bonferroni correction for multiple comparisons.

5. Figure Legends

Figure 1. Starved AstA/NaChBac flies do not have excess energy stores.

(**a**) Survival of AstA/NaChBac, Crz/NaChBac, AstA,Crz/NaChBac flies and genetic controls after starvation with access to water. Single-fly activity monitors (Trikinetics) were used to track the locomotor activity of wet-starved flies, and the time of last beam crossing was considered the time of death. The survival of AstA5/NaChBac flies was not significantly different from that of +/NaChBac flies (unpaired t-test, p=0.7218), but was significantly different from that of AstA5/+ flies (unpaired t-test, p=0.0011). The survival of Gal4/+ flies was slightly longer than Gal4/NaChBac and +/NaChBac flies (one-way ANOVA, p<0.0001), which was likely due to slight toxicity caused by the UAS-NaChBac transgene. The survival of Crz/NaChBac flies was not significantly different from that of AstA5,Crz/NaChBac flies, but the survival of both genotypes was significantly decreased compared to that of all other genotypes tested (one-way ANOVA, p<0.0001). See Supplementary Figure 1c for survival curves. Crosses were UAS-NaChBac x Corazonin-Gal4, UAS-NaChBac x AstA5-Gal4, Corazonin-Gal4, and *w* x AstA5-Gal4, Corazonin-Gal4. (n=15–32) (**b**) Whole-body triglyceride levels of unstarved and 24-hour wet-starved AstA/NaChBac flies and genetic controls. Twenty male flies were homogenized, sonicated, and centrifuged, and the triglyceride levels and protein levels of the supernatant were measured. Plotted are the average triglyceride/protein ratios. The triglyceride levels of starved AstA/NaChBac flies were not significantly different than those of starved controls (p=0.3009, N=3). Triglyceride levels were significantly depleted after 24 hours starvation within each genotype (unpaired t-test, p< or = 0.029). Although triglyceride levels of AstA/+ were significantly elevated in unstarved flies, the levels in unstarved AstA/NaChBac and +/NaChBac flies were not significantly different from each other (unpaired t-test p=0.4008, N=3). (**c**) Whole-body glucose levels of unstarved and 24-hour wet-starved AstA/NaChBac flies and genetic controls. Glucose levels of the supernatants described in (**b**) were measured. Plotted are the average glucose/protein ratios. Glucose levels of AstA/NaChBac flies were not significantly different from those of controls, in both starved and unstarved conditions (p=0.8384, p=0.0787, N=3). One-way ANOVA with Bonferoni correction was used to analyze data unless otherwise stated. Averages and

standard error are plotted unless otherwise stated. AstA/UAS-reporter are always plotted in orange, +/UAS-reporter in blue, and AstA/+ in white or black.

Figure 2. AstA/NaChBac flies do not exhibit normal starvation-induced changes in sucrose responsiveness. The proboscis extension phenotype does not account for the feeding phenotype of AstA/NaChBac flies.

(**a**) Average fraction of flies that extended their proboscis in response to different concentrations of sucrose. Responses of 24-hour wet-starved flies is plotted using solid lines and those of unstarved flies using dotted lines. The proboscis extension reflex (PER) assay [24] involves briefly touching stepwise increasing concentrations of a sucrose solution to the retracted proboscis of a fly and noting the presence or absence of proboscis extension in response to the stimulus. See Supplementary Figures 2a and 2b for statistical analysis. Data points in (**a**) represent the average percentage of flies extending their proboscis at each concentration, whereas in (**b** and **c**) the lowest concentration of sucrose that elicited a proboscis extension in individual flies is plotted (PER threshold). (**b**) The average PER threshold of unstarved and (**c**) starved flies. The PER threshold of unstarved AstA/NaChBac flies was not significantly different from that of unstarved genetic controls ($p=0.4015$, $n>68$), whereas (**c**) the PER threshold of starved AstA/NaChBac flies was significantly higher than that of starved genetic controls ($p<0.0001$, $n>52$). (**d**) Average fraction of flies feeding on 800 mM sucrose. Significantly fewer starved AstA/NaChBac flies fed compared to genetic controls ($p<0.0001$, $N=5–9$). Feeding assay parameters were the same as those described in Figure 1a, except for sucrose concentration and that feeding was scored after 20 minutes of feeding. (**e**) Average volume of 800 mM sucrose consumed when “bottle-fed”. Individual flies were immobilized and fed to satiation with 800 mM sucrose containing dye. Volume consumed by starved AstA/NaChBac flies was significantly less than that of control genotypes ($p<0.0001$, $n= 17–19$). Food intake was measure as described in Figure 1b, except that single flies were processed in a smaller final volume. (**f**) Average performance index of flies in a two-choice feeding assay using 5 mm sucrose and 1 mm sucrose. Wet-starved flies were given the opportunity to feed on either 1 mM or 5 mM sucrose which contained either red or blue food coloring. Preference did not differ significantly between genotypes ($p=0.3922$, $N=4$), although a significantly smaller

fraction of AstA/NaChBac flies fed (p=0.0005, 0.28 +/- 0.03% versus 0.71 +/- 0.04% and 0.74 +/- 0.10%). Half of the experiments were conducted using red 1mM, blue 5mM and the other half used the reverse combination to ensure that there was no bias introduced from the dyes. After 1 hour of feeding, the abdomens of flies were scored for the presence of red, blue, or purple dye. Performance index (PI), which was calculated as [(# that eat 5 mM sucrose) + (0.5)*(# that eat both)] / (total # that eat).

Figure 3. Starvation-induced hyperactivity of AstA/NaChBac flies was normal.

(**a**) Average activity levels of AstA/NaChBac flies and genetic controls during the first and second mornings after wet starvation. Flies were starved on 1% agar in single-fly activity monitors (Trikinetics, n=32). Starvation-induced hyperactivity refers to the increase in average locomotor activity that becomes evident after ~ 30 hours of wet starvation, and is represented graphically as a widening of the activity peaks, i.e., the area under the curves of both the morning peak and the evening peak of activity increases with time. Similar results were observed in population monitors (data not shown).

Supplementary Figure 1. Activity levels and locomotor agility were normal when AstA neurons were activated.

(**a**) and (**b**) Population monitors (Trikinetics) were used to measure the total activity, or total beam crosses, of groups of 20 flies per fly vial. (**a**) Activity levels of AstA/NaChBac flies and genetic controls during a 24 hour starvation period (p=0.1333, N=2–3). (**b**) Activity levels of AstA/TRPA1 flies and genetic controls during a 24 hour starvation period, during which the temperature was kept at 22°C for the first 22 hours and then shifted to 28°C for an additional 2 hours. (**c**) The survival curves of Crz/NaChBac (pink line), AstA/NaChBac (orange line), AstA5,Crz/NaChBac flies (red line), and genetic controls (blue and black lines). The survival curves of AstA5,Crz/NaChBac and Crz/NaChBac were not significantly different from each other (p=0.4225), but were significantly different from all other genotypes (p<0.0001). The survival curve of AstA5/NaChBac flies was not significantly different from that of +/NaChBac flies (p=0.0753), but was significantly different from that of the AstA5/+ flies (p=0.0012). Log-rank (Mantel-Cox) Test was used to compare survival curves, (n=15–32). Crosses were

UAS-NaChBac x Corazonin-Gal4, UAS-NaChBac x AstA5-Gal4, Corazonin-Gal4, and *w* x AstA5-Gal4, Corazonin-Gal4.

Supplementary Figure 2. Excessively depleting energy stores does not induce AstA/NaChBac flies to feed.

(**a)** Average fraction of flies feeding and (**b**) total volume consumed by 13-hour wet-starved Crz/NaChBac flies, AKH/NaChBac flies, and genetic controls on 100 mM sucrose plus 500 mM NaCl. Both Crz/NaChBac and AKH/NaChBac flies fed significantly more than genetic controls. (**a**–**d**) Feeding assay parameters were the same as those described in Figure 1a, except for the nutrients provided and that starvation time was reduced to 13 hours. Total volume consumed was measured as described in Figure 1b. (**c)** Average fraction of flies feeding and (**d**) total volume consumed after 13 hours wet-starved on 10 mM sucrose. (**c**) The fraction of AstA/NaChBac flies that fed was not significantly different from the fraction of AstA,Crz/NaChBac flies that fed, but both were significantly reduced compared to all other genotypes ($p<0.0001$, N=3–11). (**d**) The average food intake of Crz/NaChBac flies was significantly increased under these feeding conditions relative to control genotypes. (Note: multiply total nL consumed on the y-axis by 100). UAS-NaChBac was crossed to two independent insertions of a Crz-Gal4 contruct (referred to as Crz4 and Crz6) and resulted in similar effects on feeding behavior. All previous experiments used Crz4 flies.

Supplementary Figure 3 Starved AstA/NaChBac flies behave as though sated with respect to the PER assay.

(**a** and **b**) The average fraction of flies that extended their proboscis in response to 50 mM (**a**) and 100 mM (**b**) sucrose. At both these concentrations, the PER of starved AstA/NaChBac flies was significantly lower than that of either starved +/NaChBac and AstA5/+ flies (one-way ANOVA, $p<0.0001$). Moreover, at these concentrations of sucrose, the PER of starved AstA/NaChBac flies was not significantly different from that of unstarved AstA/NaChBac flies or unstarved control genotypes (two-way ANOVA, $p<0.0001$). These bar graphs replot the data described in Figure 2a. (**d**) Performance of flies in a maze that tests whether flies are attracted to light (phototaxis assay). Unstarved

flies were allowed 10 seconds to enter one of two arms of a T-maze, in which one of the arms was brightly illuminated. Performance index was calculated as [(# of flies in the illuminated arm) – (# of flies in the un-illuminated arm)] / (total # of flies in both arms). The performance index of AstA/NaChBac flies was not significantly different from that of control genotypes (p=0.4683, N=4–7). (**e** and **f**) Performance of flies in a negative geotaxis locomotor assay. Twenty unstarved flies enter a maze that branches vertically a number of times, so that flies traveling through the maze have to choose to positively or negatively geotax at least eight times before they exit the maze. The innate negative geotaxis behavior of flies is so robust, that the majority of wild-type flies will exit the maze from the uppermost exits within an average of two minutes. Experiments were done in the dark. Geotaxis scores reflect the average level at which flies exit the maze, where nine corresponds to the uppermost exit and 1 to the lowest exit. +/NaChBac flies performed significantly more poorly in the geotaxis assay (one-way ANOVA, p=0.0224), but AstA/NaChBac flies did not differ from AstA/+ flies in performance (t-test, p=0.8899, N=3–5). Time to finish was computed as (total time it took for the last of ~ 20 flies to exit the maze) / (total # flies).

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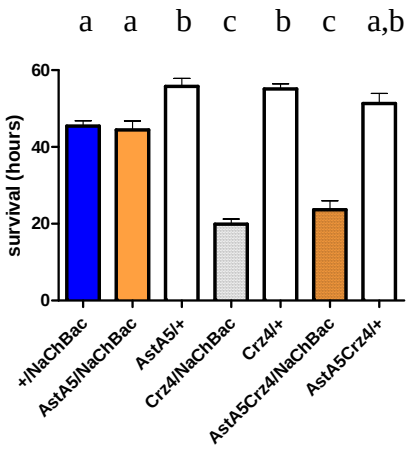
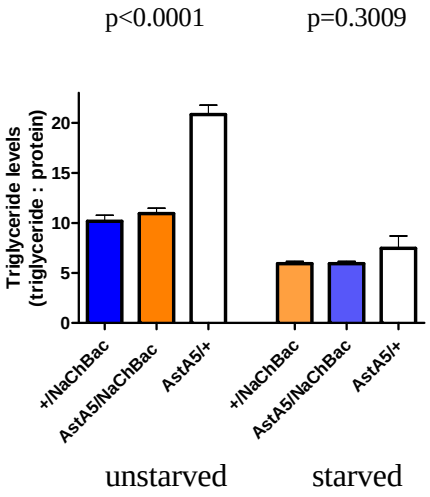
Figure 1

a.

p<0.0001



b



c.

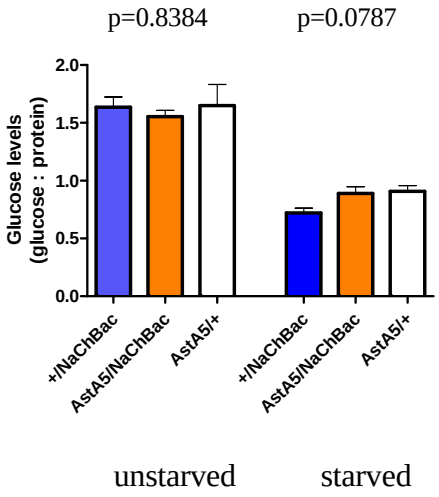
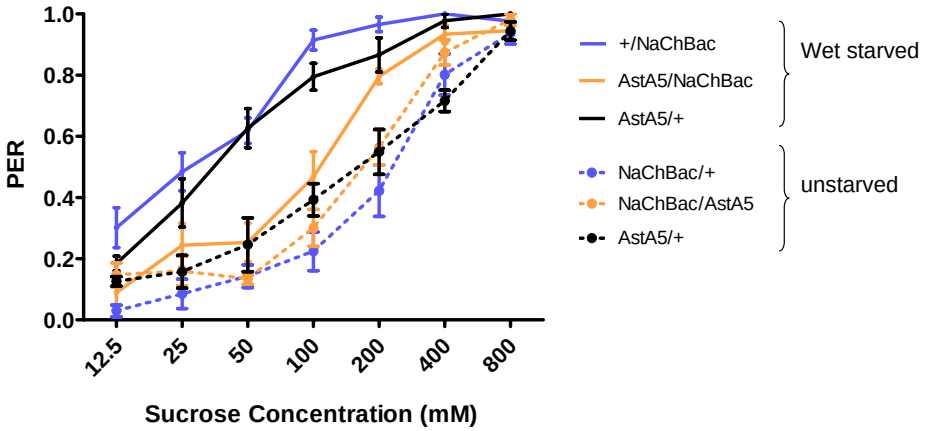


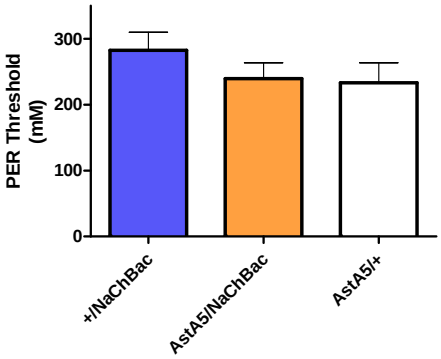
FIGURE 2

a.



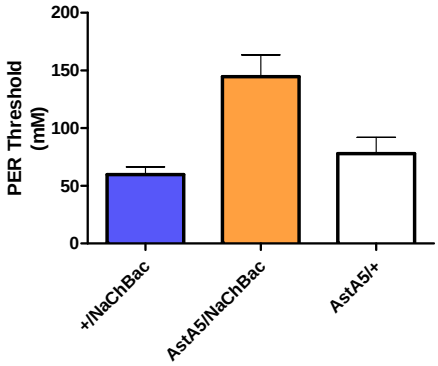
b.

Unstarved
p=0.4015



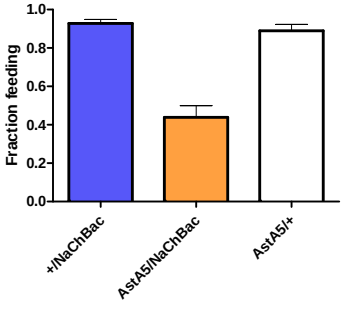
c.

