Title
A Mechanism for Behavioral Cross-Inhibition in Drosophila

Author
Mann, Kevin

Publication Date
2013

Peer reviewed|Thesis/dissertation

A Mechanism for Behavioral Cross-Inhibition in *Drosophila*

By

Kevin Mann

A dissertation submitted in partial satisfaction of the

requirements for the degree of

Doctor of Philosophy

in

Neuroscience

in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:

Professor Kristin Scott, Chair
Professor Daniel Feldman
Professor Ehud Isacoff
Professor David Bilder

Fall 2013

Abstract

A Mechanism for Behavioral Cross-Inhibition in *Drosophila*

By

Kevin Mann

Doctor of Philosophy in Neuroscience

University of California, Berkeley

Professor Kristin Scott, Chair

How animals generate stable, appropriate decisions in a noisy sensory environment is a fundamental question in neuroscience. Animals must correctly appraise the sensory world, taking into account their internal state and previous experiences. Feeding behavior in the fruit fly, *Drosophila melanogaster,* is an ideal model system to investigate the neural basis of decision-making. While feeding initiation in *Drosophila* is mostly described as the simple product of the palatability of a given substance, this behavioral decision is actually quite complex and is influenced by sensory stimuli, internal states, prior encounters, and other factors.

As with other animals, feeding behavior in the fruit fly is likely influenced by the commitment to other behaviors, as the engagement in one behavior typically suppresses the initiation of others. The first part of this thesis describes the interaction between two mutually exclusive behaviors in the fly, feeding and locomotion. This study characterizes a single pair of interneurons in the nerve cord of the fly that mediates the suppression of feeding initiation by locomotion. These neurons are activated by mechanosensory input from the legs of the fly and suppress feeding initiation through the inhibition of the proboscis extension response. This work shows that these behaviors are indeed mutually exclusive and identifies a single pair of neurons that mediates part of this interaction.

The second part of this thesis attempts to place these proboscis extension-inhibiting neurons in the larger context of the feeding circuit. Anatomical, functional, and behavioral experiments identify potential synaptic partners with these neurons as well as other, as of yet unidentified, neurons that modulate feeding initiation in the fly. This work identifies candidate neurons that influence feeding decisions in *Drosophila*, and provides a starting point for the characterization of neurons in the *Drosophila* feeding circuit.

TABLE OF CONTENTS

Chapter 4

CHAPTER 1

Introduction

Introduction

The fitness of any animal depends on its capacity to make appropriate decisions in response to a changing and often dangerous environment. As the consumption of toxic compounds or the failure to avoid predators will result in an untimely end, animals have evolved a nervous system that detects relevant sensory stimuli and generates appropriate behavioral responses.

The decision to engage in a behavior is not made in a vacuum. Multiple stimuli promoting different decisions are often present simultaneously when an animal makes a decision. Likewise, once an animal has initiated a behavior, it behooves it to ignore non-salient stimuli. In 1905, Charles Sherrington stated the concept that the selection of one behavior may result in the inhibition of other behaviors:

> "we recognize that the usual thing in nature is not for one exciting stimulus to begin immediately after another ceases, but for an array of environmental agents acting concurrently on the animal at any moment to exhibit correlative change in regard to it, so that one or other group of them becomes-generally by increase in intensity-temporarily proponent. Thus, there dominates now this group, now that group in turn. ...some reflexes, as mentioned above, are antagonistic one to another and incompatible. These do not mutually reinforce, but stand to each other in inhibitory relation. One of them inhibits the other, or a whole group of others."

Sherrington, The integrative action of the nervous system, Yale Press, Lecture IV, p. 118. This work put forth two important notions: behaviors may be exclusive and this exclusivity may be achieved by cross-inhibition of incompatible behaviors.

Advances in our understanding of how behavioral exclusivity is achieved have come from studies of organisms with simple nervous systems and robust behaviors. Invertebrates such as sea slugs, leeches and crayfish have all been used as model organisms to study the neural basis of behavioral choice. Animals such as these possess nervous systems orders of magnitude smaller than that of a mammal; for example, there are 10,000 neurons in the leech versus tens of millions in a mouse. These simple organisms still exhibit rich behavioral repertoires, including predation, escape, and reproduction. These factors allow the reliable recording and manipulation of neurons while monitoring behavioral readouts. Significant progress has been made in this field identifying the different mechanisms of behavioral choice in different invertebrate species.

Behavioral hierarchies

Decision-making experiments in invertebrates determined that when presented with opposing stimuli, certain behaviors were more likely to occur than others. These works have shown that not only do certain behaviors inhibit the expression of others, but that they are also arranged in hierarchies.

Experiments performed in the sea slug *Pleurobranchaea*, in which opposing stimuli were delivered simultaneously to a freely behaving animal, demonstrated that feeding behaviors suppress the initiation of other behaviors (Davis et al., 1974).

In these experiments, food was presented to the slug during various behavioral manipulations that normally elicit other behaviors. If the animal was inverted (a manipulation which typically results in righting of the animal) during the application of food, the slug fed and did not right itself, sometimes for as long as an hour. Tactile stimulation of the oral veil typically results in its retraction, but if food was present during tactile stimulation, feeding occurred and retraction of the oral veil was suppressed. The presentation of a conspecific, which normally elicits mating behavior, simultaneously with food resulted in feeding behavior. Similar dominance was observed when the slug was already engaged in non-feeding behaviors. A mating slug would cease to mate when presented with food. Therefore, these behaviors exhibit hierarchical expression, with certain behaviors dominating others, even in cases when opposing stimuli are present.

Behavioral hierarchies exist in other invertebrates, suggesting that it is a common strategy in decision-making. Work in the leech *Hirudo* also identified feeding as a dominant behavior (Misell et al., 1998). In these experiments, leeches were allowed to feed while electrical stimuli were applied to the animals' bodies. Electrical stimulation of the anterior portion of the leech typically resulted in shortening of the body, whereas stimulation of the posterior portion produced either crawling or swimming behavior. When the leech was feeding, however, the stimulation threshold required to elicit shortening or swimming was two to three fold greater, indicating that feeding dominates these behaviors in the leech as it does in the sea slug.

These experiments demonstrate that animals exhibit hierarchies in which certain behaviors are more likely to be initiated when the animal is presented with multiple stimuli, with the engagement in these dominant behaviors further suppressing the initiation of others.

Investigation into the neural basis of behavioral hierarchies have led to two different models for decision-making, cross-inhibition of a small number of neurons that exclusively drive specific behaviors, or the reconfiguration of circuitry shared by multiple behaviors.

Cross-inhibition of command neurons

Command neurons are single neurons whose activation can elicit the expression of coordinated behavior. They were first described in the crayfish over fifty years ago, and have since been described in sea slugs, leeches, and even fruit flies. The observations that the activity of a few neurons can generate whole behavioral repertoires led to a model of decision-making in which the competition among these neurons ultimately leads to a behavioral choice. In this cross-inhibition of command neurons model, the activation of a command neuron for one behavior leads to the inhibition of command neurons for other behaviors.

In order to test this model, the interactions between feeding and withdrawal in the sea slug were examined by recording from withdrawal neurons during the stimulation of feeding behavior (Kovac and Davis, 1977). Using partially dissected preparations, feeding activity was initiated by stimulation of the stomatogastric nerve, and withdrawal was measured by activity in the large oral veil

nerve (LOVN), which contains motor neurons that drive withdrawal. Consistent with the previously described hierarchy (Davis et al., 1974), feeding activity suppressed output from the LOVN during tactile stimulation of the oral veil. Recordings from neurons in the withdrawal circuit identified a pair of neurons, the corollary discharge neurons (CD), which were activated upon feeding, and whose activity suppresses withdrawal. Therefore, the feeding circuit in the sea slug directly inhibits the withdrawal circuit through the activation of these CD neurons.

Similar results have been observed in other behavioral decisions in the sea slug. Active swimming can suppress feeding behavior as part of an escape response (Davis and Gillette, 1978). To determine how this occurs, different parts of the swimming motor program were stimulated during feeding, while noting any effect on withdrawal (Jing and Gillette, 1995). In these experiments, it was discovered that an element of the swimming motor circuit, a pair of neurons known as the A1 neurons, which was active during swimming, could directly induce withdrawal of the mouthparts even when the animal was feeding.

Studies in different species have also identified cross-inhibition as a possible mechanism for decision-making, suggesting that this is a common strategy utilized by invertebrates. In the crayfish *Procambarus clarkii*, the engagement in feeding alters the sensitivity of the lateral giant neurons to mechanical stimulation through reduced synaptic transmission from unknown interneurons, suppressing escape behavior (Bellman and Krasne, 1983; Krasne and Lee, 1988). Taken together, these experiments in the sea slug and crayfish have shown that the mutual inhibition of different circuit elements does appear to drive behavioral choice.

This simple model provides an attractive explanation of decision-making, but further studies have revealed that many decisions are likely not made through the interactions of single neurons, but through the distributed activity of many neurons in a network.