Starved
p<0.0001
***



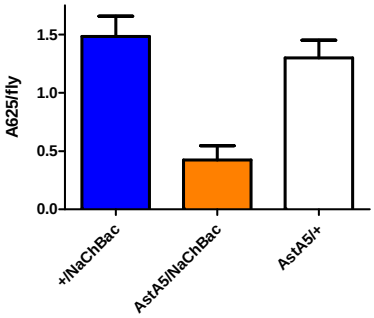
d. 800 mM (open arena)

p< 0.0001 ***



e. 800 mM feeding (hand-fed)

p< 0.0001 ***



f. Two-choice assay

p=0.3922

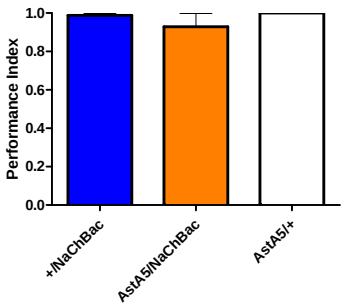
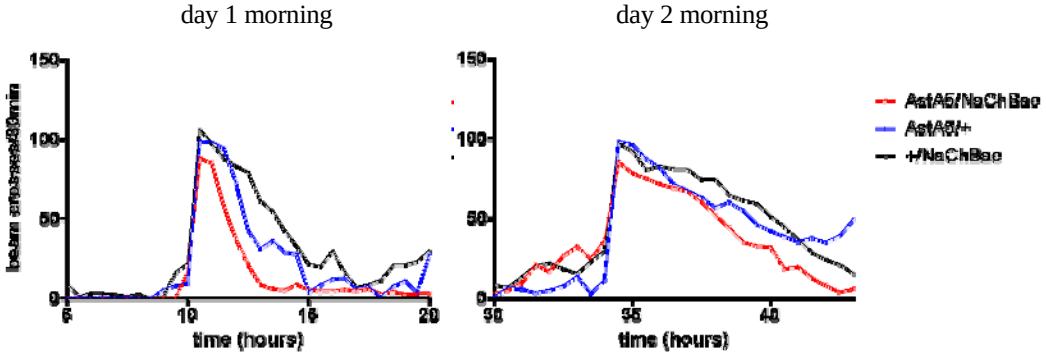


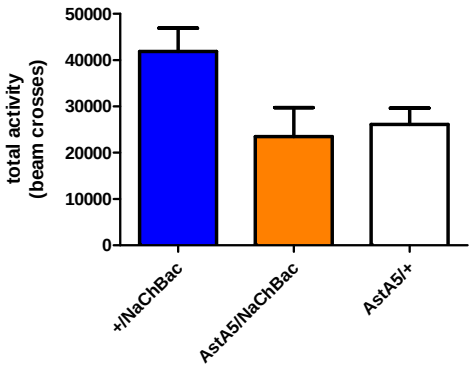
Figure 3

a.

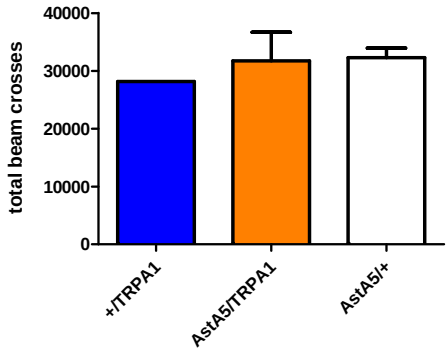

day 1 morning
day 2 morning
150
100
50
0
5
10
15
20
time (hours)
AstA6/NaChBac
AstA6/+
+/NaChBac

Supplementary Figure 1

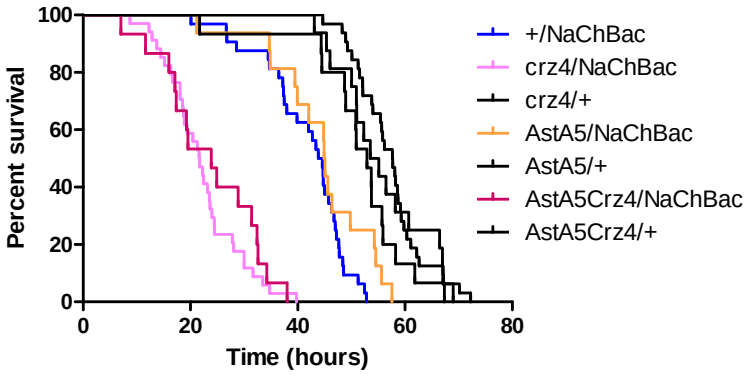
a.



b.



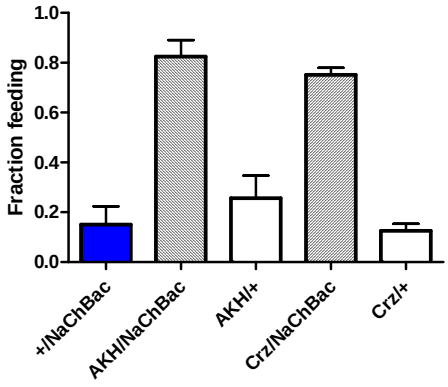
c.



Supplementary Figure 2

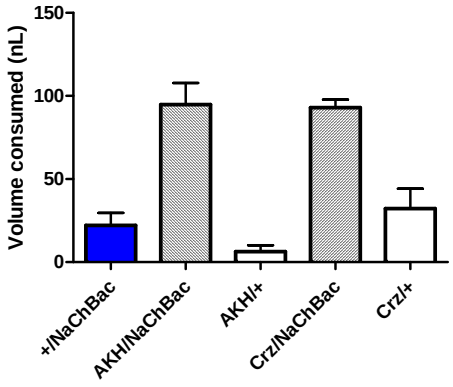
a.

100 mM sucrose + 500 mM NaCl



b.

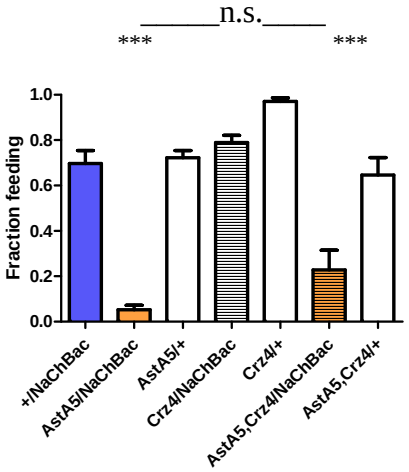
100 mM sucrose + 500 mM NaCl



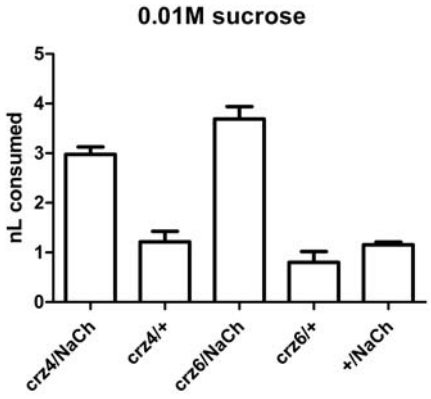
c.

10 mM sucrose

p<0.0001

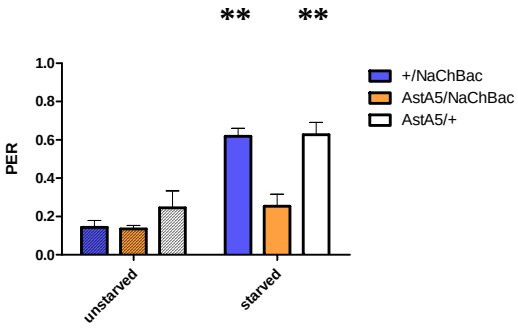


d.

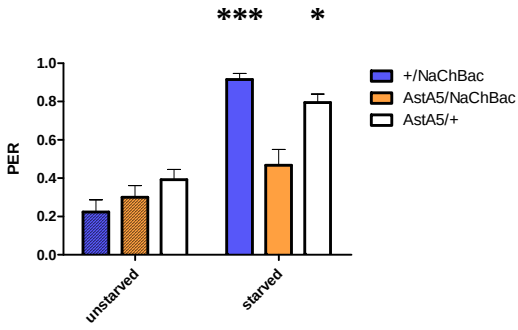


Supplementary Figure 3

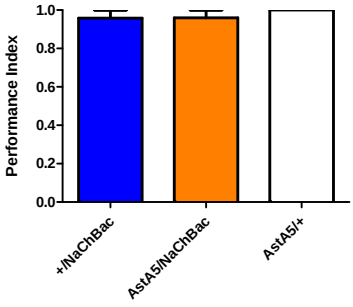
a. 50 mM sucrose



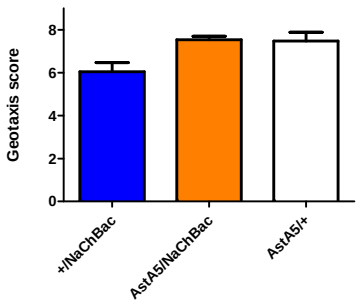
b. 100 mM sucrose



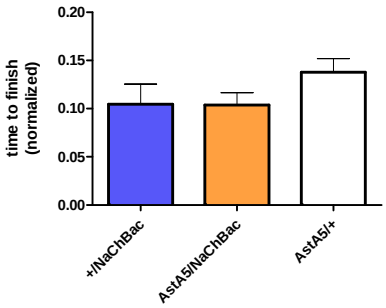
c. Phototaxis



d. Geotaxis



e. Geotaxis speed



Chapter 5

DISCUSSION AND FUTURE DIRECTIONS

Understanding the neural mechanisms that motivate us to eat is important because of the increasing rates of obesity and the consequential increasing rates of diabetes and cardiovascular disease in our society [1–3]. *Drosophila melanogaster* provides an excellent model system in which to study this complex problem because many elements of feeding regulation and homeostasis are conserved between *Drosophila* and mammals [4–6]. Here we have presented a unique model in which to study the regulation of feeding and satiety in *Drosophila*: A circuit manipulation that produces satiety-like behavior in otherwise starved flies, without affecting starvation-induced metabolic changes. While control of satiety has been extensively studied in blowflies, very few examples exist of a robust satiety-promoting signaling mechanism in *Drosophila* [7–9]. Using this model of satiety, we have demonstrated a number of important new findings in the regulation of feeding in flies. This discovery opens up countless new avenues of research that could potentially contribute to future medical interventions relevant to obesity, as well as to developing mechanisms to control agricultural pests and disease-carrying insect populations.

1. Summary of main results

1.1 Characterization of AstA-Gal4 transgenic flies

AstA has been implicated as a satiety factor and as a myoinhibitor in several other insects [8, 10]. Expression of AstA in feeding-related endocrine centers supports the possibility that AstA may play a role in the regulation of *Drosophila* satiety as well [11]. In order to test this hypothesis, we generated AstA-Gal4 transgenic flies and used them to map the distribution of AstA-expressing neurons using a UAS-mcd8::GFP reporter. In Chapter 2, we demonstrate that AstA-Gal4 is expressed in a subset of mostly adult-specific AstA-expressing neurons and neuroendocrine cells, including neurons that innervate the subesophageal ganglion (SOG), where primary gustatory information is relayed, and the

pars intercerebralis (PI), where *Drosophila* insulin-like peptide is synthesized.

Enhancers that are sufficient to promote specific expression in a subset of AstA-expressing neuroendocrine cells are contained within 2.1 kb of 5' flanking sequence of the predicted AstA coding sequence. This short promoter sequence is sufficient to drive expression in 3/8 neurons in the brain, ~ 30 optic lobe neurons that innervate the medulla, 3/13 neurons in the VNC that innervate the midgut, hindgut, and rectum, single peripheral neurons that sit on each wing and haltere nerves, neuroendocrine cells of the lower midgut, and in muscle tissue on either end of the midgut. We also confirmed by in situ hybridization, that the expression pattern of AstA mimics the labeling pattern by *Diploptera* AstA immunostaining.

In addition to further characterizing the role of AstA neurons in feeding behavior, which I will discuss in later sections, we will use mosaic analysis with a repressible cell marker (MARCM, [12]) to confirm the projection patterns of each of the three brain AstA-Gal4 neurons, since the origin of the projections in the protocerebrum and cervical connective could not be confirmed. We will also co-immunostain AstA-Gal4-expressing neurons with various antibodies in order to determine which neurotransmitters are co-released. There are a number of transgenic tools that manipulate specific neurotransmitters, and learning which neurotransmitter is co-expressed in AstA neurons would inform us of which tools we could use in the future to better understand the function of AstA neurons.

We would like to generate AstA-Gal4 transgenic flies that express Gal4 in all AstA-expressing neurons by amplifying a longer upstream sequence to try to capture additional enhancers that we might have missed. If successful, we could generate AstA-promoter::Ricin fusion transgenic flies using the shorter, 2.1 kb promoter sequence, and combine this tool with the complete-enhancer-Gal4 to probe the role of a subset of centrally expressed AstA neurons.

1.2 Activation of AstA neurons inhibits multiple starvation-induced feeding behaviors.

Previously, there was no known function for AstA or for AstA neurons in *Drosophila* [9, 10]. We used transgenic AstA-Gal4 flies to demonstrate that activation of AstA neurons inhibits starvation-induced feeding behaviors. A starved fly will normally accept lower concentrations of food and ingest larger meals [13, 14]. We have shown that activation of AstA neurons prevents these starvation-induced changes without preventing other starvation-induced behavioral changes, such a starvation-induced hyperactivity and starvation-motivated associative learning.

The only other case of a manipulation that causes starvation-induced behavioral changes in the absence of metabolic effects is NPF, which affects all starvation-induced behavioral changes and therefore seems to promote a "hunger" state [15, 16]. Investigations into the neural circuits and neuromodulators that regulate homeostasis and behavior in *Drosophila* have revealed that regulatory control is a result of opposing push-pull forces and not of individual master regulators [17]. In terms of the regulation of feeding behavior, there is no known counter-regulatory mechanism to NPF that inhibits starvation-induced feeding behaviors without altering energy metabolism.

1.3 Changes in feeding behavior are not secondary to metabolic changes.

We used metabolic and behavioral assays to demonstrate that activation of AstA neurons specifically results in the loss of behaviors typically resulting from starvation, in the absence of overt metabolic deficits (see Chapter 4). Fly mass, triglyceride stores, glucose stores, protein stores, and starvation resistance were no different from starvation-matched animals. In agreement with these findings, activation of AstA neurons in animals with substantially depleted energy stores and heightened sensitivity to starvation (Crz/NaChBac flies), was not sufficient to prolong the survival of these flies. Therefore there is no evidence supporting an underlying metabolic cause for differences in feeding behavior when AstA neurons are activated.

1.4 Tentative conclusions

AstA neurons negatively regulate starvation-induced changes in feeding behavior, by inhibiting mechanisms that promote behavioral responses to starvation, either downstream of, or in parallel to, metabolic responses to starvation. During our investigations into the mechanisms responsible for the loss of starvation-induced feeding behavior, we discovered that the starvation-induced inhibition of increased sugar-sensitivity of PER and inhibition of food intake can be experimentally uncoupled. This suggests that AstA neurons coordinately control several behavioral responses to starvation.

Gene or circuit manipulations that decrease feeding behavior usually also alter metabolism [9, 18]. Many studies have described gene mutations or neuronal silencing that result in decreased feeding behavior, but few cases of gene overexpression and no cases of neuronal activation have been shown to decrease feeding behavior [9]. Neuromodulators that promote feeding behavior include NPF and AKH [9]. Conversely, DILP overexpression results in decreased feeding behavior and increased energy stores [19, 20]. Loss of function studies have identified several potential neuromodulators that may also promote starvation-induced feeding behavior, because these loss of function manipulations led to increased feeding behavior [21, 22], but a gain of function feeding phenotype (exhibiting decreased feeding behavior) has not been demonstrated for these neuromodulators.

Starvation-induced changes in behavior can be uncoupled from the hunger state in *Drosophila,* i.e., these changes occur downstream of metabolic changes) [16]. Our results have demonstrated that activation of AstA neurons is capable of preventing starvation-induced behavioral changes without inhibiting corresponding metabolic changes. Many neuroendocrine mechanisms promote feeding behavior, and these have been documented in the literature [9], but we focus here on the promotion of satiety in order to piece together the divergent studies that might give us insight into this behavioral state. Whereas the neural control of feeding behavior has been studied in the blowfly using nerve lesions and injections, this has been studied in *Drosophila* by genetically manipulating neurons and gene expression [7, 8]. I will address whether regulatory mechanisms are similar between blowflies and *Drosophila* and how studies in each affect the interpretation of our results.

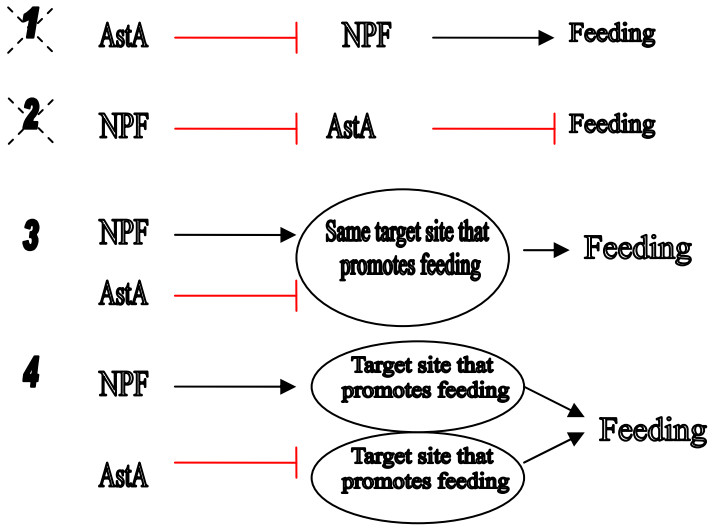
2. What do my results contribute to what is known about satiety, or the control of starvation-induced changes in feeding behavior, in *Drosophila*?