Network reconfiguration

An alternative model for how behavioral choice is achieved is that behaviors are generated by largely overlapping populations of active cells and that the dynamics of activation or the activity of the entire ensemble dictates behavioral selection (Briggman and Kristan, 2008). As neurons cannot simultaneously be recruited into two network states, this would allow for selection of one behavior over another. As described below, both the identification of putative command neurons that participate in multiple behaviors and the examination of population activity during two behaviors supports the model that reconfiguration of networks is another mechanism that allows for behavioral choice.

If competition between command neurons produces behavioral decisions, this would imply that a single (or a few) dedicated command neuron(s) would be active during one behavior and remain quiescent during all other behaviors. This is not always the case, as some previously identified command neurons are active in multiple behaviors.

Four known swimming command neurons were recorded from in the leech while eliciting the shortening response via mechanical stimulation of the animal's

head (Shaw and Kristan, 1997). If the inhibition between command neurons model is correct, the induction of shortening (a dominant behavior) should suppress command neurons for swimming. Remarkably, the induction of shortening behavior activated three of the four swimming command neurons tested, as well as several cells that are part of the central pattern generator for swimming. This work suggests that although activation of these command neurons can drive swimming, the neurons are also active in other contexts and thus their activity cannot dictate the decision of whether to swim or shorten. Instead, shortening recruits significant portions of the swim network and reconfigures its activity to produce the behavior.

To further test the reconfiguration of networks hypothesis, voltage sensitive dyes were applied to a large number of neurons in isolated preparations of the leech nervous system (Briggman et al., 2005). A stimulus was delivered that evoked swimming half of the time and crawling the other half, as measured from motor neuron activity. By monitoring 130-150 neurons simultaneously during stimulus delivery, it was possible to view the activity of almost every neuron in a ganglion during a behavioral choice. This experiment identified a small number of neurons whose activity preceded either behavior, suggesting that they were responsible for the ultimate decision. These neurons were then manipulated to determine if they could influence the choice between swimming or crawling. Of the fifty neurons tested, none of them could elicit a behavior alone, and only one of them could bias this decision during stimulation. This work suggests that rather than a few, dedicated command neurons, the activity of significant portions of the network participates in the ultimate decision to swim or crawl.

Monitoring activity in these neurons during the execution of the chosen behavior also revealed a significant overlap, with some 93% of swimming neurons also active during crawling. The patterns of oscillation differed between the two states, indicating that the overall circuitry between these two behaviors is largely shared, and network activity is reconfigured to produce different behaviors (Briggman and Kristan, 2006).

Overall, these studies showed that the activity of a population of neurons, rather than competition between command neurons, is an additional strategy used by nervous systems to generate behavioral decisions.

Feeding in *Drosophila*

Further investigation of the neural basis underlying decision-making is needed in order to determine if these mechanisms of behavioral choice apply to other systems**.** Feeding behavior in the fruit fly *Drosophila melanogaster* is an ideal system for further study of decision-making in invertebrates. The fly nervous system is intermediate in complexity between other invertebrate models such as the leech and vertebrate models such as the mouse, with approximately 100,000 neurons in the fly brain. More importantly, well-established molecular genetic approaches such as the Gal4-UAS, LexA-LexAop, and QF-QUAS binary expression systems, combined with the relative ease with which transgenic animals can be produced, enable the selective expression of transgenes in discrete cell populations in the adult nervous system (Brand and Perrimon, 1993; Lai and Lee, 2006; Potter et

al., 2010). Multiple neuronal silencers and activators have been developed in *Drosophila*, such as tetanus toxin, which prevents synaptic vesicle release, a temperature sensitive allele of dynamin (shibiri^{ts}), which blocks synaptic vesicle reuptake, Kir2.1, a potassium channel which prevents cell depolarization, dTRPA1, which depolarizes cells in response to heat, and channelrhodopsin-2, which depolarizes cells in response to blue light (Baines et al., 2001; Hamada et al., 2008; Kitamoto, 2001; Nagel et al., 2003; Sweeney et al., 1995). These tools allow for the selective manipulation of populations of neurons in the fly brain.

Feeding is executed by a stereotyped series of behaviors in the fly, consisting of foraging, identification of food, extension of the proboscis and ingestion. This process begins with the initiation of foraging. When flies become depleted of food reserves, they increase locomotion, seeking out new sources of food guided by olfactory cues (Dethier, 1976). Upon arrival at a food source and subsequent stimulation of gustatory sensory neurons with an appetitive substance such as sucrose, the fly will stop moving, bend its legs, and extend its proboscis in an attempt to feed. The proboscis extension response is a simple and quantitative readout of taste detection (Edgecomb et al., 1994). Flies will increase the probability of extension as a function of sugar concentration and will decrease extension when bitter compounds are included. Similarly, hunger and satiety influence the probability of extension. After making contact with a food source, the fly will ingest food until it reaches a state of satiety (Dethier, 1976; Manzo et al., 2012).

Taste detection in *Drosophila*

Drosophila detects potential food sources with gustatory bristles on the proboscis, internal mouthparts, legs, wings, and genitals (Stocker, 1994). These bristles contain two to four gustatory neurons that respond to nonvolatile chemicals in the environment. These sensory neurons are located at the base of the gustatory bristle and send a dendrite into the shaft of the bristle where it comes into contact with chemicals in food sources (Falk et al., 1976). Electrophysiological recordings of gustatory bristles indicate that different classes of neurons in a bristle respond selectively to either bitter compounds, sugars, salt, or water (Fujishiro et al., 1984; Hiroi et al., 2004; Meunier et al., 2003).

Work over the past decade has made significant progress in understanding how different taste compounds are detected in the periphery. One class of taste receptors in the fly is the Gustatory Receptor (GR) family containing 68 genes (Clyne et al., 2000; Robertson et al., 2003; Scott et al., 2001). GRs are novel hydrophobic proteins that show sequence similarity to *Drosophila* odor receptors, and are found only in insects and *C elegans*. Many GRs participate in the detection of sugars and bitter compounds (Chyb et al., 2003; Dahanukar et al., 2007; Jiao et al., 2007; 2008; Lee et al., 2009; Slone et al., 2007; Weiss et al., 2011).

GRs are segregated into two different cells underneath a chemosensory bristle on the proboscis, one cell that detects many different sugars and another that detects many bitter compounds (Marella et al., 2006; Thorne et al., 2004). A second class of taste receptors is encoded by a few members of the Pickpocket (PPK) ion

channel family (Adams et al., 1998; Zelle et al., 2013), the *Drosophila* version of the Epithelial sodium channel/Degenerin ion channel family. There are 30 PPK genes and four have been found to participate in chemosensory detection: PPK28 acts as an osmosensitive ion channel and detects water (Cameron et al., 2010; Chen et al., 2010) and PPK23, PPK25 and PPK29 participate in contact pheromone detection (Liu et al., 2012; Lu et al., 2012; Thistle et al., 2012; Toda et al., 2012). PPK28 is expressed in one cell underneath each proboscis chemosensory bristle and PPK23/PPK29 are expressed in a second cell. Thus, receptors and molecular markers have been identified for the four cells underneath a chemosensory bristle. These studies suggest that the four taste modalities in the fly are sweet, bitter, water and pheromones.

Interestingly, recent studies have identified a third family of receptors that is also expressed in gustatory neurons. The ionotropic glutamate receptor (IGluR) family contains 61 genes and many are expressed in olfactory and gustatory neurons (Benton et al., 2009; Cameron, unpublished). One member of this family, IR76B, has recently been shown to be expressed in gustatory neurons on the proboscis and be necessary for low salt attraction (Zhang et al., 2013). How the expression pattern of this gene fits in with the expression of other receptors is not resolved.

Overall, the organization of taste detection in the fly periphery is very similar to what is seen in mammalian taste (Yarmolinsky et al., 2009). In both cases, there are just a few different categories of taste cells and activation of these cells drives innate acceptance or rejection behavior. This organization allows for the detection of many compounds (by having many receptors) to drive the execution of a few innate behaviors (by having the activation of different cells tethered to different behaviors).

Central circuits that process gustatory cues

How taste information from the periphery is processed in the central nervous system is just beginning to be elucidated. Gustatory receptor neurons from the wings project their axons to the ventral nerve cord (VNC), gustatory neurons from the legs project to the VNC and the subesophageal ganglia (SOG) of the fly brain, and gustatory neurons from the proboscis and mouthparts send axons to the SOG. In both in the SOG and in the ventral nerve cord, taste projections are segregated based on taste modality (Rajashekhar and Singh, 1994a; Stocker and Schorderet, 1981; Thorne et al., 2004; Wang et al., 2004; Kim, unpublished).

How taste information from the SOG is processed to lead to feeding behavior is currently being investigated. The SOG is a complex brain region containing approximately 4,000 neurons. It is not a dedicated taste relay but instead acts as a major conduit between the VNC and the central brain. Although the function of most neurons in the SOG is unknown, work from Rajashekhar and Singh showed that the cell bodies and dendritic arborizations of the motorneurons (MNs) involved in proboscis extension and feeding reside in the SOG (Rajashekhar and Singh, 1994b). There are 12 pairs of muscles involved in proboscis extension, each innervated by 1-3 MNs. Molecular genetic approaches have been used to label and manipulate the

functions of single SOG motorneurons (Gordon and Scott, 2009; Manzo et al., 2012). These studies have revealed a pair of MNs that innervate the protractor muscle of the rostrum, drive proboscis extension and are activated by sugars and inhibited by the inclusion of bitter compounds (Gordon and Scott, 2009). In addition, three pairs of MNs innervate muscle 11 and 12 and mediate pumping of the cibarial organ during ingestion (Manzo et al., 2012). These studies uncovered the function of MNs involved in feeding. The observation that both sensory and motor neurons for feeding reside in the SOG suggests that there may be local circuits in the SOG that process gustatory cues from taste detection to behavior.

Only a few additional components of taste circuits have been identified. An intriguing recent study identified an SOG local interneuron that may act as a command neuron for feeding. This neuron is activated by sugar only when the animal is starved and activating a Gal4 line containing this neuron results in increased proboscis extension and feeding behavior (Flood et al., 2013). Although the authors proposed a command function for this neuron, more studies will be required to determine whether this single neuron drives the entire program for feeding behavior.

No other direct components of taste pathways have been identified, although many modulators that regulate feeding have been described (see below). In most cases, broadly-expressed Gal4 lines labeling neuropeptide/neuropeptide receptors have been used to study the function of these modulatory systems in feeding. Only in one case has the site of modulation been mapped with cellular resolution to the causal cells in the Gal4 line (Marella et al., 2012). This study showed that dopaminergic neurons promote feeding and that activation of a single dopaminergic neuron in the SOG elicits proboscis extension. This neuron arborizes extensively in the SOG, well-positioned to modulate feeding. Interestingly, the activity of this neuron varied with satiety state, with high activity when animals were food-deprived and low activity when they were sated. This argues that this dopaminergic neuron translates a global signal of hunger into a local change in the activity of feeding circuits via dopaminergic modulation.

While the architecture of the taste circuit in *Drosophila* is still being elucidated, these data suggest that the SOG is a region in the fly brain responsible for the production of feeding behaviors. All of these elements of the taste circuit generate a relatively simple behavior that is tightly regulated by many factors, making it an excellent system for the study of behavioral modulation.

Modulation of feeding behavior in *Drosophila*

Feeding behavior in the fly is regulated by more than just the palatability of substances. Nutritional state, circadian rhythms and previous experiences all influence the probability of feeding.

Nutritional state alters feeding behavior through long-term metabolic changes, as well as short-term changes in neuronal activity. The neuropeptides *Drosophila* insulin-like peptide (DILP) and adipokinetic hormone (AKH) act in a manner similar to mammalian insulin and glucagon, respectively, and function to maintain sugar homeostasis in insects (Ikeya et al., 2002; Kim and Rulifson, 2004;

Noyes et al., 1995; Wu et al., 2005). Several of the DILP genes show greater expression in fed animals compared to starved ones and overexpression of DILPs in the nervous system of fly larvae is sufficient to suppress hunger-induced behaviors (Ikeya et al., 2002; Wu et al., 2005). Conversely, AKH is released when the animals are starved and deletion of the AKH receptor increases hunger induced feeding behaviors (Bharucha et al., 2008; Kim and Rulifson, 2004). Similarly, the targeted ablation of AKH-producing cells suppresses behaviors associated with starvation such as increased foraging (Lee and Park, 2004). As manipulations of DILP and AKH peptides alter metabolism in the fly, with AKH promoting the release of energy stores and DILPs promoting a reduction in circulating hemolymph sugar, these neuropeptides do not likely alter feeding behavior directly, but rather operate on longer time scales (hours to days) via alterations in circulating nutrients (Kim and Rulifson, 2004; Rulifson et al., 2002).

Several classes of neurons have also been identified that respond to metabolic changes, translating nutritional state into neural activity to alter behavior on shorter time scales. The neuropeptide allatostatin A (AstA) suppresses feeding behavior in flies in a manner similar to DILP overexpression, but without altering metabolism or energy reserves, suggesting that AstA-expressing cells do not influence metabolic states, but sense them. In line with this assumption, acutely activating AstA neurons using dTRPA1 suppressed feeding behavior on the time course of seconds, suggesting that they act downstream from DILP or AKH manipulations (Hergarden et al., 2012). The neuropeptide leucokinin also suppresses feeding behaviors in the fly by limiting the size of meals that the fly consumes (Al-Anzi et al., 2010). As deletion of either the gene for leucokinin or for the leucokinin receptor resulted in increased meal size, but not overall caloric intake, leucokinin does not alter nutrient states in the fly. Rather, it likely responds to acute feeding signals from the gut, as leucokinin-expressing neurons innervate the foregut of the fly (Al-Anzi et al., 2010). As noted above, dopamine also acutely affects feeding behavior and a single dopaminergic cell in the SOG of the fly promotes feeding initiation within seconds of activation (Marella et al., 2012). This neuron, TH-VUM, elevates its basal firing rate in response to starvation. Therefore, rather than altering nutrient homeostasis, AstA and dopamine likely respond to satiety signals, or gut stretch in the case of leucokinin, to acutely modulate feeding behaviors.

Feeding behavior is also modulated by circadian rhythms (Chatterjee et al., 2010; Xu et al., 2008). Eliminating circadian rhythms in the fat body of the fly, a tissue analogous to the mammalian liver (Scott et al., 2004), promotes feeding behavior, likely through reduced energy stores (Xu et al., 2008). Similarly, circadian clocks in the gustatory receptor neurons themselves promote increased proboscis extension and sensitivity of gustatory receptor neurons to sucrose during the daytime. Therefore, circadian rhythms likely affect feeding behaviors both through alterations in metabolic stores as well as through sensitivity in primary sensory neurons to tastants.

A fly's previous experience also modulates feeding behavior by changing the probability of proboscis extension (Masek and Scott, 2010; Kirkhart, unpublished). Flies that have been stimulated on their legs with an appetitive substance, but

subsequently given a bitter substance on the proboscis, learn to stop extending the proboscis to sugar. This modulation can occur with one pairing of sugar and bitter and learning is of short duration, decaying in minutes. The gamma lobes of the mushroom bodies, a structure in the fly brain required for short-term olfactory memory formation, is required (Heisenberg, 2003). Thus, feeding behavior is influenced by the fly's memory and modulation of proboscis extension occurs via the same structure involved in olfactory memory formation.

While it well established that feeding behavior is tightly modulated by both gustatory and non-gustatory cues, the influence of other behaviors on feeding is unknown. Do feeding behaviors suppress the initiation of other behaviors? Do other behaviors, in turn, suppress feeding initiation? While feeding decisions are influenced by the engagement in other behaviors in other invertebrates, it is unclear if and how other behaviors modulate feeding in *Drosophila*.

In this thesis, I investigated the modulation of proboscis extension by neurons in the Gal4 line E564. I identified a single pair of neurons that arborize in the ventral nerve cord and SOG that elicit constitutive proboscis extension when they are silenced. Monitoring the activity of these neurons in a variety of conditions revealed that these neurons do not modulate feeding behavior in response to nutritional state or gustatory stimulation. Remarkably, they are activated by movement of the legs, and suppress proboscis extension when the animal is moving. Additionally, I determined that engagement in proboscis extension suppresses locomotion, demonstrating that these two behaviors are mutually exclusive. This work has identified a novel mechanism for the modulation of feeding behavior in *Drosophila*, the suppression of proboscis extension by locomotion, and identified interneurons that participate in behavioral choice.

CHAPTER 2

A pair of interneurons influences the choice between feeding and locomotion in *Drosophila*

Summary

The decision to engage in one behavior often precludes the selection of others, suggesting cross-inhibition between incompatible behaviors. For example, the likelihood to initiate feeding might be influenced by an animal's commitment to other behaviors. Here, we examine the modulation of feeding behavior in the fruit fly, *Drosophila melanogaster*, and identify a pair of interneurons in the ventral nerve cord that is activated by stimulation of mechanosensory neurons and inhibits feeding initiation, suggesting that these neurons suppress feeding while the fly is walking. Conversely, inhibiting activity in these neurons promotes feeding initiation and inhibits locomotion. These studies demonstrate the mutual exclusivity between locomotion and feeding initiation in the fly, isolate interneurons that influence this behavioral choice, and provide a framework for studying the neural basis for behavioral exclusivity in *Drosophila*.

Introduction

Sensory stimuli promoting different behaviors are often present simultaneously in the environment. An animal must evaluate these cues in the context of internal physiological state and prior experience to select one behavior and exclude others. How animals assess their environment to generate behavioral decisions and allow for behavioral exclusivity is not well understood.