Although a number of neuropeptides have been implicated in the regulation of feeding, the mechanism by which they act has not been clearly elucidated [9, 18]. Neuropeptides and neuropeptide receptors are often expressed in both central and peripheral sites, making it difficult to establish how their behavioral effects are achieved [9]. For example, NPF regulates starvation-induced feeding behaviors and is expressed in the both the CNS and in gut neuroendocrine cells [9]. Furthermore, the NPF receptor (NPFR) is expressed in both the crop and the malphigian tubules, which are involved in osmoregulation and excretion [18, 23]. Therefore, NPF could regulate feeding behavior by either modulating gustatory input or by regulating proprioceptive feedback. A large number of neuropeptides and neuropeptide receptors are expressed in the stomatogastric nervous system and in the gut, but their function peripherally has been largely ignored [9, 24]. In this section, I will discuss how our results relate to other neuropeptides that regulate feeding behavior, and will discuss potential central and peripheral mechanisms in a later section.

2.1 AstA neurons and NPF neurons co-regulate feeding behavior in opposing directions.

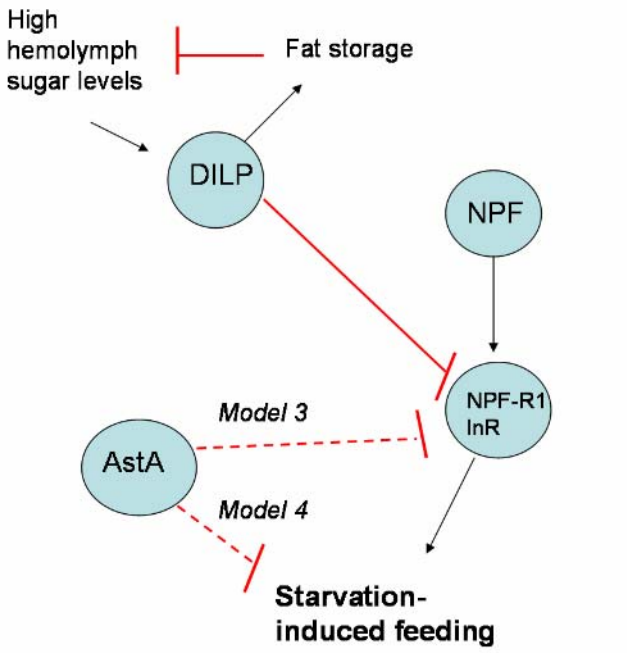
Activation of AstA neurons inhibits starvation-induced changes in feeding behavior. Conversely, NPF neurons promote starvation-induced changes in feeding and foraging activity [15, 25]. The opposite actions of NPF and AstA neuron activation brings up the question of how these two neuronal circuits are related. There are four possibilities: AstA neurons could be upstream of NPF neurons (Figure 1, Model 1), NPF neurons could be upstream of AstA neurons (Model 2), NPF and AstA neurons could act on a common target (Model 3), or they could act on independent targets (Model 4).

Figure 1. Models of how NPF and AstA neurons regulate feeding behavior



Since co-activation of AstA and NPF neurons partially rescues the AstA feeding phenotype, it rules out the first two models. If either of the first two models were correct, we would have expected that co-activation mimicked the effect of either AstA or NPF activation on feeding behavior. Both activation of AstA neurons and co-activation of AstA and NPF neurons resulted in significantly reduced feeding behavior, but co-activation resulted in significantly increased feeding versus AstA neuron activation. If AstA and NPF neurons modulate a common post-synaptic target (Model 3), then the physiological effects caused by AstA and NPF neurons would need to be opposing, since the behavioral effects are opposing. Potential common post-synaptic targets include primary gustatory neurons and peripheral proprioceptors that monitor gut activity. Finally, these two mechanisms could target independent sites that regulate feeding behavior (Model 4). For example, NPF neurons could modify gustatory input and AstA neurons could be promoting peripheral inhibitory feedback. For this model to be correct, constitutive activation of NPF neurons would need to result in significantly increased NPF signaling than that caused by increased endogenous NPF signaling as a result of 24 hours of starvation. This is likely true, since constitutive activation of NPF neurons resulted in increased feeding compared to starved controls.

Figure 2. Possible mechanisms by which NPF and AstA neurons regulate starvation-induced feeding behavior



NPF signaling is indirectly regulated by nutritional state. Hemolymph sugar levels regulate *Drosophila* insulin-like peptide (DILP) levels, and DILP signaling blocks NPF signaling. After feeding, hemolymph sugar levels rise, which increases DILP levels, and DILP inhibits neurons that express the NPF receptor (NPFR) via a co-expressed DILP receptor (InR) (Figure 2) [15]. This means that in fed flies, the ability of NPF to promote feeding behavior is suppressed. If Model 3 is correct, AstA and NPF neurons may antagonistically co-regulate NPFR neurons, which promote starvation-induced feeding (Figure 2).

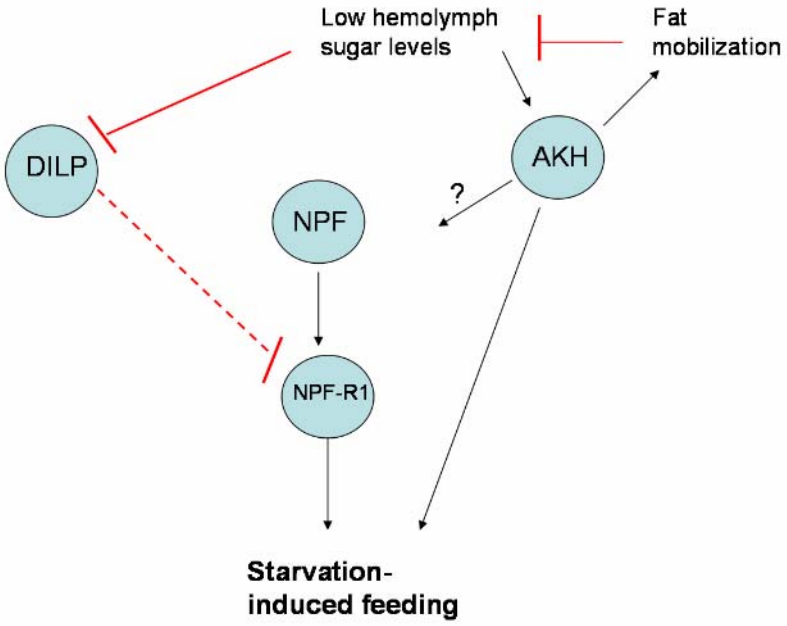
Future directions

In order to determine whether Model 3 is correct, that AstA and NPF regulate a common target in an opposing fashion, we could determine whether NPFR and AstA neuron co-activation phenocopies the behavioral effects of NPFR neuron activation. If we instead see partial rescue due to co-activation, then it would imply that NPF and AstA neurons do not target the same neurons (Model 4).

2.2 Are AstA neurons involved in the regulation of feeding under normal conditions?

DILP and adipokinetic hormone (AKH) signaling inversely modulate metabolism, and also inversely modulate starvation-induced behavioral effects. AKH neurons are sensitive to blood sugar levels [26] and low levels result in AKH secretion, which promotes the mobilization of fat stores in *Drosophila* [27, 28]. AKH mutants feed normally under ad libitum conditions but do not exhibit starvation-induced increases in food intake (Figure 3) [28, 29].

Figure 3. AKH signaling regulates fat mobilization and feeding in starved flies

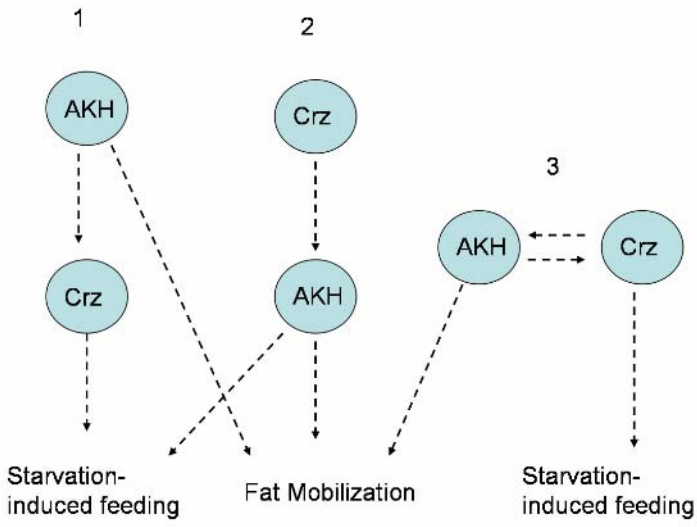


The mechanism by which AKH regulates starvation-induced feeding behavior is not known. Both AKH and AKHR mutants do not exhibit starvation-induced increases in feeding behavior [28, 29]. Interestingly, arborizations of AKH-R neurons overlap with sweet-sensing primary gustatory neurons in the SOG, which suggests that AKHR neurons may mediate starvation-induced changes in sugar responses [29]. Alternatively, AKH may regulate feeding behavior by regulating NPF signaling. Since AKH and AKHR mutants have altered energy stores, it cannot be ruled out that the feeding behavior of these mutants is due to some aspect of their altered metabolism compared to controls.

Activation and silencing of Crz neurons mimics the metabolic effects of AKH

signaling, and Crz and AKH projections overlap in the corpus collosum, which is a peripheral endocrine release site [30, 31]. This suggests an interaction between Crz neurons and AKH signaling. In males, Crz neurons are involved in responding to environmental stress, since activating Crz neurons results in increased sensitivity to osmotic, oxidative, and nutritional stress (starvation). Since both Crz activation and AKH overexpression result in reduced triglyceride levels and starvation sensitivity, we presumed that these flies with compromised energy stores would be more motivated to feed. Indeed, we found that activating either AKH or Crz neurons resulted in increased starvation-induced feeding (see Chapter 4).

Figure 4. Several models that explain the interactions between Crz and AKH

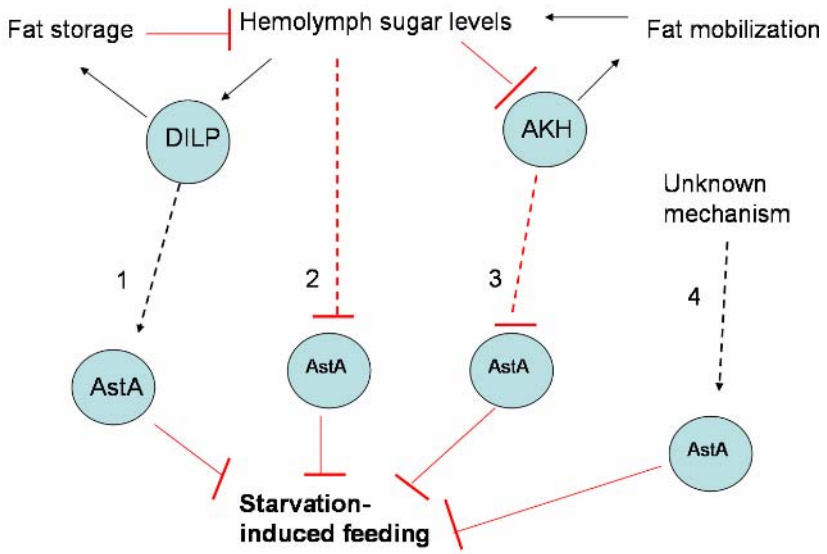


There are several models to explain the interaction of Crz and AKH neurons (Figure 4). Since it is well established that AKH signaling is directly involved in fat mobilization, Crz neurons cannot be downstream of AKH neurons in mediating this metabolic effect. The first possibility is that AKH neurons promote starvation-induced feeding behavior by activating Crz neurons (Model 1). Behavioral and metabolic effects could also be explained by a case where AKH and Crz neurons co-activate each other to promote common metabolic and behavioral effects (Model 3). Another possibility is that Crz neurons activate AKH neurons in times of stress, as proposed by Zhao et al. [30] (Model 2). Finally, as stated earlier, the feeding behavior due to AKH and Crz neurons

may be indirect effects of depletion of energy stores (not shown).

Since AstA/NaChBac flies have behavioral deficits that do not accompany metabolic changes, AstA neurons may be acting downstream of AKH and DILP neurons. If AstA neurons are involved in the normal regulation of feeding behavior, four different types of interactions could occur (Figure 5). High DILP levels may activate AstA neurons in order to inhibit starvation-induced feeding behavior (Model 1). Alternatively, AKH neurons may inhibit AstA neurons in order to disinhibit starvation-induced feeding behavior (Model 3). A third possibility is that AstA neurons monitor energy stores and act independently of DILP and AKH (Model 2). Finally, either some other unknown mechanism or an intermediate signal that is downstream of DILP or AKH could regulate AstA neurons (Model 4). If we can establish an interaction between AstA neurons and DILP signaling, AKH signaling, or nutritional state, then it would demonstrate that AstA neurons are involved in the regulation of feeding under normal conditions.

Figure 5. Four potential mechanisms by which AstA neurons are regulated



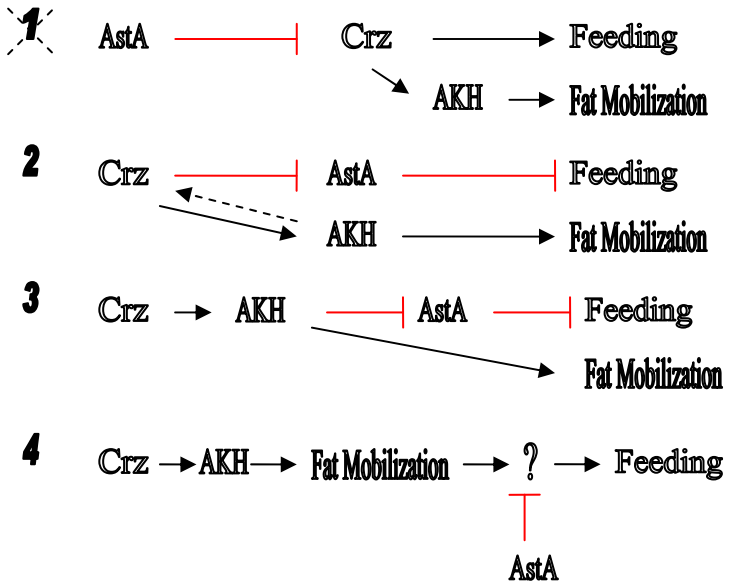
Future directions

There are a number of established transgenic tools that can be used to probe the relationship between AstA neurons and DILP signaling. Overexpression of both InR and

the dominant negative version of InR in AstA neurons could teach us about a potential relationship between DILP/AstA neurons. Since AstA/NaChBac flies behave as though sated, it is possible that DILP functionally activates AstA neurons.

In order to determine whether AstA neurons monitor hemolymph nutrient levels, we could take advantage of transgenic tools that alter the target of rapamycin (TOR) pathway, which is involved in nutrient sensing and requires S6 kinase (S6K), a target of TOR [6]. Using transgenic tools to overexpress either wild-type, dominant negative, or constitutively active S6K in AstA neurons, we could determine whether AstA neurons are sensitive to hemolymph sugar levels, and whether we can use these manipulations to alter feeding behavior.

Figure 6. There are several models to explain the interactions between Crz and AstA.



Activation of AstA neurons fully suppressed the increased food intake phenotype due to activating Crz neurons. There are several models that explain the interaction between AKH, Crz, and AstA neurons (Figure 6). Our results imply that Crz may not have a direct role in regulating feeding behavior, unless it does so via a mechanism that is overruled by activation of AstA neurons (Model 1). Another possibility is that either AKH neurons or Crz neurons are upstream of AstA neurons, and disinhibit starvation-induced feeding behavior in response to stress (Models 2 and 3). Finally, it is possible that the feeding behavior due to manipulation of Crz and AKH neurons is an indirect metabolic

effect (Model 4).

We have also demonstrated that starvation-induced changes in feeding behavior occur downstream of, or in parallel with, the metabolic effects of starvation. Interpreting the behavioral effects of manipulating DILP, AKH, or Crz neurons is confounded by the simultaneous effect of these manipulations on energy stores. Additional mechanisms that potentially alter starvation-induced changes in feeding behavior independent of changes in metabolism include NPF, leucokinin, and hugin neurons, though evidence for or against a role for these neurons in metabolism has not been demonstrated [9].