Studies of decision-making in invertebrates argue that behavioral choice is in part guided by the ability of one behavior to suppress the initiation of others. In the sea slug, *Pleurobranchaea californica*, and in the medicinal leech, *Hirudo verbena,* behaviors are ranked in a hierarchy and the selection of one behavior inhibits others (Kristan and Gillette, 2007). In a few cases, this inhibition occurs by interactions between command neurons for different behaviors. For example, in *Pleurobranchaea*, swimming is dominant over feeding and interactions between command neurons for swimming and feeding generate behavioral selection (Jing and Gillette, 1995; 1999). More commonly, behavioral selection occurs in distributed networks. Swimming and crawling are mutually exclusive behaviors in the leech that are executed by reconfiguration of partially shared circuitry (Briggman et al., 2005; Briggman and Kristan, 2006). In addition, the multiple pathways that inhibit swimming while the leech is feeding provide evidence for distributed decisions, with ingestion altering the response of sensory neurons to mechanosensory stimuli (Gaudry and Kristan, 2009) and gut distension inhibiting swimming downstream of sensory neurons (Gaudry and Kristan, 2010). These studies suggest that behavioral exclusivity can be achieved by distributed networks, but the generality of these findings remains to be explored.

Feeding decisions in the fruit fly, *Drosophila melanogaster*, afford an excellent opportunity to examine the hierarchy of behavioral decisions in a genetically tractable model. The relative simplicity of the fly brain with 100,000 neurons, as well as the molecular genetic approaches available in the fly to selectively manipulate identified neurons and examine the effect on animal behavior, provides a powerful platform to study the neural basis of behavioral exclusivity. In *Drosophila*, feeding behavior begins with detection of taste compounds on the legs or proboscis, resulting in proboscis extension and feeding initiation (Edgecomb et al., 1994). The probability that an animal performs the proboscis extension response (PER) is influenced by the palatability of the taste compound, the energy requirements of the animal and previous associations (Dethier, 1976; Inagaki et al., 2012; Marella et al., 2012; Masek and Scott, 2010).

The neural circuits for proboscis extension and feeding are just beginning to be elucidated. Chemosensory neurons on the legs, proboscis and mouthparts are modality-selective, detecting sugars, bitter compounds, water, or pheromones (Cameron et al., 2010; Chen et al., 2010; Lu et al., 2012; Marella et al., 2006; Thistle et al., 2012; Thorne et al., 2004; Toda et al., 2012; Wang et al., 2004). Sensory neurons from the legs project to the ventral nerve cord (VNC) and subesophageal ganglion (SOG) of the fly brain whereas those from the proboscis and mouthparts project to the SOG (Stocker, 1994; Wang et al., 2004). Motor neurons that drive

proboscis extension as well as modulatory neurons that influence proboscis extension are also found in the SOG (Gordon and Scott, 2009; Manzo et al., 2012; Marella et al., 2012; Rajashekhar and Singh, 1994b), suggesting that the SOG contains local circuits that process gustatory cues from detection to behavior. Whether the circuits that control proboscis extension are influenced by other behaviors or influence the probability of other behaviors has not been examined.

Here, we describe a pair of interneurons in *Drosophila* that is activated upon stimulation of mechanosensory neurons and inhibits feeding initiation, suggesting that these neurons suppress feeding while the animal is walking. Conversely, when the neurons are inhibited, the animal continuously engages in feeding initiation at the expense of locomotion. Thus, our studies suggest that feeding initiation and locomotion are mutually exclusive behaviors and identify neurons that participate in the coordination of this behavioral choice.

Results

Neurons in the *E564-Gal4* line inhibit proboscis extension

We previously performed a behavioral screen that identified Gal4 lines with proboscis extension defects (Gordon and Scott, 2009). In this study, we examined the proboscis extension phenotype associated with the Gal4 line, *E564-Gal4*, and its neural basis. E564 neurons were chronically silenced in the adult fly by expressing the inward-rectifying potassium channel Kir2.1 under the control of the *E564-Gal4* driver (Baines et al., 2001). The temperature-sensitive Gal4 repressor Gal80^{ts} was utilized to restrict Kir2.1 expression to the adult stage upon a temperature shift (McGuire et al., 2004). Nearly 100% of flies with chronically silenced E564 neurons exhibited constitutive proboscis extension (Figure 2.1AB). This phenotype was completely absent in genetically identical flies without Kir2.1 induction and *E564-Gal4* controls and nearly absent in *UAS-Kir2.1, tub-Gal80^{ts}* controls (Figure 2.1B). Acute silencing of E564 neurons, using a temperature-sensitive shibire^{ts} (Shi^{ts}) (Kitamoto, 2001) that acts on the timescale of minutes to prevent synaptic vesicle reuptake at elevated temperature, promoted spontaneous proboscis extensions and retractions rather than constitutive extension and greatly enhanced sucrose-induced responses (Figure 2.1BC). Although the two neural inactivation strategies act on different timescales (days for Kir2.1 and minutes for Shi^{ts}), act by different means (prevent depolarization or prevent endocytosis) and may also differ based on copy number, the behavioral phenotypes upon neural inactivation are consistent. These experiments demonstrate that inhibiting activity in E564 neurons promotes proboscis extension in the absence of sensory stimuli as well as in response to taste compounds.

To determine whether these neurons influenced feeding as well as proboscis extension, we measured intake of sucrose solutions in freely feeding flies, as well as the time spent consuming sucrose applied directly to the proboscis. E564 flies expressing Kir2.1 consumed the same amount of sucrose as control flies in both consumption assays (Figure 2.2), indicating that the neurons influence feeding initiation but not consumption.

To assess whether increased activity in E564 neurons inhibits proboscis extension, we expressed the heat-activated cation channel dTRPA1 (Hamada et al., 2008) in E564 neurons and monitored proboscis extension to sugar at temperatures that activate the channel. Activating E564 neurons using dTRPA1 suppressed the proboscis extension response (PER) over a range of sucrose concentrations (50-1000mM). Interestingly, suppression occurred upon leg stimulation but not upon proboscis stimulation, showing that E564 neurons selectively inhibit responses to gustatory stimuli detected on the legs (Figure 2.1D). The activation and inactivation experiments demonstrate that E564 neurons modulate the threshold of PER, with high activity suppressing and low activity promoting proboscis extension.

Inactivation of a single pair of neurons produces constitutive proboscis extension

The *E564-Gal4* line is expressed in 10-12 neurons in the central nervous system of the fly, including the ventral nerve cord (VNC) and central brain (Figure 2.3AB; Figure 2.4AB). To determine the neurons causal for the constitutive proboscis extension phenotype, Kir2.1 was expressed in E564 neural subsets using a genetic mosaic approach. A ubiquitously expressed Gal80 flanked by FRT recombination sites was stochastically excised using an inducible Flp recombinase, allowing the Gal4-dependent expression of Kir2.1 and mCD8-GFP in a subset of E564 neurons (Gordon and Scott, 2009). Adult flies were then assayed for constitutive extension, and the frequency distributions of cell-types in extenders and non-extenders were compared (Figure 2.3C). Cell-type #1 was highly enriched in extenders and rarely labeled in non-extenders, whereas the other cells were present at similar frequencies in extenders and non-extenders. Additionally, in five animals that displayed constitutive proboscis extension, cell-type #1 was exclusively labeled, demonstrating that silencing of these neurons produces the aberrant behavior.

The neurons that inhibit proboscis extension (which we name PER_{in}) have cell bodies and processes in the first leg neuromeres of the VNC and projections to the subesophageal ganglion (SOG), the brain region that contains gustatory sensory axons and proboscis motor neuron dendrites (Figure 2.3D-G). Labeling with the presynaptic synaptotagmin-GFP marker (Zhang et al., 2002) and the postsynaptic DenMark marker (Nicolai et al., 2010) indicated that the dendrites of PER_{in} neurons are restricted to the first leg neuromeres, whereas axons are found in both the SOG and the first leg neuromeres (Figure 2.3HI). The anatomy of these neurons suggests the possibility that they convey information from the leg neuromeres to a region of the fly brain involved in gustatory processing and proboscis extension. Anatomical studies examining the proximity of PER_{in} fibers to gustatory sensory dendrites or proboscis motor axons revealed that PER_{in} neurons do not come into close contact with known neurons that regulate proboscis extension (Figure 2.4CD).

PER_{in} neurons are not modulated by satiety state or gustatory cues

There are several different contexts in which PER_{in} neurons might modulate feeding initiation. PER_{in} activity might reflect the satiety state of the animal, such that high activity inhibits feeding initiation when the animal is fed and low activity promotes feeding when the animal is food-deprived. Alternatively, PER_{in} neurons might directly process gustatory sensory cues, increasing activity in response to bitter compounds to suppress proboscis extension or decreasing activity upon sucrose stimulation to promote extension. A third possibility is that they regulate proboscis extension in response to other cues, such as mechanosensory or somatosensory cues, to inhibit proboscis extension while the animal is engaged in other behaviors.

To test whether PER_{in} neurons influence extension probability based on satiety state, we performed cell-attached electrophysiological recordings to monitor the basal firing rate of PER_{in} neurons under fed and food-deprived conditions (Marella et al., 2012). In both conditions, PER_{in} neurons exhibited constant basal activity of ~14 Hz, indicating that tonic activity in these neurons is not altered by satiety state (Figure 2.5AB).

To evaluate whether PER_{in} neurons respond to taste stimuli, legs were stimulated with a sugar (350mM sucrose) or a bitter substance (10mM quinine) and activity of PER_{in} was measured by electrophysiology. Taste stimulation had no effect on the firing rate of PER_{in} neurons in either fed or food-deprived animals (Figure 2.5C). These studies argue that PER_{in} neurons are not modulated by satiety state or gustatory cues.

PER_{in} neurons are activated by mechanosensory input to the VNC

Because the dendrites of PER_{in} neurons reside in the first leg neuromere, we wondered whether inputs into the first leg neuromere would activate PER_{in} neurons. We therefore stimulated the major nerves of the ventral nerve cord and monitored responses of PER_{in} by G-CaMP calcium imaging (Tian et al., 2009), using a dissected brain plus ventral nerve cord preparation and electrical nerve stimulation (10V). PER_{in} dendrites responded to stimulation of nerves of the first leg neuromere and were also excited by the stimulation of nerves from all legs, wings and halteres, but not the abdominal nerve (Figure 2.6A-C). Of these nerves, the posterior dorsal nerve in segment 2 (PDN2), and the dorsal nerve in segment 3 (DN3) do not contain any gustatory neurons (Demerec, 1994), consistent with the notion that non-gustatory input activates PER_{in}.

Because mechanosensory neurons are a major sensory input carried by all nerves into the VNC, we tested whether PER_{in} was activated by stimulation of mechanosensory neurons. The blue light-activated ion channel, channelrhodopsin-2 (ChR2), was expressed in mechanosensory neurons under the control of the nompC promoter using the QF/QUAS transgenic system (Nagel et al., 2003; Petersen and Stowers, 2011; Potter et al., 2010) and G-CaMP3 was expressed in PER_{in} using the Gal4/UAS system. Light-induced activation of mechanosensory neurons produced robust calcium increases in PER_{in} neurons (Figure 2.6DE). Activating sugar, bitter or water gustatory inputs with ChR2 did not elicit responses in PER_{in} (Figure 2.7A-C). These results argue that PER_{in} selectively responds to activation of mechanosensory neurons.

In the adult, *nompC-Gal4* drives expression in mechanosensory neurons in external sensory bristles and chordotonal organs (Cheng et al., 2010; Petersen and Stowers, 2011). In larvae, NompC-positive neurons respond to touch, whereas different neurons detect noxious heat and harsh mechanosensory stimuli (Cheng et al., 2010; Tracey et al., 2003; Yan et al., 2013). As the repertoire of stimuli that activate NompC neurons in the adult has not been rigorously examined, we tested whether heat or mechanosensory cues would activate PER_{in} similar to optogenetic stimulation of NompC neurons. Neither temperature increases nor an airpuff to a single leg activated PER_{in} (Figure 2.7FG). To test whether more rigorous movement would activate PER_{in}, we monitored G-CaMP changes in PER_{in} axons in animals that could freely move their legs (Figure 2.8). Bouts of PER_{in} activity were highly correlated with bouts of leg movement (Figure 5AC). When legs of the same animals were the immobilized with wax, PER_{in} activity changes were abolished (Figure 5A). Control experiments expressing GFP rather than G-CaMP in PER_{in} neurons showed no fluorescent changes upon movement, showing that responses are not motion

artifacts (Figure 2.8B). Taken together, these experiments argue that PER_{in} is activated upon movement, likely by mechanosensory inputs from multiple legs.

The observation that mechanosensory inputs activate PER_{in} neurons suggests that movement of the legs might activate PER_{in} to inhibit proboscis extension while the animal is engaged in other behaviors. If leg inputs inhibit proboscis extension through PER_{in}, then one prediction would be that removing leg inhibition would promote extension and that this would require PER_{in}. Flies whose legs were either removed (stumps) or immobilized with wax (wax) showed increased spontaneous proboscis extension, demonstrating that leg inputs inhibit extension (Figure 2.9AB). Extensions were further enhanced in *E564-Gal4, UAS-Shi^{ts}* flies, suggesting that tonic activity in PER_{in} or non-leg inputs may also inhibit extension. Importantly, activation of PER_{in} neurons with dTRPA1 in flies with stumps or immobilized legs prevented the increased spontaneous proboscis extension, suggesting that PER_{in} neurons act downstream of leg inputs to inhibit extension (Figure 2.9CD). These studies suggest that PER_{in} neurons function to inhibit extension while the animal is participating in other behaviors, such as locomotion.

Engagement in proboscis extension inhibits locomotion

As PER_{in} promotes behavioral exclusivity by altering the threshold for feeding initiation in response to mechanosensory-driven behaviors, we hypothesized that commitment to one behavior might more generally prevent other behaviors. Since *E564-Gal4; UAS-Kir2.1, tub-Gal80^{ts}* flies display constitutive proboscis extension, we wondered whether engagement in this behavior might alter the probability of other behaviors. To test this, we monitored the activity of *E564-Gal4; UAS-Kir2.1, tub-Gal80^{ts}* flies in a closed arena. Control flies, as well as *E564-Gal4; UAS-Kir2.1, tub-Gal80^{ts}* flies not expressing Kir2.1, showed robust walking activity, whereas flies expressing Kir2.1 in E564 neurons had greatly reduced activity, with some flies not taking a single step in the 60 seconds assayed (Figure 2.10AB). All flies were able to move when presented with a startle stimulus.

To test whether the movement impairment was a consequence of silencing PER_{in}, we generated mosaic animals in which Kir2.1 and mCD8-GFP were expressed in subsets of E564 neurons, screened for constitutive proboscis extension and assayed the extenders and non-extenders for movement (Figure 2.10AB). Flies with extended proboscises displayed impaired locomotion. To ensure that the locomotion defect was a result of inactivating PER_{in}, we screened mosaic animals for locomotor defects, and determined the frequency distribution of neural classes in flies with normal locomotion (>250mm/min traveled) or impaired locomotion (<200mm/min traveled). PER_{in} was enriched in flies with locomotor defects and no other cell-type correlated with locomotor defects (Figure 2.10C). These experiments show that silencing PER_{in} both promotes proboscis extension and inhibits movement. A second Gal4 line, *E605-Gal4*, contains PER_{in} and displays the same behavioral phenotypes upon neural inactivation or activation (Figure 2.11). These data suggest that there is a reciprocal balance between feeding initiation and locomotion mediated by PER_{in} activity.

To test whether the act of proboscis extension sufficed to inhibit locomotion, we immobilized the proboscis in an extended or retracted position with wax. Wild-type flies with extended proboscises moved significantly less (Figure 2.10D), arguing that motor activity or proprioceptive feedback from the proboscis inhibits locomotion. Consistent with this, immobilizing the proboscis in a retracted state partially rescued the locomotor defect of flies with inactivated PER_{in} neurons (Figure 2.10D). Thus, proboscis extension feeds back onto circuits to inhibit locomotion, allowing for mutually exclusive behaviors.

Conclusions

Many behaviors are mutually exclusive, with the decision to commit to one behavior excluding the selection of others. Here, we show that feeding initiation and locomotion are mutually exclusive behaviors and that activity in a single pair of interneurons influences this behavioral choice. PER_{in} neurons are activated by stimulation of mechanosensory neurons and activation of PER_{in} inhibits proboscis extension, suggesting that they inhibit feeding while the animal is walking. Consistent with this, leg removal or immobilization enhances proboscis extension probability and this is inhibited by increased PER_{in} activity. The opposite behavior is elicited upon inhibiting activity in PER_{in} neurons: animals show constitutive proboscis extension at the expense of locomotion. This work shows that activity in a single pair of interneurons dramatically influences the choice between feeding initiation and movement.

The precise mechanism of activation of PER_{in} neurons remains to be determined. PER_{in} dendrites reside in the first leg neuromere, suggesting that they process information from the legs. Stimulation of leg chemosensory bristles with sucrose or quinine or activation of sugar, bitter or water neurons using optogenetic approaches did not activate PER_{in} neurons, nor did satiety state change tonic activity. Stimulation of sensory nerves into the thoracic ganglia and stimulation of mechanosensory neurons, using a nompC driver, activated PER_{in}. In addition, by monitoring activity of PER_{in} while flies moved their legs, we demonstrated that activity was coincident with movement. These studies argue that PER_{in} is activated by non-gustatory cues in response to movement, likely upon detection of mechanosensory cues. Additional cues may also activate PER_{in}.

Studies of behavioral exclusivity in other invertebrate species suggest two mechanisms by which one behavior suppresses others (Kristan and Gillette, 2007). One strategy is by competition between command neurons that activate dedicated circuits for different behaviors. More common is a strategy in which decision-making occurs by distributed activity changes across neural populations. While our studies are a starting point to begin to examine these models in *Drosophila*, the circuits for proboscis extension and locomotion drive different motor neurons, muscles and behaviors, suggesting that they may be connected by a few links rather than largely overlapping circuitry. PER_{in} is likely to inhibit feeding initiation while the animal is moving and is one critical link. The observation that simply gluing the proboscis in an extended state, but not in a retracted state, inhibits locomotion suggests that motor activity or proprioceptive feedback from the proboscis acts as a reciprocal link to locomotor circuits.