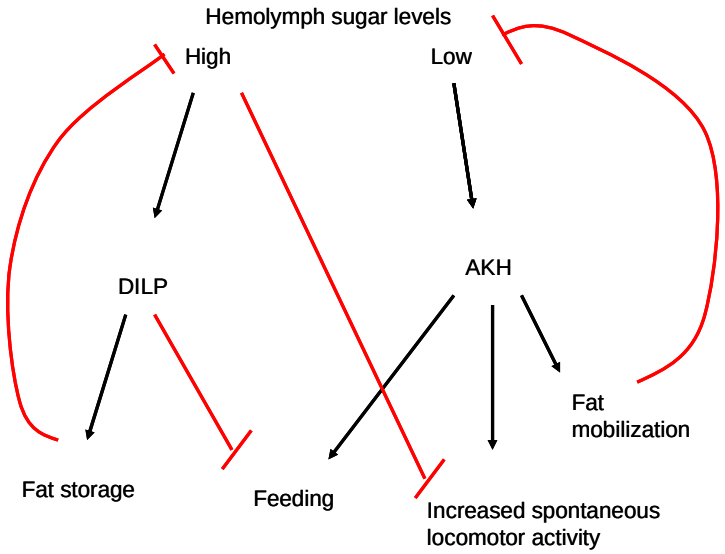
2.3 The regulation of starvation-induced feeding can be uncoupled from the regulation of starvation-induced hyperactivity.

Interestingly, activation of AstA neurons affected only starvation-induced feeding behavior and not other starvation-induced behaviors, such as starvation-induced hyperactivity. This suggests that starvation-induced feeding and hyperactivity occur via independent mechanisms, since activation of AstA neurons abolishes the former but not the latter. This conclusion is supported by the behavioral phenotype of AKH receptor (AKHR) mutants. Whereas both AKH and AKHR mutants display defects in starvation-induced feeding behavior, only AKH mutants exhibit defects in starvation-induced hyperactivity [29, 32]. Ablation of AKH neurons results in the absence of normal starvation-induced hyperactivity, yet overexpression of AKH in *Drosophila* does not cause increased locomotor activity. Two explanations for these findings are that either AKH neurons produce a neuromodulator other than AKH that is required for starvation-induced hyperactivity, or that a second AKH receptor that has not yet been identified could be mediating these effects. Contrary to the results of AKH overexpression in *Drosophila*, AKH injection increases locomotor activity in other insects. One candidate neuromodulator that may coordinate starvation-induced hyperactivity is octopamine. Octopamine injection increases spontaneous locomotor activity in several other insects [33] and octopamine feeding promotes locomotor activity in *Drosophila* larvae [34]. Nevertheless, AKH mutants provide support for our observation that the coordination of starvation-induced feeding and hyperactivity occur via independent mechanisms.

Our findings that activation of AstA neurons has no impact on the regulation of

energy stores, results in severely reduced feeding behavior despite excessively depleted energy stores (in Crz,AstA/NaChBac flies), and does not impact starvation-induced hyperactivity, support a mechanism by which AstA neurons inhibit feeding behavior downstream of or independent of AKH signaling.

Figure 7 The interactions between nutritional state, neuromodulators, energy homeostasis, and behavior



Future directions

We could utilize AstA/NaChBac flies to determine whether there are discrete sets of genes that independently regulate starvation based changes in metabolism and behavior. Using cDNA microarrays, genes that modulate starvation-induced changes in behavior would be upregulated in starved wild-type flies versus unstarved wild-type flies, but remain unchanged in starved versus unstarved AstA/NaChBac flies. Analysis of genome-wide transcript expression could also aid us in identifying interactions between AstA and other neuropeptide signaling systems, as has been demonstrated using mutants defective in feeding behavior. Larvae with mutations in *klumpfuss* display defective food-oriented behavior and feeding; microarray analysis of these mutants revealed upregulation of several neuropeptides including hugin, Crz, NPF, and AKH, and resulted in the discovery that hugin neurons regulate the acceptance of novel foods [22]. This experiment could also identify or rule out additional mechanisms by which activation of AstA neurons is inhibiting feeding behavior, i.e., stress responses, untested metabolic differences, or

additional neuromodulators mediating the effects of activating AstA neurons.

2.4 Can we integrate molecular genetic approaches and neuroethological approaches to studying the regulation of feeding behavior?

Most of what we know about the neural regulation of insect satiety has come from blowfly studies [7, 35]. Studies in the blowfly demonstrate that in starved animals, the most salient cues come from inhibitory proprioceptive feedback from the foregut, crop, and abdomen, via the recurrent nerve (RN), median abdominal nerve (MAN), and ventral nerve cord (VNC). Each of these nerves provides different strengths and types of inhibition, and severing any of these nerves results in hyperphagia. Regulation of feeding and satiety in *unstarved* blowflies, however, involves only a minor contribution from inhibitory proprioceptive feedback; Severing the RN only had a mild effect on acceptance threshold and severing the MAN had no effect on acceptance threshold [36] (refer to Chapter 1 for a thorough discussion).

In *Drosophila*, lesioning studies to demonstrate any effect of inhibitory proprioceptive feedback from the periphery have not been reported. But otherwise, behavioral studies in *Drosophila* have demonstrated that the regulatory mechanisms in *Drosophila* and blowflies are similar. Similar to blowflies, in *Drosophila* carbohydrates have a powerful stimulatory effect on food intake, the regulatory mechanisms that control the feeding behavior of unstarved and food-deprived animals is different, and the regulation of protein and carbohydrate intake are independently regulated.

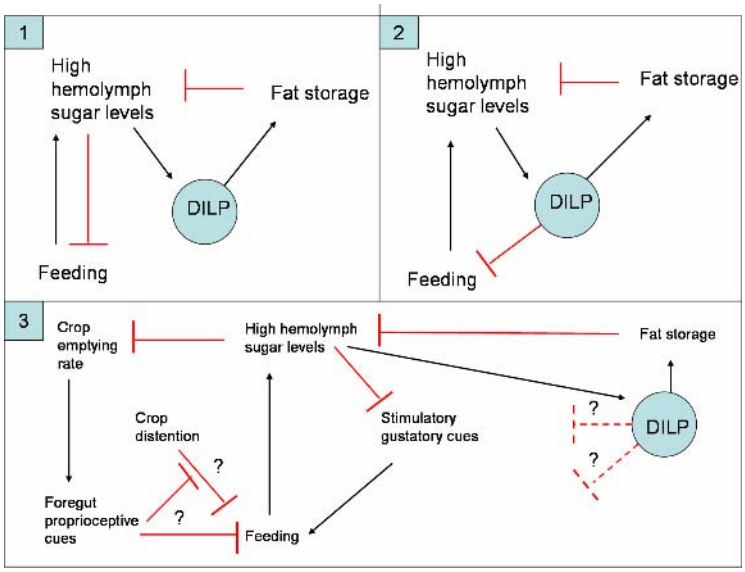
Taken together, this suggests three possible mechanisms by which activation of AstA neurons regulates feeding behavior. Either this manipulation is activating a mechanism that promotes ad libitum fed regulatory mechanisms, is inhibiting starvation-induced feeding behavior, or is inhibiting feeding behavior by some alternative mechanism that is not involved in the normal regulation of feeding.

Neuropeptides that have been implicated in regulating starvation-induced feeding behavior include AKH and NPF, but these neuropeptides are only required to regulate starvation-induced feeding behavior, and are not involved in the feeding behavior of unstarved flies [15, 28]. The only neuropeptides that have been shown to modulate the feeding behavior in ad libitum fed flies are DILP and hugin [20, 22, 26]. Because DILP

signaling also regulates metabolism and growth, the direct effects of DILP on feeding behavior have been difficult to determine. The role of DILP signaling in feeding behavior, moreover, has been overshadowed by its role in sugar metabolism, since studies that interrogate the latter could provide insight into human diseases such as diabetes. Given the strong foundation of behavioral and genetic studies in *Drosophila*, it is surprising that so little is known about the neuromodulators and circuits that regulate feeding behavior under ad libitum feeding conditions.

Although a few studies have demonstrated a role for DILP signaling in feeding behavior, it is difficult to differentiate the effects on energy stores from the effects on feeding behavior as a result of manipulate DILP signaling. If feeding behavior were exclusively regulated by the inhibition and disinhibition of NPF signaling, then we would expect a much more drastic phenotype due to manipulation of NPF neurons. Three potential additional mechanisms by which feeding behavior could be regulated include (Figure 8, Model 1) a negative feedback loop occurs between hemolymph sugar levels and feeding behavior, (Model 2) DILP signaling mediates this negative feedback loop, or (Model 3) feeding is regulated by feedback from the gut about nutritional state and gut content, as studies suggest in the blowfly (refer to Chapter 1).

Figure 8. Mechanisms by which feeding behavior may be regulated in unstarved flies.



3. Are the observed feeding behavioral effects due to the action of AstA signaling?

The function of AstA or AstA-expressing neuroendocrine cells has not previously been demonstrated in *Drosophila*. Three of the four splice variants of AstA have been isolated from the adult VNC (but not neural sheath) of adult *Drosophila* by MALDI-TOF mass spectrometric analysis, and at least one of these was isolated from the pars intercerebralis and SOG, suggesting that the isoforms may have differential tissue specificity [37]. It would be interesting to determine whether the AstA-Gal4 expression pattern reflects the expression pattern of one of these isoforms.

Drosophila Allatostatin Receptor-1 and -2 (DAR-1, DAR-2) are G-protein-gated inwardly rectifying potassium channels (GIRK) and are orthologues of the mammalian galanin receptor family, which is a subfamily of the somatostatin receptor family [38, 39]. DAR-1 is highly expressed in the adult brain, whereas DAR-2 is expressed in the periphery,[1] within the midgut, hindgut, crop, and the neural sheath [38, 40]. The differential expression of the two *Drosophila* homologues suggests that they may be associated with discrete functions of AstA signaling. The function of these receptors may also be differentially regulated by the four putative splice variants of AstA, since they have

[1] Contrary to several independent studies, Veenstra, 2009, reported co-expression of DAR-2 with Corazonin (Crz) in the larval brain, despite using the same antibody and the same experimental protocol as a previous study [38., 40,, 41].. I also immunostained adult (but not larval) brains with anti-DAR-2, and my results support previous reports that DAR-2 is expressed in the neural sheath and not in the brain (data not shown).

differential binding properties to the receptors [42].

The simplest interpretation of our experimental results is that the behavioral effects of activating AstA neurons are mediated through the release of AstA itself. However, our attempts to demonstrate this produced negative results (see Section 3.1). It is possible that other neuromodulators or neurotransmitters co-expressed with AstA are required for the phenotypes we observed due to activation of AstA neurons. Neuropeptides are co-expressed with classical neurotransmitters and sometimes with other neuropeptides, but whether they are co-released depends on the neuron and/or the firing frequency of the neuron [43]. Despite this caveat, there is a precedent for AstA in the regulation of feeding behavior in other insects (see Section 3.3).

3.1 Experiments I have conducted to address this question

I have made several attempts to address the role of AstA signaling in mediating the effects of activating AstA neurons. Since our AstA-Gal4 is not expressed in all AstA neurons, using this tool to silence or ablate AstA neurons does not eliminate AstA expression in many CNS neurons. Ablation eliminates only three of eight pairs of AstA neurons in the brain, and only three of 13 pairs of neurons in the VNC. These remaining AstA neurons may compensate for the loss of a subset of AstA neurons in silencing or ablation experiments. One approach to this question is to ask whether the effects of activating AstA neurons can be overridden by simultaneously knocking down AstA expression. To knock down AstA expression, we could use several UAS-RNAi transgenic tools.

A transgenic library of inducible UAS-RNAi lines was generated by the Vienna *Drosophila* RNAi Center (VDRC) and includes UAS-AstA-RNAi transgenic lines (two independent insertions), UAS-DAR1-RNAi (3 constructs, two independent insertions each), and UAS-DAR2-RNAi (two independent insertions) [44]. I have crossed these transgenic flies to pan-neuronal drivers, including elav-Gal4 and appl-Gal4, which are also expressed in gut neuroendocrine cells. In order to improve cleavage of the dsRNA into RNAi fragments, I also co-expressed UAS-dicer. I tested these flies and genetic controls in several feeding assays, and did not observe any feeding phenotypes. Q-RT-PCR experiments indicated, however, that levels of AstA mRNA were not diminished in

elav/AstA-RNAi flies. I also tried using a stronger ubiquitous driver, daughterless-Gal4, to drive AstA-RNAi expression, but did not see a behavioral phenotype or knock down of transcript expression. It is possible that the particular sequences used to generate these RNAi lines were not ideal or that transcript expression compensated for knockdown. There are additional available collections of UAS-RNAi that we can test in the future.

Another way to determine whether AstA signaling is responsible for the feeding phenotype, would be to phenocopy the feeding phenotype using AstA peptide injections. We had *Drosophila* AstA peptide synthesized and we also obtained synthetic *Diploptera* AstA. I injected starved, cold-anesthetized flies with either saline or with varying concentrations of AstA. After a brief recovery period (flies wake up in under 5 minutes), I tested either food intake or the PER of the injected flies. Neither food intake nor PER to sucrose was impaired in AstA-injected starved flies.

The failure of AstA injections to phenocopy the effect of AstA neuron activation could be due to either methodology or to mechanism. By methodology, I mean that either the synthesized peptide may not have been pure or concentrated enough, that injections or cold-anesthesia adversely affected starvation-induced feeding responses, or that the injected peptide may not have had access to relevant sites of action. Degradation may also be a problem, as injected synthetic *Diploptera* AstA has been reported to degrade rapidly and to only be moderately effective (to inhibit JH synthesis in *Diploptera*) [10]. Several *Diploptera* AstA mimetics have been developed that are not so rapidly degraded [10], and we can instead use these in future injection experiments. By mechanism, I mean that we might have synthesized an AstA isoform that does not normally regulate feeding. There are four putative AstA isoforms, and the one we had synthesized may not be expressed in the brain. Another isoform of AstA has been isolated from both the pars intercerebralis and SOG by MALDI-TOF mass spectrometric analysis, and we will have this isoform synthesized for future injection experiments as well. Alternatively, these negative results may indicate that the effects of AstA neuron activation on feeding may require co-release of another neuromodulator, or that another neuromodulator, and not AstA, is responsible for the behavioral phenotypes observed.

3.2 Future experiments to address this question

Besides peptide injection, there are other ways to address the role of AstA signaling in feeding behavior. Since most neuromodulators involved in feeding behavior are regulated in response to nutritional state, we would like to determine whether AstA levels are sensitive to nutritional state as well. Transcript levels of neuropeptides that are normally upregulated due to starvation, such as hugin, Crz, NPF, and AKH, are also elevated in larval *klumpfuss* mutants, which have impaired feeding behavior and die of starvation [22]. If AstA levels change in response to starvation, it would support a function for AstA signaling in the regulation of feeding. Furthermore, looking for changes of AstA levels in the brain versus the gut could illuminate which regions of the gut/brain are involved in sensing nutritional state. We will use an antibody to quantify relative levels of AstA expression and simultaneously use UAS-ANF::GFP to visualize peptide release [45, 46]. In addition to comparing AstA levels in starved versus unstarved flies, we would also like to look at the effects of altering nutritional regime on AstA expression in various tissues. There are several transgenic tools I would like to generate to establish a requirement of AstA in feeding behavior. We could use UAS-AstA transgenics in order to overexpress AstA in various tissues, which would also allow us to determine whether AstA signaling is sufficient to regulate feeding behavior. Furthermore, we could limit overexpression using various Gal4 drivers in order to determine whether effects of AstA are central or peripheral. Since DAR-1 and DAR-2 are exclusively expressed centrally or peripherally, respectively, generating Gal4 enhancer traps of these receptors would be useful to determine the site of action of AstA. We will address additional experiments that could differentiate the site of action of AstA as well as determine whether other neuromodulators are required, in later sections.

3.3 Orthologues of AstA signaling regulate feeding behavior

3.3.1 In arthropods

As mentioned in Chapter 1, AstA has been demonstrated to promote satiety in other insects. It is involved in signaling nutritional stress, promoting a metabolic switch towards fat storage, and decreasing food intake. Many studies support a role for AstA in sensing nutritional or environmental stress, since AstA is regulated in response to

starvation [10]. Some of the reported effects of AstA signaling may be indirect, as a result of the inhibition of Juvenile Hormone (JH) [10]. Since DAR-1 is expressed in the nervous system in *Drosophila* and AstA does not regulate *Drosophila* JH, suggests that AstA has retained other neuromodulatory functions [11, 38]. The co-expression of AstA and serotonin in the SGS of crabs and lobsters [47, 48], and the studies implicating serotonin to inhibit feeding in several insects including *Drosophila* [49, 50], suggests that serotonin may be mediating the effects of AstA neuron activation.