Neurons act over different time scales and in response to different sensory cues to influence behavior. The powerful molecular genetic approaches available in

Drosophila enable the precise manipulation of individual neurons and allow for the examination of their function in awake, behaving animals. Modulatory neurons such as PER_{in} are difficult to identify by calcium imaging or electrophysiological approaches because they influence gustatory-driven behavior but are not activated by gustatory stimulation. The ability to probe the function of neurons in unbiased behavioral screens facilitates the identification of neurons that act as critical nodes to influence behavior. The identification and characterization of PER_{in} as a significant modulator of feeding initiation provides a foundation for future studies determining how PER_{in} influences proboscis extension circuits to alter behavioral probability and how mechanosensory inputs activate PER_{in}. In addition, examining how proboscis extension suppresses locomotion will provide important insight into the links between different behaviors.

Neural circuits for a given behavior do not work in isolation. Information from multiple sensory cues, physiological state and experience must be integrated to guide behavioral decisions. Our work uncovers a pair of interneurons that influences the choice between feeding initiation and locomotion. The discovery of the PER_{in} neurons will aid in examining the neural basis of innate behaviors and the decision-making processes that produce them.

Acknowledgements

Mike Gordon performed the initial behavioral screen that isolated the proboscis extension phenotype. Heesoo Kim and Chris Rodgers provided crucial assistance in the analysis of electrophysiology and calcium imaging data. David Harris developed the heat probe used for dTRPA1 activation. Brendan Mullaney developed the blue-dye feeding assay used in this study. Priscilla Kong generated the *lexAop-ChR2* flies.

Materials and Methods

Fly strains

w^B flies were used as control wild type flies. The following fly lines were used: *E564-Gal4* (from the Gal4 collection kindly proved by Ulrike Heberlein), *hs-flp, MKRS* (Bloomington stock collection), *UAS-Kir2.1* (Baines et al., 2001), *tub-Gal80ts* (McGuire et al., 2004), *ptub-FRT-Gal80-FRT, Gr5a-LexA, UAS- CD4::spGFP1-10, LexAop-CD4::spGFP11, E49-Gal4* (Gordon and Scott, 2009), *UAS-mCD8::GFP* (Lee and Luo, 1999), *UAS-dTRPA1* (Hamada et al., 2008), UAS-GCamP3 (Tian et al., 2009), *UAS-DenMark*; *UAS-Syt::GFP* (Nicolai et al., 2010), *UAS-Shi^{ts}* (Kitamoto, 2001), *LexAop- dTRPA1* (vectors described in (Pfeiffer et al., 2010) a gift from Barret Pfeiffer, Rubin lab), *NompC-QF* and *QUAS-ChR2* (Petersen and Stowers, 2011), *ppk28-LexA* and *Gr66a-LexA* (Thistle et al., 2012), *UAS-mCD8:: dsRed* (Ye et al., 2007), *E605-Gal4* (Gohl et al., 2011), *UAS-C3PA* (Ruta et al., 2010), *UAS-GCamP5* (Akerboom et al., 2012). Flies were grown on standard fly food.

Transgenic flies

LexAop-ChR2 flies were generated by PCR amplification of the ChR2 sequence from *UAS-ChR2* flies (a gift from Steve Stowers) and cloning into the pLOT vector. Primers for amplification were from pUAST (5' AGAACTCTGAATAGGGAATTGGG and 3'AAATCTCTGTAGGTAGTTTGTCCA). The functionality of *LexAop-ChR2* was validated by behavioral experiments showing that *Gr5a-LexA, LexAop-ChR2* flies extend the proboscis to light (not shown).

Behavioral Experiments

Proboscis extension: PER was performed as previously described (Marella et al., 2012), except that each individual stimulation of each animal was treated as an independent data point. For the *UAS-Kir2.1, tub-Gal80^{ts}* experiments, Kir2.1 was induced by a 2-day temperature shift to 30^{o}C then returned to 22^{o}C for one day prior to testing. Uninduced flies remained at 22^{o}C. For the Shibire^{ts} inducible silencing experiments and the dTRPA1 inducible activation experiments, flies were transferred to a heating block at 32^{o}C for 5 minutes and then assayed for behavior. The Shibire^{ts} flies were raised at 19^{o}C. All UAS control flies were crossed to w^b in order to produce animals isogenic to experimental flies. Constitutive extension was determined as a complete extension of the proboscis (with both the rostrum and haustellum fully extended) maintained over several seconds in the absence of stimulus. The number of spontaneous extensions and retractions were measured in individual flies over a 30 second window.

Walking assay: Flies were gently aspirated into a circular chamber 4cm in diameter. Freely moving flies were videotaped for 1 minute at 12fps using a Sony DCR-HC38 camera. Video was subsequently analyzed using the ctrax software suite version 0.3.9 (Branson et al., 2009). The total distance walked was computed and

subsequently used to generate a mean distance traveled for each genotype assayed. Flies were shifted to 30°C for 48 hours to inactivate Gal80^{ts}, then placed at room temperature for 24 hours before assaying. *w^{b}* flies crossed to *UAS-Kir, Gal80^{ts}* were used as isogenic controls. All flies assayed were females 5-8 days old. Manual proboscis manipulations were performed by melting wax over the sides of the proboscises of CO_2-anethetised flies in either the extended or retracted position. Flies were allowed 2 hours of recovery in food vials before assaying movement. Wild type flies used were *W^{b}*.

Feeding assay: Flies were put into vials containing 300µl of 200mM sucrose mixed with blue dye (Erioglaucine, Sigma, 0.25mg/ml) on a piece of Whatman filter paper (2.5cm circular paper, grade 1). 25-50 flies were allowed to feed for 30 minutes, after which they were put on ice. Flies were scored in the following manner: Flies with no blue dye visible in their abdomen were scored as 0, flies with blue dye in less than half of their abdomen were scored as 1, and those with blue dye in more than half of their abdomen were scored as 2. Starved flies were put on wet kimwipes for 24 hours prior to experimentation.

For the temporal consumption assay, flies were starved for 24 hours on wet kimwipes and then mounted on glass slides using nail polish. After 2 hours of recovery in a humidified chamber, the time spent consuming 1M sucrose was measured for each fly. Flies were considered non-responsive if they failed to consume sucrose upon 10 consecutive stimulations.

Inducible activation

For channelrhodopsin-2 experiments, flies were prepared as previously described (Gordon and Scott, 2009), except that flies were not starved prior to experimentation. For stimulation, 10ms light pulses were applied at 30Hz for a total of 3 seconds using a 50mW 473nm diode pumped solid state laser (Shanghai Dream Lasers).

Genetic mosaics

Genetic mosaics were generated as previously described (Gordon and Scott, 2009), except that flies were of the genotype *tub>Gal80>; E564-Gal4,UAS-mCD8::GFP/UAS-Kir2.1; MKRS, hs-FLP*. Flies were heat-shocked at 37.5°C for 55 minutes during late larval to pupal stages.

Immunohistochemistry

Antibody staining and imaging was carried out as previously described (Wang et al., 2004). The following antibodies were used: rabbit anti-GFP (Invitrogen, 1:1,000), mouse anti-GFP (Invitrogen, 1:1000), mouse anti-nc82 (Hybridoma bank, 1:500), rabbit anti-dsRed (Biovision, 1:1000). Brightness or contrast of single channels was adjusted for the entire image using ImageJ software.

Photoactivation

Photoactivation of GFP was performed essentially as previously described (Ruta et al., 2010). Dissected brain plus ganglia preparations were secured to the bottom of a

plastic dish in AHL. PER_{in} axons were photoactivated through the cervical connective, and E49 motor neurons were photoactivated at the cell body on the surface of the SOG using a 710nm two-photon laser. The anterior portion of the SOG was then imaged using a 925nm two-photon laser.

Electrophysiology

Experiments were performed as previously described (Marella et al., 2012), except that flies were immobilized ventral side up, with cover glass separating the front tarsi and head of the fly from the recording chamber. E564 neurons were labeled with GFP and PER_{in} neurons identified for recordings based on their fluorescence and anatomical position. For taste stimulations, tastants were delivered to the ipsilateral tarsus using a glass capillary. A stimulus artifact in the recording indicated when stimulation occurred. Data was band-passed filtered between 10 and 300Hz using a butterworth-type filter. Pre-stimulus spike rates were calculated using 15s of recording prior to stimulation; post-stimulus spike rates were calculated using 1s of recording after stimulation.