3.3.2 In nematodes

An allatostatin/galanin-like receptor in *Caenorhabditis elegans*, NPR-9, promotes food-leaving behavior in the presence of food cues without affecting gustatory discrimination [51]. NPR-9 is expressed exclusively in one pair of neurons, named AIB, that determine sensory-dependent locomotor behavior. These neurons are required for the stereotyped enhanced forward motion exhibited in the absence of food. NPR-9 is necessary and sufficient to promote food-leaving behavior.

3.3.3 In mammals

Although the peptide sequence of AstA has not been conserved in mammals, six of seven transmembrane sequences of DAR-1 have 50–70% amino acid similarity with the mammalian somatostatin family of GPCRs, and are most similar to the mammalian galanin subfamily of GPCRs [38]. Galanin and galanin receptors are expressed in brain regions that regulate satiety in mammals (including the hypothalamic arcuate nucleus), and are involved in the regulation of feeding behavior [52–54]. Knockouts of galanin exhibit normal feeding behavior on low-fat foods, and have normal responses to starvation, but become obese on a high-fat diet [52]. This suggests that galanin is not involved in the normal regulation of feeding, but is instead involved in only context-specific regulation of feeding behavior.

Further support for a context-specific function in feeding, is that leptin injection induces galanin expression, whereas starvation has no effect on galanin expression [55]. Interestingly, galanin knockouts are much more sensitive to the effects of leptin, which suggests that galanin antagonists may have the potential to be used to treat obesity. The

exact function of galanin in feeding behavior remains to be determined, but one hypothesis has been proposed that the function of galanin signaling may be to reduce the negative side effects produced by by-products of fat metabolism (when fed on a high-fat diet), by reverting to increased carbohydrate metabolism [52].

3.4 Conclusions and future directions

Multiple animal models suggest a conserved role for allatostatin/galanin signaling to modulate food intake in response to nutrient-based stress. Our results demonstrate that in *Drosophila,* AstA neuron signaling represses certain behavioral responses to nutritional stress. In future studies, we would like to determine whether AstA signaling is mediating the behavioral effects we observed upon AstA neuron activation. Since the AstA-Gal4 transgenic flies that we generated were only expressed in a minority of AstA neurons in the brain and VNC, we cannot determine from our silencing and ablation studies whether AstA signaling is required in feeding behavior. The finding that DAR-1 and DAR-2 are expressed centrally and peripherally, respectively, affords us the opportunity to distinguish between a role for central versus peripheral AstA neurons to mediate the feeding phenotype of AstA/NaChBac flies (assuming that the feeding effect is not due to a co-expressed neuromodulator with different targets.)

4. What is the circuit-level mechanism that underlies the effect of AstA neuron activation to inhibit starvation-induced feeding behaviors?

4.1 Neural mechanisms that regulate blowfly and *Drosophila* satiety

In the unstarved blowfly, MAN transection has no effect on feeding behavior, suggesting that crop distention has no effect on the feeding behavior of unstarved flies [36]. In unstarved *Drosophila*, the crop is barely utilized for food storage unless fed flies are nutrient-deprived due to a diluted or unbalanced food source [36]. If proprioceptive feedback from crop distention is disregarded in fed flies, it would explain why MAN transection in blowflies has no effect on feeding, and why in *Drosophila* a low concentration (low stimulating) food source could overcome inhibitory proprioceptive feedback from the crop to induce feeding. Nutritional state determines the mechanisms that regulate feeding behavior (refer to Chapter 1).

4.2 Does activation of AstA neurons inhibit feeding behavior by promoting proprioceptive feedback?

Starved AstA/NaChBac flies behaved as though unstarved in that they minimally utilized crop storage even though all metabolic assays indicated that starvation-induced metabolic changes were no different than controls. If we assume that the mechanisms to promote satiety in blowflies are conserved in *Drosophila*, since we observed such a robust inhibition of starvation-induced feeding behavior upon activation of AstA neurons, then one obvious interpretation is that activation of AstA neurons in *Drosophila* directly or indirectly transmits inhibitory proprioceptive feedback to the CNS.

In several species of moth, AstA is expressed in the frontal ganglion and in nerves projecting to the brain and through the RN [8]. The larvae of these moths exhibited decreased feeding when injected with AstA during a feeding stage [8]. Since AstA is released at neurohemal release sites, it is possible that even though expression of AstA within the RN may not have been conserved in *Drosophila*, the target neurons expressing AstA receptors may have been conserved.

The mechanisms by which AstA neuron activation could potentially mimic inhibitory proprioceptive feedback in *Drosophila* is by inhibiting either foregut or crop contractions, or by inhibiting proprioceptive information from being transmitted. Peripheral sites of AstA expression that could inhibit feeding behavior include midgut muscle, midgut neuroendocrine cells and motor neurons innervating the midgut and hindgut. AstA is not expressed in sensory neurons in the gut, but since DAR-2 is expressed in the midgut and crop [40], it would be informative to determine whether DAR-2 is expressed in peripheral sensory neurons.

4.2.1 Proventriculus

In the midgut, we observed co-labeling of muscle near the proventriculus. The proventriculus, also called the cardia, is the stomach in insects, and serves as a valve permitting transfer of food from the foregut to the midgut [18]. AstA is expressed below the proventriculus in a similar region as another another peptide, leucokinin, that is required for regulating meal size ([21], see Chapter 1). If AstA neuron manipulation were involved in only modulating meal size, we would expect all flies to feed, but imbibing

smaller volumes. After 24 hours of starvation, 100% of leucokinin mutants consumed food, whereas a smaller percentage of AstA/NaChBac flies feed compared to controls.

We have ruled out the possibility that the feeding phenotype of AstA/NaChBac flies is due to an inability to transfer food to the midgut or the crop by visual inspection. An impairment in cardiac valve function does not prevent hungry flies from feeding, as the crop duct is anterior to the proventriculus and food can enter the crop. Support for this argument lies in the feeding behavior of flies carrying mutations in the gene *drop-dead.* These mutant flies are unable to transfer food into the midgut yet their crops are bloated with food [56]. These mutants eventually die of starvation among other problems.

4.2.2 Motorneurons

Since AstA is expressed in motor neurons innervating the gut, we addressed the possibility that AstA/NaChBac flies were impaired at moving food through the gut. Despite this expression in motor neurons, I saw no evidence of constipation, decreased excretion, decreased regurgitation, etc. After 24 hours of starvation, very little food remains in the gut of AstA/NaChBac flies, implying that excess food is not impairing their ability to imbibe food. Although this does not rule out the possibility that AstA is inhibiting gut contractions, it suggested that constipation/ability to ingest was not affected.

4.2.3 Midgut neuroendocrine cells

AstA expression in neuroendocrine cells in the posterior midgut could secrete neuromodulators that target gut muscle or sensory neurons (Figures 2d–f). It is not known whether neuroendocrine cells are excitable or how overexpression of a sodium channel would affect the function of these cells. There are a variety of neuroendocrine cells in the midgut of *Drosophila* and they express a large number of neuropeptides, including NPF [57].

4.2.4 Humoral factor

Activation of AstA neurons could release a neuromodulator into the hemolymph which could target peripheral tissues, the SGS, or the CNS. In blowflies, paracrine gut/brain signals were not sufficient to inhibit feeding behavior in transfusion or parabiotic

studies [35]. Contrary to these findings, injection of several neuromodulators was sufficient to inhibit feeding, including dopamine, serotonin, and sulfakinin [58, 59].

Future experiments

Activation of AstA neurons could be affecting feeding behavior by promoting inhibitory proprioceptive feedback from the gut. If this is the case, a requirement for AstA neurons in the gut for normal feeding behavior is not necessary or is redundant, because silencing or ablation of these neurons has no effect on feeding. Potential targets include gut muscles, proprioceptive sensory neurons, and the SGS.

Although I saw no evidence of overt effects on gut motility in AstA/NaChBac flies, I will quantify gut motility in more detail in these flies. To address potential myoinhibitory properties of AstA neurons, I will try bath application of synthetic AstA alone or in combination with serotonin and proctolin. In other insects, bath application of AstA could only inhibit proctolin-induced gut contractions.

We could attempt nerve transection in *Drosophila* to determine whether inhibitory proprioceptive feedback regulates *Drosophila* satiety and if so, whether feeding is rescued in AstA/NaChBac flies due to nerve transection. Since octopamine injection mimics the effects of RN/MAN transection [60], we could test whether octopamine injection rescues the AstA/NaChBac feeding phenotype.

Experiments and transgenic tools described in Section 2 could also address whether activation of peripheral AstA neurons is responsible for the feeding phenotype. Determining whether starvation, feeding, or feeding regime alters AstA signaling in the gut could demonstrate whether AstA normally functions in the regulation of feeding. We could drive UAS-AstA expression peripherally or silence DAR-2 neurons to determine whether these modulations phenocopy the effects of AstA neuron activation.

4.3 How could central sites of AstA expression contribute to the behavioral effects of activation of AstA neurons?

The activation of AstA neurons that innervate the SOG, the Pars intercerebralis, the protocerebrum, and the optic lobes could impair feeding behavior. In addition to being potential downstream targets of inhibitory proprioceptive feedback, these brain centers have each been implicated in the regulation of feeding behavior.

4.3.1 Subesophageal ganglion

The SOG receives primary gustatory input and also contains motor neurons and SGS neurons that control various aspects of feeding behavior [61]. How gustatory information is processed and how this is integrated with nutritional state to execute feeding behaviors is not well understood [18, 62]. We have shown that gustatory acuity and discrimination is intact in AstA/NaChBac flies. AstA/NaChBac flies are also capable of executing the motor patterns that control feeding behavior. We have discussed mechanisms by which activation of AstA neurons could affect gustatory integration/feeding decision centers in a previous section.

4.3.2 Pars intercerebralis

AstA neurons project to the Pars intercerebralis (PI), which contains a number of neurosecretory cells that project to peripheral neurosecretory sites [63]. The PI is involved in regulating growth and metabolism, egg development, and color change [64, 65]. DILP-expressing neurons that are critical for growth and development have cell bodies located in the PI [19, 27]. These neurons monitor hemolymph sugar levels and DILP signaling regulates fat storage and feeding behavior (see Section 2 for a discussion of DILP signaling).

4.3.3 Protocerebrum

AstA neurons have extensive projections in the protocerebrum, which contains higher processing centers. The superior lateral neuropile is “invaded” by secondary-to- quaternary visuo-, chemo-, mechanosensory systems [66, 67]. Several studies have demonstrated that the mushroom bodies in the protocerebrum integrate gustatory inputs [68, 69].

4.3.4 Optic lobes

AstA is expressed in ~ 30 optic lobe neurons that innervate the medulla [11]. While this is not a feeding-related center per se, feeding is affected by light exposure and by internal circadian rhythm [70].

Flies exhibit two peaks of locomotor activity daily, when lights are turned on and

when lights are turned off, with relatively little activity in between these peaks [71]. Cycling of clock genes not only regulates circadian rhythm, but is autonomously required for the proper functioning of a surprising number of tissues [72, 73]. *Drosophila* exhibit cycling in their feeding behavior, with a peak in feeding behavior in the late morning, and abolishing clock genes in the fat body abolishes this cycling in feeding behavior [70].

Flies exposed to constant light feed more than flies exposed to a 12:12 light:dark cycle. Exposing flies to constant light causes arrhythmia [74] and results in excess feeding due to either constant activity or to interferences with sleep patterns [35].

Diapause is a state of dormancy employed by insects to survive harsh environmental conditions [75] such as low temperatures and short length of day. In *Drosophila,* diapause is impaired in mutants in *couch potato.* These mutants are hypoactive and exhibit only weak phototaxis and negative geotaxis [76].

Conclusions

We demonstrated that activity levels of flies during the 24 hours prior to starvation of AstA/NaChBac and AstA/TRPA1 flies did not differ from controls. The decreased feeding phenotype of AstA/NaChBac flies persisted despite time of day tested. Given that we were testing starved flies, any differences in circadian dependent patterns of ad libitum feeding behavior ought to be overwhelmed by the state of deprivation. There was no evidence that AstA/NaChBac flies were in a state of diapause, because energy stores were not increased, starvation resistance was not increased, geotaxis and phototaxis was normal, and egg production and fertility was normal (data not shown). Therefore the decreased feeding behavior of AstA/NaChBac flies cannot be explained by circadian influences.

5. Other potential neuromodulators mediating the effects of activating AstA neurons

5.1 Serotonin

Given the function of serotonin in both invertebrates and vertebrates to decrease food intake, it is possible that AstA neurons are serotonergic [77]. In the blowfly, serotonin injection inhibits both sugar and protein intake [58, 78]. Injection also resulted in an increased acceptance threshold as well as weight loss. Activation of AstA neurons does not

result in weight gain, but this might be explained by the fact that AstA-Gal4 is expressed in only a small number of neurons, whereas serotonin is expressed in around 100 neurons in the brain. Also, serotonin is found in *Drosophila* pharyngeal muscle, proventriculus, and gut [49]. It is also found in the stomatogastric nervous system of other invertebrates, and allatostatin has been found to be co-expressed with serotonin in the stomatogastric nervous system of *Crustacea* [43]. Allatostatin is co-expressed with serotonin in stretch receptor neurons innervating the stomatogastric nervous system of crab and lobster, and in vitro studies have demonstrated that allatostatin and serotonin inhibited gastric mill rhythm/contractions [43]. Interestingly, the effects of allatostatin on the spiking of stretch receptors in the crab, *Cancer borealis*, was dependent on prior activity; inhibition of spiking was more pronounced at low stretch amplitudes and with prolonged exposure [79]. In *Drosophila*, a null mutation in one of the enzymes required for serotonin production also exhibited a decreased feeding phenotype [49]. Based on these studies, it seems likely that AstA and serotonin are coexpressed in *Drosophila* as well. Manipulation of serotonin levels may be responsible for the feeding phenotype we observe in AstA/NaChBac flies. Alternatively, AstA and serotonin levels may jointly regulate feeding behavior.

PDF neurons, which coordinate circadian rhythms, project to the medulla, and it has been speculated that they may receive input from serotonergic neurons in this region. AstA neurons in the optic lobe project to the base of the medulla, and it would be interesting to determine whether these neurons are also serotonergic, and to determine their pre-synaptic inputs and post-synaptic outputs.

Future directions

If we determine that serotonin and AstA expression overlap, it could give us an opportunity to 1) determine if we might be able to target a subset of AstA neurons, 2) determine whether excess serotonin levels are responsible for the feeding phenotype, 3) identify a silencing phenotype, and 4) confirm a role for serotonin in feeding behavior.

In order to determine whether the two are co-expressed in the same neurons, we will use immunohistochemistry in order to co-label AstA-Gal4/UAS-mcd8::GFP brain, VNC, and gut tissue with antibodies against GFP and serotonin. If no overlap occurs, we would then attempt co-labeling with antibodies to serotonin receptors. If we see overlap in expression, it is possible that only a subset of AstA-Gal4+ neurons overlap with serotonin

expression. This would give us the opportunity to identify which subset of neurons are responsible for the feeding phenotype.

Various tools have been used in *Drosophila* to successfully manipulate serotonin levels and alter behavior, including feeding of serotonin agonists and antagonists, null mutations in enzymes required for serotonin synthesis (Tph1 and 2), and UAS-Tph transgenic flies [49, 80]. In addition, we can use THGal80 to discriminate serotonin from dopamine requirement [16].

5.2 Hugin

Activation of AstA neurons may represent the reverse of silencing hugin neurons. Flies in which hugin neurons are silenced exhibit an increased rate of acceptance of a novel food source [22]. Silencing hugin neurons in unstarved adult flies resulted in feeding sooner than but not more than controls flies, but only when transferred from regular fly food to yeast paste. Silencing did not alter the rate of accepting a novel food source in starved flies, possibly because starved wild-type flies increase their rate of acceptance as well, compared to unstarved wild-type flies. Another interpretation of this data is that hugin neurons regulate protein feeding, since standard fly food is not typically rich in protein content.

To test whether the activation of AstA neurons is impairing the rate of acceptance of a novel food source, we tested feeding on normal fly food, and still saw decreased feeding behavior of AstA/NaChBac flies. We also tested whether AstA/Kir2.1 and AstA/TNT had an increased rate of acceptance of a novel food when unstarved, and did not observe differences compared to controls. We also tested whether minimizing stress levels during feeding assays was either promoting feeding in control flies or whether this was uncovering a context-dependent feeding phenotype in AstA/NaChBac flies, and still saw normal feeding behavior of control flies and decreased feeding behavior of AstA/NaChBac flies.