Nerve stimulation

Whole nervous systems (brain and ventral nerve cord) were carefully dissected in cold adult hemolymph-like solution (AHL) lacking calcium and magnesium, then transferred to a room temperature dish with AHL containing calcium and magnesium, and gently pinned with the dorsal surface facing up (Wang et al., 2003). Nerves were then individually inserted into a stimulating suction electrode. Stimulus was 10V, 300μs delivered at 100Hz for 100ms (ten stimulations).

G-CaMP Imaging

G-CaMP3 responses were monitored as previously described (Marella et al., 2006), except that flies were immobilized on Scotch tape, dorsal side up. The dorsal surface of the thorax was partially dissected to expose the VNC. During nerve stimulations and heat stimulations, PER_{in} dendrites were imaged at 1.1 Hz (9 1mm Z-sections at 100ms/mm) on a 3i spinning disk confocal system, using a 20x water objective and 2x optical zoom. For the heat stimulus, a custom heat probe was placed directly under the fly, and temperature was ramped to 36^{o}C while imaging. For channelrhodopsin-2 experiments, PER_{in} dendrites were imaged on a Zeiss PASCAL microscope with a 20x water objective and digital zoom factor of 3, at a rate of ~4Hz (56.6 μM thick optical section). Heat maps were generated using ImageJ. The mean of 4 frames prior to stimulus were used as the baseline fluorescence value.

G-CaMP imaging while monitoring movement

PER_{in} axons were imaged during movement by immobilizing the fly in a manner similar to that previously described for electrophysiology (Marella et al., 2012). The distal segments of the forelegs were removed to prevent them from coming into contact with the bath solution, but otherwise the fly's legs were allowed to move freely during imaging. Calcium responses were monitored in the SOG of the fly using a 40x water objective and a 3x optical zoom at 3.3Hz (17.7μM thick optical section). Movement of the legs was monitored using a 1800USBPS Penscope

(1800endoscope.com). Only movement involving all 6 fly legs was scored as movment. Video was scored for movment using LifesongX 0.8 (Neumann et al., 1992) and resampled at 3.33Hz (to match the calcium imaging rate) using zeros and ones to indicate periods of no movement and movement, respectively. This signal was used to generate correlations (rho) between movment and ΔF/F values. All analyses and statistics were performed in Matlab. The correlation coefficient (rho) between the ΔF/F signal and the movement array showed high rho values (mean=0.4559, std=0.182). With the exception of one animal, all correlations were highly significant ($p<0.0002$). To test if significant rho values are an artifact of correlating two highly time-varying signals, we shuffled the data and computed the correlation coefficients for all possible movement array and ΔF/F combinations. The distributions of the rho values for congruent correlations (n = 10) and shuffled data (n = 10^2 – 10 = 90) were compared with a two-sided t-test.

Figure 2.1. Inducible inactivation and activation of *E564-Gal4* neurons alters the threshold for proboscis extension.

A. Example images of *E564-Gal4; UAS- Kir2.1, tub-Gal80^{ts}* flies without (left) and with (right) induction of Kir2.1.
B. Chronically silencing neurons in *E564-Gal4; UAS-Kir, tub-Gal80^{ts}* (left) produced constitutive proboscis extension in nearly 100% of animals, a phenotype almost never observed in control (*UAS-Kir2.1*, *tub-Gal80^{ts}* or *E564-Gal4* flies) and non-induced flies (*E564-Gal4; UAS-Kir, tub-Gal80^{ts}* , 22^{o}C). Kir2.1 was induced by placing the flies at 30^{o}C for two days prior to experimentation at 22^{o}C. 0 indicates that no flies showed proboscis extension. n=55-60 flies, mean±95%CI, Fisher's exact test, *** P<0.001. Acutely silencing neurons in *E564-Gal4; UAS-Shi^{ts}* flies (right) increased spontaneous proboscis extensions more than five-fold at restrictive temperature (32^{o}C, red bars) compared to permissive temperature (22^{o}C, black bars). n=25-36 flies, mean±SEM, student's t-test, *P<0.05. See also Figure S1, showing that silencing E564 neurons did not affect sucrose consumption.
C. Proboscis extension response to sucrose (10-1000mM) in *E564-Gal4, UAS-Shi^{ts}* (left) and control *E564-Gal4* or *UAS-Shi^{ts}* flies (right) at permissive (black, gray) and restrictive temperatures (red, rose). n=54-67 flies, mean±95% CI, Fisher's exact test, ***P<0.001, *P<0.05.
D. Proboscis extension response to tarsal (top) or proboscis (bottom) stimulation in *E564-Gal4, UAS-dTRPA1* (left) and *UAS-dTRPA1* control flies (right) at 22^{o}C (black) and 32^{o}C (green). n=31-44 flies/condition, mean±95% CI, Fisher's exact test, ***P<0.001, *P<0.05.

Figure 2.2. Inactivating E564 neurons does not alter sucrose consumption.

A. *E564-Gal4, UAS-Kir2.1, tub-Gal80^{ts}* and control flies were allowed to freely feed on 200mM sucrose spiked with blue dye for 30 minutes in both satiated (0H starvation) and deprived (24H starvation) conditions. Flies not feeding are given a score of 0, those with less than half of their abdomen full of blue dye are given a score of 1, and those with greater than half are given a score of 2. All flies consumed similar amounts of 200mM sucrose when Kir2.1 expression is induced (30ºC) or not induced (22ºC). n=3 groups of 30-60 flies each per genotype, mean±SEM, ANOVA with post-hoc Tukey test, ns.
B. The same genotypes used in A were mounted on glass slides and stimulated with 1M sucrose. Each fly was allowed to drink until it would no longer consume sucrose after 10 consecutive stimulations. Sucrose consumption time was not significantly different. n=27-36 flies per genotype, mean±SEM, ANOVA with post-hoc Tukey test, ns.

Figure 2.3. Mosaic analysis identifies a single cell-type responsible for constitutive proboscis extension.

A-B. Expression of *UAS-mCD8-GFP* in *E564-Gal4* neurons in VNC (A) and central brain (B). The six neural types in mosaics are labeled (arrows). See Figure S2 for single-cell clones of the six different neural types.
C. Flies expressing Kir2.1 and CD8-GFP in subsets of E564 neurons were assayed for constitutive proboscis extension (extension, red bars, n=98), or normal proboscis posture (no extension, black bars, n=116) (genotype: *tub>Gal80>; E564-Gal4,UAS-mCD8::GFP/UAS-Kir2.1; MKRS, hs*-FLP flies). The frequency of the 6 cell-types identified in A and B is shown in extenders and non-extenders. All cell-types except for cell-type #1 showed similar distribution in both groups. mean±95% CI, Fisher's exact test , *** P<0.001 .
D. A clone of cell-type #1 (PER_{in}) expressing mCD8-GFP, showing cell bodies in the first thoracic segment and projections to the SOG.
E-F. Detailed image of PER_{in} clone from D in first thoracic segment (E) and SOG (F).
G. Schematic of PER_{in} showing processes in the VNC and SOG.
H-I. *E564-Gal4* line expressing the presynaptic marker *UAS-Syt-GFP* and the postsynaptic marker *UAS-DenMark*. Images are the same regions shown in E, F (PER_{in}), indicating mixed pre-and postsynaptic fibers in the first thoracic segment (H), and presynaptic fibers in the SOG (I). The edges of the VNC (H) and SOG (I) are shown with dotted lines. Scale bars are 50μM..

Figure 2.4. The 6 cell types in the *E564-Gal4* line and proximity of PER_{in} to gustatory and motor fibers.

A. Schematic of the ventral nerve cord (VNC, top) and central brain (bottom). Each cell type in the *E564-Gal4* line is illustrated in green.
B. Confocal images of each of the cell types. Each panel corresponds to the cell type number shown in A, Figure 2A-C and Figure 7C. The top 3 panels show the VNC and the bottom 3 show the brain. In panel 3, the border of the abdominal ganglion and nerve are outlined. In panel 5, the SOG and the esophagus borders are outlined. In panel 6, the SOG and the labeller nerves are outlined. Flies were generated using the mosaic method as presented in Figure 2 and are the same genotype.
C. Contacts between gustatory dendrites and PER_{in} were examined by expressing membrane-tethered split GFP fragments in the processes, by the GRASP method as previously described (Gordon and Scott, 2009). PER_{in} is shown in magenta. No GRASP punctae (green) were observed.
D. Proximity of PER_{in} to motor neurons that drive proboscis extension was examined by double labeling PER_{in} and motor fibers. Cell-specific labeling was achieved by photoactivation of pa-GFP using *E564-Gal4*, *E49-Gal4*, *UAS-paGFP* flies. Left panel shows frontal view of PER_{in} in green and proboscis motor neurons in magenta. Middle panel show sagittal view and right panel shows the sagittal view of PER_{in} alone. There is no overlap in projections. Erroneous photoactivation and background was removed from the image by masking the motor neurons and PER_{in} axons. E49 motor neurons were then pseudocolored using ImageJ to enhance contrast. All scale bars are 50μM.

Figure 2.5. PER_{in} neurons are not modulated by satiety or taste stimulation.