5.3 DILP-7

In *Drosophila*, there are seven genes encoding DILPs. The DILPs I have been referring to throughout my dissertation are DILP-2, DILP-3, and DILP-5, which are expressed in the pars intercerebralis and have functional roles in growth and metabolism [5]. DILP-6 is expressed in the fat body and also plays a role in growth and development, as it is required to maintain growth during the non-feeding stage of metamorphosis [81, 82]. In contrast, DILP-7 is not essential for growth and development, but instead is involved in food-based decision-making.

The evidence against the involvement of DILP-7 in growth and development is that flies with a null mutation in DILP-7 exhibit normal development time, body weight, lipid and glycogen content, starvation and paraquat resistance, viability, lifetime fecundity, or median lifespan [83]. Further support for this suggestion, is that both the survival and body weight of flies with combinatorial mutations in DILP-2, -3, and -5 or in DILP-1–5 were significantly reduced, and combining these combinatorial mutants with a null mutation in DILP-7 did not further decrease survival or body weight. While not being required for growth and development, and not being sufficient to compensate for the loss of other DILPs, an independent study demonstrated that constitutive overexpression of DILP-7 using armadillo-Gal4 (a housekeeping gene, thus expressed everywhere) resulted in a increase in body weight of adults [20]. This effect on body weight was likely due to overexpressing DILP-7 at much higher levels throughout the body than might be expected from the endogenous DILP-7 enhancer. Evidence for low levels of DILP-7 endogenous expression levels is that DILP-7 expression was not detectable by in situ hybridization in adults [20].

Several studies have implicated DILP-7 in the regulation of feeding behavior. One study demonstrated a role for DILP-7 neurons in the rate of food intake. Silencing DILP-7 neurons did not have any effect on food intake unless provided a low-nutrient food source, in which case food intake was increased compared to control flies [24]. Feeding behavior was increased during 24 and 48 hours of feeding on 26 mM sucrose plus 0.6% yeast, whereas food intake was normal when provided a nutritious diet, which contained sixfold the amount of sucrose and yeast.

Another study demonstrated a role for DILP-7 in determining oviposition site.

Overexpression of DILP-7 by either using hs-DILP-7 (a heat shock promoter fused to DILP-7) or by using DILP-7-Gal4/UAS-DILP-7 reduced or eliminated the oviposition preference of females, respectively [84]. Whereas wild-type females preferentially laid eggs on lobeline when given the choice between this aversive tastant and sucrose, over-expressing DILP-7 either suppressed or eliminated this preference. The nature of this oviposition preference of wild-type flies is peculiar, given the stimulatory value of the sucrose (100 mM) and the lack of nutritional value of the lobeline (which did not contain any sucrose). Both oviposition sites contained 1% ethanol, which might have added stimulatory value to the lobeline. The effects of overexpressing DILP-7 were not further examined in this study.

In wild-type flies, nutritional state did not effect the decision to lay eggs on lobeline, and wild-type flies visited either food source an equal number of times [84]. This suggests that flies do not indiscriminately choose the location to lay eggs or that nutritional state would alter this choice. In fact, the preference *against* laying eggs on 100 mM sucrose of wild-type females was upheld when given the choice between sucrose and agarose. These findings suggest that overexpressing DILP-7 is not reflecting the behavior of a "sated" fly. When DILP-7 neurons were silenced, using DILP-7-Gal4/UAS-Kir2.1, all egg-laying motor patterns were abolished, rendering these flies sterile. In addition to the expression pattern of DILP-7 in both the female reproductive tract and the SOG, this suggests that DILP-7 signaling may be involved in integrating gustatory cues to inititate oviposition motor patterns.

The expression pattern of AstA and DILP-7 in the larval brain is surprisingly similar (data not shown). Similar to AstA expression in the VNC, DILP-7 is expressed in several pairs of large abdominal ganglion neurons that project to the hindgut and rectum, in three pairs of lateral dorsal neurons, and in a pair of central medial neurons [84]. Immunostaining images of DILP-7 in the adult brain in Yang et al. [84], shows a strong signal in the posterior SOG, in the same location that AstA and AstA-Gal4 label a pair of cell bodies. In contrast to antibody staining, DILP-7-Gal4 was not expressed in the dorsal medial SOG, which could be explained if additional enhancers of DILP-7 expression were missing in the DILP-7-Gal4 transgenic construct. It is also possible that the DILP-7 antibody is not specific, and is detecting the expression of other DILPs in the SOG. In the

adult, the DILP-7 cell bodies in the abdominal ganglion send projections through cervical connective to the SOG and also to the hindgut, rectum, and to the female reproductive tract. Although the AstA-Gal4 transgenics and the anti-AstA antibody do not show expression in the female reproductive tract, it is possible that the VNC neurons that project to the hindgut and rectum overlap with DILP-7 neurons. Both the AstA-Gal4 transgenics and the anti-AstA antibody also show expression in the ventral nerve cord and the SOG, but the origin of the projections in the cervical connective is not clear.

This similarity in expression patterns is especially intriguing due to the proximity of *Drosophila* AstA receptor 1 (DAR-1) and *Drosophila* insulin-like peptide 7 (DILP-7) on the X chromosome (DAR-1 is located only ~ 1.3 kb upstream of DILP-7 and in the same 5' to 3' orientation). The close proximity of two genes involved in feeding decision-making suggests that there may be common enhancers for these genes. Although microarray data suggest that DAR-1 is exclusively expressed in the CNS [40], the expression pattern of DAR-1 is undetermined because in situ hybridization of GPCRs has generally posed a challenge.

These observations suggest a possible overlap between DILP-7 and either AstA or DAR-1 expression. Arguing against DILP-7 and AstA co-expression though, is a report that larval DILP-7 neurons weakly express proctolin, which is a widely expressed neuropeptide that stimulates gut contractions. In several insects, AstA has been reported to inhibit proctolin-induced gut contractions [8, 85]. But since cases/examples exist where neuropeptides with counteracting functions are co-expressed, this finding may not exclude the possibility that AstA and DILP-7 are co-expressed.

Also intriguing, is the fact that DILP-7 is the most conserved DILP in *Drosophila*, and is in fact the only DILP with homology to other ILPs outside of *Drosophila* species. Similarly, AstA is found in almost all insects/arthropods studied, and at least 431 different isoforms of AstA have been observed in arthropods (!!). The extent of conservation of each of these molecules suggests an important role in evolution. Although the sequence of the neuropeptide AstA has not been conserved across the animal kingdom, the sequence of the receptors of AstA, DAR-1 and DAR-2, has been conserved. In *C. elegans* and in mammals, receptor orthologues of DAR have similarly been implicated in regulating feeding behavior. The close proximity of DILP-7 and DAR-1 on the X chromosome may

have co-conspired in the evolutionary conservation of these two signaling systems.

Evidence for a role of DILP-7 in food-based decision-making without displaying a role in growth and development, in combination with potential overlap of DILP and AstA/DAR1 expression, begs the question of whether DILP-7 signaling is driving the phenotype we observed upon AstA activation, an impairment of feeding decision-making without a role in metabolism. Another possibility is that these two signaling systems are interacting. I can address this possibility by using the feeding and oviposition assays described in the DILP-7 studies to examine whether AstA/Kir2.1 flies exhibit similar phenotypes as DILP-7/Kir2.1 flies. Further characterization of DILP-7 overexpression is necessary as well as characterization of DILP, AstA, DAR-1, and InR expression patterns. Given the number of DILP-7 tools available, we could also determine whether AstA-Gal4/UAS-DILP phenocopies the observed phenotype of activating AstA neurons, and whether AstA-Gal4/UAS-NaChBac/UAS-DILP-7-RNAi rescues the phenotype.

6. Summary

The aim of this dissertation is to gain insight into the neuromodulators and neural mechanisms that regulate satiety. Since many mechanisms that regulate mammalian feeding behavior have been conserved in *Drosophila* and other insects [4, 8], we sought to better characterize how feeding behavior is regulated in *Drosophila.* I optimized a set of assay parameters that clearly distinguishes the hunger-state of flies. I utilized this assay to identify a circuit manipulation that inhibits starvation-induced changes in feeding behavior. Activation of AstA neurons results in reduced starvation-induced changes in food responsiveness and food intake and this manipulation does not alter any other changes that normally occur in response to starvation. This suggests that we have tapped into a mechanism that regulates a specific subset of starvation-induced changes in feeding behavior and that is independent from general starvation-induced behavioral responses and energy metabolism. A mechanism that promotes satiety but that does not alter energy metabolism has not previously been identified in *Drosophila*. This may be because studies in *Drosophila* have focused on energy metabolism and not feeding behavior, or because studies have focused on larvae, which are in a growth phase and feed continuously.

Given the precedent of AstA to act as a satiety factor in other insects, and given the precedent of orthologues of AstA receptors in the regulation of feeding behavior, suggests that my findings that activation of AstA neurons inhibits feeding behavior are evolutionarily relevant. Identifying the mechanism by which activation of AstA neurons reduces feeding behavior has been complicated by the fact that AstA-expressing neurons are located in multiple anatomical locations that could regulate feeding behavior. Furthermore, I found a gap in the literature, between studies that characterized the molecular genetics underlying the regulation of feeding behavior in *Drosophila* and studies that used nerve lesions to characterize the regulation of feeding behavior in other insects. Many of the neuropeptides and neuropeptide receptors that have been implicated in the regulation of feeding behavior and that were identified in *Drosophila* studies, are expressed in the nervous system, in the gut, and also at potential neurohemal release sites, yet the majority of these studies have ignored a potential peripheral mechanism of action of these neuropeptides. A number of reviews have pointed out this gap in the literature: the adult SGS is not well characterized in terms of its role in the regulation of feeding, digestion, and metabolism, and a large number of neuropeptides and neuropeptide receptors are expressed in various regions of the gut. The neural mechanisms that regulate feeding behavior have been extensively studied in blowflies, and suggest that peripheral feedback from the gut plays an important role in the regulation of feeding behavior, and this finding is consistent with studies conducted in other insects.

Since this discrepancy in the literature, between a focus on central mechanisms in *Drosophila* studies and peripheral mechanisms in blowfly studies, has made it difficult to interpret my findings, I have used this opportunity to develop several models that could integrate the discrepancies between the role of neuromodulators in the regulation of feeding behavior, the role of central and peripheral mechanisms in the regulation of feeding behavior, and the role of nutritional state in the regulation of feeding behavior.

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A p p e n d i x

A SINGLE POPULATION OF OLFACTORY SENSORY NEURONS MEDIATES AN INNATE AVOIDANCE BEHAVIOUR IN *DROSOPHILA*

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A single population of olfactory sensory neurons mediates an innate avoidance behaviour in *Drosophila*

Greg S. B. Suh[1,2], Allan M. Wong[1,3], Anne C. Hergarden[1,2], Jing W. Wang[1,3], Anne F. Simon[2*], Seymour Benzer[2], Richard Axel[1,3] & David J. Anderson[1,2]

[1]*Howard Hughes Medical Institute,* [2]*Division of Biology 216-76 and 156-29, California Institute of Technology, Pasadena, California 91125, USA*
[3]*Columbia University College of Physicians and Surgeons 701 West 168th Street, New York 10032, USA*

*Present address: Brain Research Institute University of California, Los Angeles, California, USA

All animals exhibit innate behaviours in response to specific sensory stimuli that are likely to result from the activation of developmentally programmed neural circuits. Here we observe that *Drosophila* exhibit robust avoidance to odours released by stressed flies. Gas chromatography and mass spectrometry identifies one component of this '*Drosophila* stress odorant (dSO)' as CO_2. CO_2 elicits avoidance behaviour, at levels as low as 0.1%. We used two-photon imaging with the Ca^{2+}-sensitive fluorescent protein G-CaMP to map the primary sensory neurons governing avoidance to CO_2. CO_2 activates only a single glomerulus in the antennal lobe, the V glomerulus; moreover, this glomerulus is not activated by any of 26 other odorants tested. Inhibition of synaptic transmission in sensory neurons that innervate the V glomerulus, using a temperature-sensitive *Shibire* gene (Shi^{ts})[1], blocks the avoidance response to CO_2. Inhibition of synaptic release in the vast majority of other olfactory receptor neurons has no effect on this behaviour. These data demonstrate that the activation of a single population of sensory neurons innervating one glomerulus is responsible for an innate avoidance behaviour in *Drosophila*.

We observed that *Drosophila* tend to avoid chambers in which other flies have previously been subjected to stress by mechanical shaking or electric shock. To investigate the basis of this behaviour, about 70 'emitter' flies were subjected to mechanical stress by vortexing them in a test tube (see Methods). We removed the stressed flies, and allowed naive 'responder' flies to choose between this 'conditioned tube' and a fresh tube, in a T-maze apparatus[2]. The majority (80–95%) of responder flies avoided the conditioned tube in a one-minute trial (Fig. 1a). A similar avoidance response was observed when emitter flies were stressed using electric shock (Fig. 1a). To determine whether the mere presence of flies in a tube causes emission of the avoidance-promoting substance, flies were gently introduced into a tube using positive phototaxis, and removed after several hours of occupancy. Despite the evident presence in the tube of fly waste, responder flies showed no avoidance response to the tube (Fig. 1a, 'no stress'). This suggests that avoidance is elicited by a substance emitted in response to mechanical or electrical stress. The emission of the substance is not observed when anaesthetized flies are vortexed, indicating that such emission requires neural activity.

Surgical removal of the third antennal segment, which houses the olfactory receptor neurons (ORNs), eliminated the avoidance response (Fig. 1b). By contrast, removal of the aristae or maxillary palps had no effect. These data suggest that the olfactory system mediates the avoidance response. We therefore operationally refer to the substance evoking the avoidance response as *Drosophila* stress odorant (dSO).

Olfactory sensory neurons in the antennae project axons to glomeruli in the antennal lobe. Projection neurons then connect the antennal lobe to the mushroom body and lateral protocerebrum[3]. Conditioned olfactory avoidance responses produced by associative learning require the mushroom body[4], whereas unconditioned avoidance responses to chemical repellents do not[5,6]. We asked whether the mushroom body is required for the avoidance response to dSO, by ablating this structure through hydroxyurea (HU) treatment at a critical stage of development[7]. Alternatively, we have used a mushroom-body-specific Gal4 enhancer trap line to drive the expression of UAS-Shi^{ts}, a dominant-negative mutant of dynamin that inhibits neurotransmitter release at a non-permissive temperature[1]. Neither treatment impaired the avoidance response to dSO (Fig. 1c), although a deficit in olfactory learning confirmed that the lesion was successful (Fig. 1c, lower panel). These data suggest that the avoidance response to dSO does not require brain structures necessary for learned olfactory avoidance.

Analysis of the chemical components of dSO by gas chromatography and mass spectrometry (GC/MS) revealed a small peak of 44 daltons that corresponds to CO_2, which was present in samples of air from conditioned tubes (Fig. 2a, left panel, arrow). Further analysis using a CO_2 respirometer indicated that flies emit about three- to fourfold more CO_2 during shaking than do undisturbed flies (Fig. 2b). By comparison to signals obtained by injecting known amounts of pure CO_2 into the respirometer, the concentration of CO_2 in conditioned tubes was estimated at ~0.2% (data not shown). We next asked whether CO_2 alone evoked avoidance behaviour in a T-maze assay. Flies avoid CO_2 in a dose-dependent manner, at concentrations far below anaesthetic levels (30%) (Fig. 2c; see also Supplementary Fig. S1). A concentration as low as 0.1% above the ambient CO_2 level (0.0376%) evoked a statistically significant avoidance response (performance index, $PI = 29.6 \pm 10.9$, $P < 0.001$ by ANOVA, $n = 3$, see Supplementary Fig. S1). A concentration comparable to that estimated in dSO (~0.2%), although it did elicit significant avoidance, evoked a weaker response than did dSO itself (Fig. 2c, '$+ CO_2$ 0.02 ml' versus 'CS shaken', where 'CS' indicates the wild-type Canton S *Drosophila* strain), suggesting that stressed flies release additional repellent compound(s) together with CO_2.

We analysed the pattern of glomerular activity in the antennal lobe elicited by CO_2, using a calcium-sensitive fluorescent indicator protein (GCaMP) and two-photon microscopy[8]. Electrophysiological recordings have previously identified CO_2-responsive ORNs in the ab1 basiconic sensilla of the antenna[9–11], but the receptor expressed in these neurons, and their projections, have not been established. Experiments in Lepidoptera[12,13] and Diptera[14,15] have traced the glomerular targets innervated by axons from sensilla containing CO_2-responsive ORNs, but these sensilla contain other ORNs as well. Moreover, the studies in Dipteran species have differed with respect to the number and identity of the glomeruli innervated[14,15]. Ca^{2+} imaging permits an analysis of the glomerular activation pattern of CO_2-responsive sensory neurons in the antennal lobe. We first examined flies in which the GCaMP indicator (UAS-GCaMP) is driven in all neurons by the Elav-GAL4 activator. At concentrations of CO_2 up to 10%, only the most ventral pair of glomeruli, the V glomeruli[2], were activated (Fig. 3a). Activation was detected by as little as 0.05% CO_2 (Supplementary Fig. S1). These glomeruli were not activated by any of 26 other odorants tested (data not shown).

We have previously shown[16] that axonal projections to V originate from antennal sensory neurons expressing the candidate gustatory receptor GR21A (Fig. 3d, left). GR21A$^+$ neurons are located in the dorso-medial portion of the antenna (Fig. 3d, right), the region where CO_2-responsive ab1 neurons are positioned in basiconic sensilla[10,11]. Calcium imaging was thus performed with flies in which the UAS-GCaMP reporter was driven by a GR21A promoter-Gal4 activator (Fig. 3b). CO_2 (Fig. 3b), as well as air from a tube conditioned by traumatized flies (Fig. 3c, left), activated GR21A sensory termini in the V glomeruli. Air from a tube that had contained undisturbed flies produced significantly lower levels of

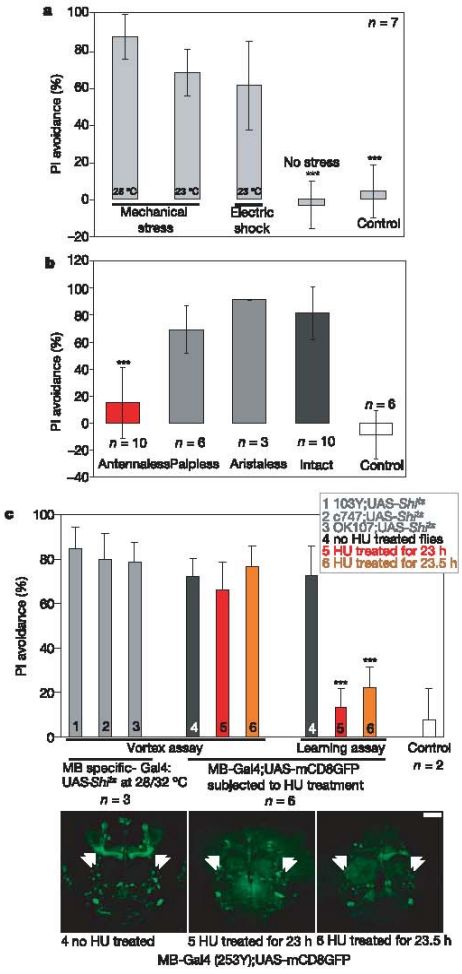

Figure 1 *Drosophila* exhibits innate avoidance of odorants released by stressed flies. **a**, Avoidance of tubes containing air from stressed flies in a T-maze, quantified as the PI (see Methods) of avoidance. The 'No stress' tube was conditioned by flies gently introduced and removed by phototaxis. The 'Control' is an empty tube. Bars indicate the mean + s.e.m. of seven independent experiments. ***, $P < 0.001$ in this and all subsequent figures (ANOVA). **b**, Effect of ablating different sensory organs on dSO avoidance. **c**, dSO-avoidance is MB-independent. Three MB-specific Gal4 lines, 103Y (1; http://www.fly-trap.org), c747 (ref. 29) (2), and OK107 (ref. 29) (3), each crossed with UAS-*Shi*^{ts} and tested at 28 or 32 °C. Flies were also treated with HU (5, 6) or without HU (4) to chemically ablate the MB. The HU ablation prevents classical olfactory avoidance conditioning ('Learning assay'). Fluorescent micrographs indicate successful HU-mediated MB ablation (arrowheads), visualized in 253Y;UAS-mCD8GFP brains. Scale bar, 100 μm. Error bars in **b** and **c** indicate the s.e.m.

activation (Fig. 3c, right). These results indicate that both CO_2 and dSO activate neurons that express the GR21A receptor and project to the V glomerulus.

We next asked whether the GR21A$^+$ sensory neurons are necessary for the avoidance responses to CO_2 and dSO. For this, we employed *Shibire*^{ts} to reversibly inactivate these neurons at increased temperature[1]. Flies bearing either of two independent GR21A-Gal4 insertions and UAS-*Shi*^{ts} no longer exhibited the avoidance response to ~1% CO_2 at a non-permissive temperature (that is, a temperature at which neurotransmitter release cannot occur), but revealed a normal response at a permissive temperature (Fig. 3e, red versus blue bars labelled '2', respectively). Control flies expressing either of the GR21A-Gal4 drivers, but not UAS-*Shi*^{ts}, showed normal CO_2 avoidance at the non-permissive temperature (Fig. 3e). Furthermore, flies expressing UAS-*Shi*^{ts} and either of two Gal4 drivers broadly expressed in other ORNs, but not in GR21A$^+$ neurons (OR83b-Gal4, expressed by ~80% of ORNs, or Or47b-Gal4; L. Vosshall, personal communication), exhibited normal CO_2 avoidance at the non-permissive temperature (Fig. 3e). Similarly, flies expressing UAS-*Shi*^{ts} and another Gal4 driver, GH146-Gal4, which is expressed in about two-thirds of antennal lobe projection neurons[5,17] (but not in those innervating V), also showed robust CO_2 avoidance at the non-permissive temperature (Fig. 3e). These data indicate that GR21A$^+$ sensory neurons that project to the V glomerulus are probably the sole population of ORNs responsive to

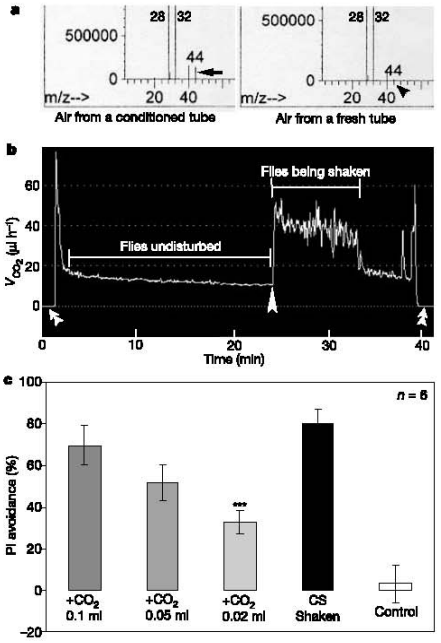

Figure 2 CO_2 is a component of dSO. **a**, Mass spectrometry of air sample derived from ~250 shaken flies. Arrow (44 daltons) indicates CO_2. **b**, Respirometer analysis of CO_2 emission from undisturbed and shaken flies. Double arrowheads indicate the CO_2 level of an unoccupied tube. **c**, Flies avoid CO_2 in a dosage-dependent manner. The indicated volumes of pure CO_2 were infused into a 15-ml tube immediately before the choice tests. 'CS shaken' indicates avoidance PI obtained with tube conditioned by ~70 Canton S flies. 'Control' indicates empty tube. Data represent mean + s.e.m. ($n = 6$).

CO_2, and are required for the avoidance response. Flies expressing UAS-Shi^{ts} in GR21A$^+$ neurons still avoided dSO (Fig. 3e, red bars labelled '1'). Although a reduced response was observed using one of the two driver lines (Fig. 3e, GR21aG4(2);UAS-Shi^{ts}), this reduction did not reach statistical significance. These data support the notion that dSO contains other repellent(s), in addition to CO_2.

To characterize further the neural substrates mediating dSO-responsiveness, we conducted a screen for UAS-Shi^{ts}-dependent defects in dSO-avoidance, using a collection of Gal4 enhancer trap lines (K. Kaiser). A pilot screen of ~250 lines yielded 12 exhibiting reduced dSO avoidance at non-permissive temperatures (G.S.B.S., unpublished work). Several of these dSO-unresponsive lines also exhibited a strong and specific reduction in CO_2 avoidance in subsequent tests, including one designated c761 (Fig. 4a, b). Analysis of the c761 expression pattern revealed that it includes a subset of ORNs in the third antennal segment (Fig. 4c, right), but

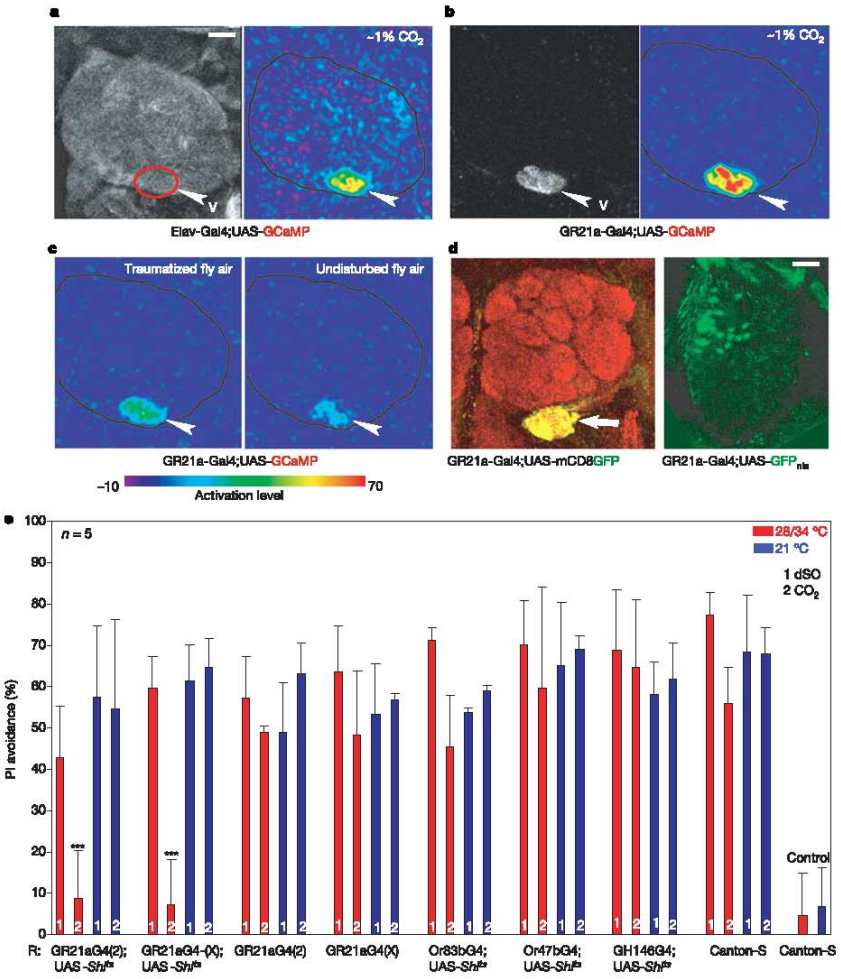

Figure 3 CO_2 avoidance is mediated by ORNs that project to the V glomerulus. **a**, Calcium response to CO_2 imaged using a pan-neuronally expressed GCaMP reporter. Left, prestimulation image; right, ~1% CO_2. Colour scale indicates activation level (red is the highest). Arrowheads in **a**–**c** indicate V glomerulus. Scale bar, 10 μm. **b**, Activation by CO_2 of presynaptic terminals of GR21A$^+$ neurons innervating V glomerulus. **c**, Activation of GR21A$^+$ neurons by dSO (left) versus control (right). **d**, GR21A$^+$ ORNs project to V (arrow). Left, double-labelling of antennal lobe with anti-GFP and mAb nc82 (see also ref. 16). Right, GR21A$^+$ sensory neuron cell bodies in the dorso-medial region of the antenna. Scale bar, 15 μm. **e**, Inhibition of synaptic transmission in GR21A$^+$ neurons using Shi^{ts} blocks CO_2 avoidance. Red and blue bars indicate non-permissive and permissive temperatures, respectively. Bars labelled '1' and '2' are responses to dSO and CO_2, respectively, of flies of the indicated genotypes. ***, $P < 0.001$, by ANOVA. Error bars indicate the s.e.m.

not projection neurons (not shown). That this line is deficient in avoidance of CO_2, as well as of dSO, suggested that these ORNs might include those projecting to V, and others projecting to additional glomeruli. The projections of c761$^+$ neurons to the antennal lobe were consistent with this expectation; labelling was observed both in the V glomerulus, and in several other glomeruli (Fig. 4c, left; Fig. 4d, left). Calcium imaging of c761;UAS-GCaMP flies revealed activation of V by CO_2 (Fig. 4d) as well as by dSO (not shown), confirming expression of this enhancer trap in GR21A$^+$ neurons.

Together, these results indicate that c761 is expressed in, among others, CO_2-responsive sensory neurons that project to V (Fig. 4c). Because c761 was isolated in a screen for dSO-unresponsive lines, these data provide additional evidence that CO_2 is a behaviourally relevant component of dSO. Moreover, the observation that c761 expresses in additional populations of ORNs besides GR21A$^+$ neurons suggests that these ORNs may respond to other active components of dSO.

We have shown that *Drosophila*, when stressed, emits an odorant mixture that elicits avoidance in other flies, and have identified CO_2 as one active component of this mixture. Calcium imaging data suggest that a single population of primary olfactory receptor neurons, which projects to the V glomerulus[16], is activated by CO_2. Specific inhibition of neurotransmission in these GR21A$^+$ sensory neurons, but not in other sensory neurons, abrogates CO_2 avoidance behaviour. Together, these data identify a single population of olfactory sensory neurons that mediates robust avoidance to a naturally occurring odorant, and provide initial insight into the neural circuitry that underlies this innate behaviour.

Many insect species, when stressed or threatened, emit semiochemicals that evoke aggressive or avoidance behaviour in conspecifics[16]. Whether *Drosophila* actually uses dSO to signal stress to conspecifics in the wild, and the conditions under which they might do so, are not yet clear. We have used experimental stimuli such as mechanical agitation and electrical current to elicit release of dSO from *Drosophila*. Although such conditions are artificial, they afford us the ability to maintain tight control over the stimulus and the organism's response, as well as to apply molecular genetic tools to monitor and perturb neural activity. Although these stimuli increase physical and metabolic activity, it is possible that dSO could also be emitted in response to threats.

We have identified CO_2 as one active component of dSO. CO_2 is known to be an important chemical messenger for many insect species[19]. In mosquitoes, for example, CO_2 is an attractant that directs the insect towards warm-blooded animals[20]. At all the concentrations of CO_2 that we tested, we detected only avoidance responses. The different behavioural responses to CO_2 exhibited by mosquitoes and *Drosophila* are likely to reflect hard-wired, species-specific differences in neural circuitry; nevertheless, we cannot exclude that these behavioural differences may be context-dependent. The behavioural and physiologic responses to CO_2 that we have measured in the laboratory are seen at several-fold increases above ambient (~0.0376%) that are well within the range measured for other insects[19]. The role of CO_2 in the ethology and ecology of *Drosophila* remains to be explored, but the highly specific olfactory circuitry revealed by our experiments suggests that it may be important.

Current data suggest that in *Drosophila*, most odorant compounds excite multiple populations of olfactory sensory neurons, each expressing a single olfactory receptor gene. A given odorant will therefore activate multiple glomeruli in the antennal lobe[8,21]. By contrast, our data suggest that a single population of ORNs, and therefore a single glomerulus (V), are involved in sensing and avoiding CO_2. Previous electrophysiological studies identified ORNs uniquely responsive to CO_2, located exclusively in ab1

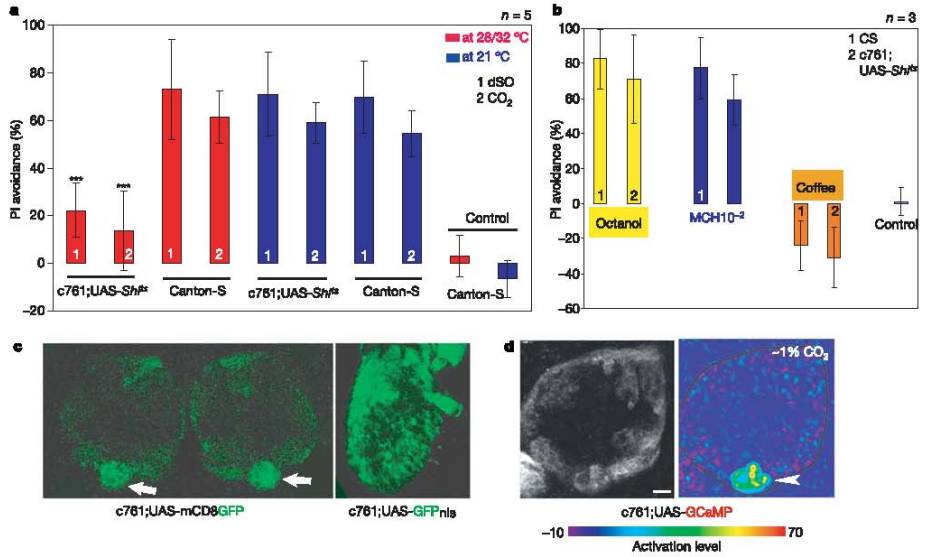

Figure 4 A dSO-unresponsive enhancer trap line is also defective in its CO_2 response. **a**, Response of c761;UAS-*Shi*^{ts} flies to dSO (labelled '1') or CO_2 (labelled '2') at the non-permissive (red bars) and permissive (blue bars) temperatures. ***, $P < 0.001$). **b**, Wild-type (1) and c761;UAS-*Shi*^{ts} (2) flies exhibit equivalent responses to other repellents (yellow and blue bars) and an attractant (orange). **c**, c761-Gal4 is expressed in antennal sensory neurons (right panel, nuclear-GFP reporter) that project to V (arrows), and other glomeruli. **d**, Calcium imaging of CO_2 responses in c761;UAS-GCaMP flies reveals activation in V (arrowhead).

basiconic sensilla[10,11]. These and the present data suggest that CO_2 activates a single population of ORNs, and that these ORNs respond only (or primarily) to CO_2. We cannot exclude, however, that other ORNs, (or antennal lobe projection neurons innervating glomeruli other than V), are also activated by CO_2 at levels below the detection limit of our imaging technology. Nevertheless, previous studies using this method have shown that multiple glomeruli are activated by most odorants tested[8], so it is striking that just a single glomerulus is activated by CO_2. Furthermore, the behavioural response to CO_2 is extinguished by genetic silencing of GR21A$^+$ sensory neurons. The fact that the avoidance response to CO_2 is unaffected by genetic silencing of Or83b or GH146 neurons further suggests that large populations of ORNs and projection neurons not directly innervating V are unnecessary for CO_2 avoidance. Taken together, these data suggest that a dedicated circuit, which involves a single population of ORNs, mediates detection of CO_2 in *Drosophila*. The simplicity of this early-stage olfactory processing offers a great advantage in further tracing the circuits that translate CO_2 detection into an avoidance response.

In general, recognition of many odorants in insects probably requires the decoding of combinatorial patterns of glomerular activation[8,22–24], perhaps combined with complex temporal dynamics in the antennal lobe[25]. Nevertheless, our data suggest that there is also a set of olfactory stimuli, including CO_2, that release innate behaviours by activating a single class of primary sensory neurons and their associated glomerulus. In *Drosophila*, these stimuli may also include mating pheromones which, in other insect species, are known to activate specialized glomeruli[26,27]. In *Caenorhabditis elegans*, the activation of a single chemosensory neuron can elicit a repulsive behaviour[28]. Uni-glomerular circuits dedicated to the detection of certain odorants may have evolved to provide innate behavioural responses to these stimuli, which are essential to survival or reproduction of the species. □

Methods

Flies

Unless otherwise indicated, Canton-S flies (Caltech stock) were used for all experiments. Flies carrying the UAS-*Shi*^{ts} transgenes on the X and third chromosomes were reared at 20 °C. For reversible neuronal silencing, flies were incubated in a 28 °C room for ~90 min and then in a 32° or 34 °C water bath for 5 min, before performing the behavioural experiments at 28 °C. The permissive temperature used in experiments with *Shi*^{ts} was 21 °C. To generate antennaless, palpless or aristaless flies, extirpations were performed using fine tweezers under CO_2 anaesthesia, and the animals were allowed to recover for ~24 h before testing.

Behavioural tests

For behavioural testing, ~40 'responder' and 70 'emitter' adult flies (3- to 6-days post-eclosion, mixed gender) were anaesthetized using CO_2 and sorted into separate vials 24–48 h before use, or were sorted using an aspirator. A 16-inch 15-W fluorescent bulb was horizontally centred on the bench-top, behind the testing apparatus, in an otherwise dark room. Responder flies were transferred into the T-maze by first placing them into a 15-ml plastic tube (Fisher no. 149598), and tapping them into the elevator of the T-maze. While the responders were in the elevator, emitter flies were vortexed in a clean 15-ml tube in 3-s bouts, at 5-s intervals, over a 1-min period. As an alternative method of stressing flies, electric shocks were applied across a copper grid at 60 V (direct current) for 1 min. The emitter flies were discarded, the conditioned tube immediately inserted into one side of the T-maze, a fresh tube being inserted on the other side. The elevator containing responder flies was lowered, and the flies given one minute to choose between the two tubes, after which the elevator was partially lifted to block any further choices, and the number of flies in each tube counted. For testing responses to other odorants, 10 µl of 0.01% octanol, 10 µl of 0.01% methylcyclohexanol (MCH) or 10 µl of 0.3 mg ml^{-1} freshly prepared freeze-dried instant coffee (Taster's Choice) were dripped onto pieces of Whatman filer paper (0.5 × 0.25 in), placed at the end of the 15-ml plastic tube inserted into the T-maze apparatus. The control tube contained filter paper and vehicle alone. The avoidance response was analysed by calculating the PI, that is, the percentage of flies avoiding minus the percentage of flies entering. PI = 0 indicates an equal distribution of flies between the two tubes. PI = 100% indicates that all flies avoided the conditioned tube. Statistical significance was calculated using analysis of variance, ANOVA.

Ca^{2+} imaging and odour delivery

Sample preparation and calcium imaging were as described in ref. 8. CO_2 was diluted to 1% and delivered at a flow rate of 81 ml min^{-1}. dSO and air from non-traumatized flies were prepared as described above, and delivered undiluted.

Gas chromatography and mass spectrometry

Air samples from tubes containing dSO and from fresh tubes were analysed using a gas chromatograph (Agilent 6890) interfaced with a quadruple mass spectrometer (Agilent 5970B). Five microlitres of air from a tube in which 250 flies had been shaken, or from a fresh tube, were injected with a Hamilton syringe into the GC column at 50 °C. The column was then heated to 270 °C at a rate of 10 °C min^{-1} with normal inlet temperature of 250 °C (splitless mode). The GC column was equipped with a column from J&W, DB5-MS, 30 cm × 0.25 mm (i.d.) × 0.25 µm film thickness. Each molecule eluted from the GC column was detected and its molecular mass and abundance measured by the MS. The operating conditions for the MS were:10 to 500 *m/z*; 1.64 scans s^{-1}; ionization energy 70 eV.

Respirometer measurements

Emission of carbon dioxide was measured using a Sable System TR-2 carbon dioxide gas respirometry system (Model LI-6251). Groups of 20 flies were placed in a 2.2-ml glass chamber, which was flushed with a constant flow of CO_2-free air through a CO_2 detector. The amount of CO_2 produced by each group of flies was calculated by using DATACAN software (Sable Systems International).

Visualization of murine CD8GFP

Adult fly brains were dissected, fixed in 2% paraformaldehyde, and mounted in Vectashield (Vecta Labs). Native green fluorescence protein (GFP) fluorescence of whole-mount brains was visualized by confocal microscopy (Leica). Olfactory axonal projections of flies bearing GR21A-Gal4 and UAS-mCD8GFP were visualized by fluorescent immunohistochemistry, as described in ref. 16.

Hydroxyurea treatment

The HU protocol[7] was used to block development of the mushroom body (MB) structure. To check the ablation of the MB, we used the 253Y enhancer trap line carrying UAS-mCD8GFP, which expresses in the MB, as well as in other regions. The survival of the other structures after treatment serves as an internal control for the specificity of HU ablation.

Learning assay

The 'Long Program' training protocol described in ref. 30 was used to train flies. Flies were exposed to 60 s of odour A associated with a 90-V 1.5-s shock delivered every 5 s for 60 s (CS +) followed by 60 s of odor B with no shock (CS −). The trained flies were then given a choice in the T-maze between odours A and B. Both training and testing were done at room temperature (23–25 °C) and humidity (20–50%).

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Correspondence and requests for materials should be addressed to D.J.A. (wuwei@caltech.edu).

A general mechanism for perceptual decision-making in the human brain

H. R. Heekeren[1], S. Marrett[2], P. A. Bandettini[1,2] & L. G. Ungerleider[1]

[1]*Laboratory of Brain and Cognition, NIMH,* [2]*Functional MRI Facility, NIMH, NIH, Bethesda, Maryland 20892-1148, USA*

Findings from single-cell recording studies suggest that a comparison of the outputs of different pools of selectively tuned lower-level sensory neurons may be a general mechanism by which higher-level brain regions compute perceptual decisions. For example, when monkeys must decide whether a noisy field of dots is moving upward or downward, a decision can be formed by computing the difference in responses between lower-level neurons sensitive to upward motion and those sensitive to downward motion[1–4]. Here we use functional magnetic resonance imaging and a categorization task in which subjects decide whether an image presented is a face or a house to test whether a similar mechanism is also at work for more complex decisions in the human brain and, if so, where in the brain this computation might be performed. Activity within the left dorsolateral prefrontal cortex is greater during easy decisions than during difficult decisions, covaries with the difference signal between face- and house-selective regions in the ventral temporal cortex, and predicts behavioural performance in the categorization task. These findings show that even for complex object categories, the comparison of the outputs of different pools of selectively tuned neurons could be a general mechanism by which the human brain computes perceptual decisions.

Consider driving home from work in clear weather. Stopping at a light, you see pedestrians waiting to cross the street. Effortlessly, you decide whether one of them is your spouse, your boss or a stranger, and connect the percept with the appropriate action, so that you will either be waving frantically, greeting respectfully or taking another sip of coffee. During a rainstorm, however, the sensory input is noisier, and thus you have to look longer to gather more sensory data to make a decision about the person at the light and the appropriate behavioural response.

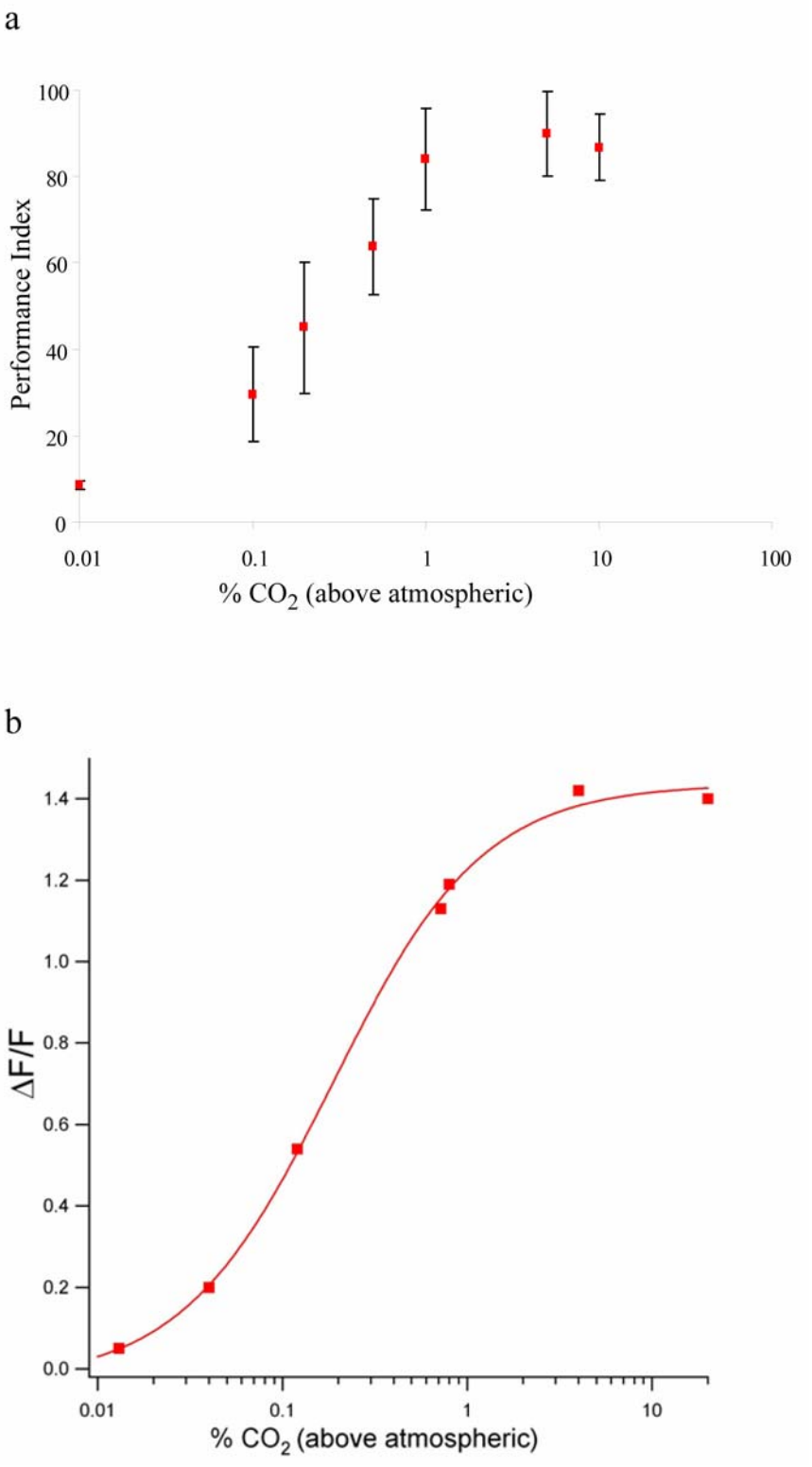
This type of decision-making has been studied in single-unit recording studies in monkeys performing sensory discriminations[5–8]. Shadlen *et al.* proposed that perceptual decisions are made by integrating the difference in spike rates from pools of neurons selectively tuned to different perceptual choices[9]. For example, in a direction-of-motion task, in which the monkey must decide whether a noisy field of dots is moving upward or downward, a decision can be formed by computing the difference in responses between lower-level neurons that are sensitive to upward motion and those sensitive to downward motion[1–4]. Similarly, in a somatosensory task, in which the monkey must decide which of two vibratory stimuli has a higher frequency, a decision can be formed by subtracting the activities of two populations of sensory neurons that prefer low and high frequencies, respectively[8,10]. These findings suggest that a comparison of the outputs of different pools of selectively tuned lower-level sensory neurons could be a general mechanism by which higher-level cortical regions compute perceptual decisions[1,2,11]. However, it is still unknown whether such a mechanism is at work for more complex cognitive operations in the human brain and, if so, where in the brain this computation might be performed.

We used functional magnetic resonance imaging (fMRI) while subjects decided whether an image presented on a screen was a face or a house (Fig. 1). Previous neuroimaging studies have identified regions in the human ventral temporal cortex that are activated more by faces than by houses, and vice versa[12–16]. Increases in the blood-oxygen-level-dependent (BOLD) signal have been shown to be proportional to changes in neuronal activity in a given region[17,18]. Therefore larger BOLD responses to faces than to houses and vice versa in specific voxels in the ventral temporal cortex reflect the change in activity in a population of neurons that are more responsive to faces than to houses, and vice versa. Our task thus enabled us to identify two brain regions, one more sensitive to faces and another to houses, and to test whether there are higher-level cortical regions whose output is proportional to the difference in activation in the face- and house-selective regions, respectively.

We based our hypotheses on results from single-unit recording studies in monkeys, which have shown that neuronal activity in areas involved in decision-making gradually increases and then remains elevated until a response is given, with the rate of increase being slower during more difficult trials[1,2]. These studies have also shown that higher-level cortical regions, such as the dorsolateral prefrontal cortex (DLPFC), might form a decision by comparing the output of pools of selectively tuned lower-level sensory neurons[4,9]. Therefore, we hypothesized that higher-level cortical regions computing a decision would have to fulfil two conditions. First, they should show the greatest activity on trials in which the evidence for a given perceptual category is greatest, for example, a greater fMRI response during decisions about suprathreshold images of faces and houses than during decisions about perithreshold images of these stimuli. Second, their activity should be correlated with the difference between the output signals of the two brain regions containing pools of selectively tuned lower-level sensory neurons involved; that is, those in face- and house-responsive regions.

To test the model of decision-making, we added noise to the face and house stimuli, which made the task arbitrarily more difficult by reducing the sensory evidence available to the subject (Fig. 1b). In the fMRI experiment, subjects viewed images that were either easy (suprathreshold, Fig. 1b top) or difficult (perithreshold, Fig. 1b bottom) to identify as faces or houses.

Supplementary Figure 1 (S1)



Supplementary Figure Legend 1

(a) Dose-response curve for avoidance behavior to CO_2. Flies were sorted without the use of CO_2 anaesthesia for this experiment. Each point represents the mean±SEM of n=3 determinations, 50 flies per determination. (b) Dose-response curve for activation ($\Delta F/F$) of GR21A+ neurons to CO_2 , using the UAS-GCaMP reporter.