A. Raster plots of 5 seconds of recording from PER_{in} in 10 different animals, satiated (0H starvation) or food-deprived (24H starvation).
B. Mean firing rate over a 30 second period is not significantly different in satiated (n=6) and food-deprived (n=5) flies by student's t-test. Individual data points as well as mean±SEM are shown.
C. Firing rates of PER_{in} neurons before (pre-stimulus) and during stimulation (stimulus) with either 350mM sucrose (left two panels) or 10mM quinine (right two panels) in satiated (0H) and food-deprived (24H) conditions. Pre-stimulus firing rate was averaged over 15 seconds prior to stimulus. Stimlulus duration was 1 second. n=5 flies for each condition, paired t-test. ns.

Figure 2.6. PER_{in} neurons receive input from multiple nerves in the VNC and respond to mechanosensory stimulation.

A. Schematic of the major nerves of the VNC. VN=ventral nerve, DN=dorsal nerve, AbN=abdominal nerve, A and P indicate anterior and posterior, respectively, subscripted numbers indicate thoracic segment corresponding to each nerve. TG1, TG2, TG3=Thoracic ganglia 1, 2, 3; AbG=Abdominal ganglion. PER_{in} dendritic region is shown in green in the first segment.
B. Summary of nerve stimulation data. Stimulation (10V) of all nerves except the abdominal nerve resulted in calcium responses in PER_{in} dendrites when compared to sham stimulation (10V applied with no nerve in the suction electrode). n=6-8 flies/condition, mean±SEM, ANOVA with Tukey post-hoc test, *P<0.05, **P<0.01 .
C. The same data as in B is shown as representative heat maps and traces of DF/F values (0-200%) in PER_{in} dendrites in *E564-Gal4; UAS-GCaMP3* flies for each of the nerves stimulated as well as a sham stimulation. Red arrows indicate stimulation time. Black line is mean DF/F value, with gray areas representing ±SEM. Scale bar is 50µM.
D. Heat map and DF/F trace as in C but upon *NompC-QF; QUAS-ChR2* stimulation. Blue bar indicates light stimulation. Scale bar is 50µM. Black line is mean DF/F value, with gray areas representing ±SEM. Scale bar is 50µM. N=6 flies/condition.
E. Summary of *NompC-Q; QUAS-ChR2* data. Only flies expressing ChR2 under the control of *NompC-Q* showed activation in PER_{in} dendrites upon blue light stimulation. n=6-8 files/condition, mean±SEM, ANOVA with Tukey post-hoc test, *P<0.05. See Figure S3, showing that activation of sugar, bitter or water gustatory projections did not activate PER_{in} nor did air puff nor heat stimulation.

Figure 2.7. PER_{in} neurons do not respond to channelrhodopsin-2 (ChR2)-mediated stimulation of gustatory neurons, related to Figure 4.

A-E. Stimulation of sugar-sensing (A), water-sensing (B), bitter-sensing (C), and no (D) sensory neurons while monitoring GCaMP3 responses in PER_{in} dendrites. Flies were prepared in an identical manner to those in NompC-ChR2 experiments (data from Figure 4D is shown as a reference in panel E), with identical light stimulation (blue bar).

F. A gentle puff of air (red arrow) was delivered to legs while monitoring GCaMP3 responses in PER_{in} dendrites. Air was delivered manually through a glass capillary.
G. Leg sensory neurons were stimulated using a heat probe to activate thermosensitive neurons. DF/F values are shown in gray (left axis), and temperature values of the heat probe are shown in red (right axis).
H. Summary data of maximum DF/F for each stimulation. n=6-8 flies per genotype, mean±SEM, ANOVA with post-hoc Tukey test, ***P<0.001.

For GCaMP3 traces, lines and shaded areas represent mean±SEM. Flies used were *UAS-GCaMP3; Sensory-LexA* (GR5a, PPK28, GR66a)/*E564-Gal4*, *UAS-GCaMP3*; *LexAop-ChR2, UAS-GCaMP3*. Control flies used lacked a sensory LexA driver.

Figure 2.8. PER_{in} activity correlates wth leg movements.

A. ΔF/F traces from PER_{in} axons of *E564-Gal4/UAS-GCaMP5; UAS-GCaMP5* for 4 individual flies (black lines), with leg movement indicated by shaded rectangles. ΔF/F traces of the same flies with immobilized legs are shown below (red lines). Calcium transients seen in moving animals are correlated with movement and are abolished in immobilized flies.
B. ΔF/F traces from PER_{in} axons of *E564-Gal4, UAS-mCD8::GFP* flies for 2 individual flies (black lines), with leg movement indicated by shaded rectangles.
C. Correlation values between movement and ΔF/F values in freely moving *E564-Gal4/UAS-GCaMP5; UAS-GCaMP5* flies (n=10), shuffled data (n=90), and freely moving *E564-Gal4, UAS-mCD8::GFP* flies (n=4). Data is mean±SEM, two-sided t-test, ***P<0.001.

Figure 2.9. Leg removal or immobilization promotes proboscis extension.

A. Control *E564-Gal4* or *UAS-Shi^{ts}* flies and *E564-Gal4; UAS-Shi^{ts}* flies with or without legs (legs vs. stumps) at permissive (22°C, black bars) and restrictive (32°C, red bars) temperatures. Removal of legs in control flies caused an increase in spontaneous proboscis extensions, which was similar at both temperatures. Removal of legs in *E564-Gal4, UAS-Shi^{ts}* flies also increased spontaneous extensions, and was greatly enhanced at restrictive temperature. n=16-20 flies/condition.
B. Fly legs were immobilized with wax and proboscis extensions were examined at permissive (black bars) and restrictive (red bars) temperatures. n=26-29 flies/condition.
C. *UAS-dTRPA1* controls and *E564-Gal4; UAS-dTRPA1* flies with or without legs (legs vs. stumps) at 22°C (black) and 32°C (green). Removal of legs in control flies caused an increase in spontaneous proboscis extensions, which was similar at both temperatures. Removal of legs in *E564-Gal4, UAS-dTRPA1* flies caused spontaneous extensions, but this effect was abolished at 32ºC upon dTRPA1 activation. n=20-21 flies/condition.
D. Activating E564 neurons abolished the increase in spontaneous proboscis extensions caused by leg immobilization. n=19-21 flies/condition. All data for Figure 6 is mean±SEM, ANOVA with Tukey post-hoc test, *P<0.05, **P<0.01, ***P<0.001.

Figure 2.10. Silencing PER_{in} neurons reduces locomotion.

A. Sample walking traces of control, heterozygous *E564-Gal4; UAS-Kir2.1*, *tub-Gal80^{ts}* and homozygous *E564-Gal4; UAS-Kir2.1, tub-Gal80^{ts}* flies (3 flies/genotype) without (22^{o}C) or with Kir2.1 (30^{o}C) induction (top 6 traces). The bottom two traces show mosaic E564 flies selected for either a retracted or extended proboscis. As with the full *E564-Gal4* line, flies with extended proboscises moved significantly less. Different colors mark movement paths of different flies. Scale bar is 10mm.
B. *E564-Gal4; UAS-Kir2.1, tub-Gal80^{ts}* flies showed severely reduced movement when compared to controls. This phenotype was also observed in mosaic flies exhibiting constitutive PER, but not in mosaic flies that did not exhibit PER. n=12-15 flies/condition for non-mosaic animals, n=25-30 for mosaics, mean±SEM, ANOVA with Tukey post hoc test, ***P<0.001.
C. Analysis of mosaic animals selected for high movement (<250mm/minute) or low movement (>200mm/minute). Only cell-type 1 (PER_{in}) neurons were enriched in flies with low locomotion (cell-types numbered in Figure 2 and S2). n=25-30 flies/condition, mean±SEM, Fisher's exact test, ***P<0.001. See Figure S4 for behavioral analyses of a second Gal4 line that contains PER_{in}.
D. Waxing the proboscis in an extended state, but not in a retracted state, impaired locomotion in wild-type flies. Waxing the proboscis in a retracted state partially rescued the locomotion defects seen upon neural inactivation of *E564-Gal4; UAS-Kir2.1, tub-Gal80^{ts}* flies.
n=12-22 flies/condition for non-mosaic animals, n=23-26 for mosaics, mean±SEM, ANOVA with Tukey post hoc test, ***P<0.001, *P<0.05.

Figure 2.11. A second Gal4 line, *E605-Gal4*, containing PER_{in} neurons displays similar behavioral phenotypes, related to Figure 7.

A. Expression of *UAS-CD8-GFP* in *E605-Gal4* neurons in VNC (top left) and central brain (bottom left). PER_{in} is labeled (top left, arrows) and dendrites in VNC are shown (right). Scale bars are 50μM.
B. Chronically silencing neurons in *E605-Gal4; UAS-Kir, tub-Gal80^{ts}* (left) produced constitutive proboscis extension in nearly 100% of animals, a phenotype almost never observed in *E605-Gal4* flies and non-induced flies (*E564-Gal4; UAS-Kir, tub-Gal80^{ts}*, 22^{o}C). 0 indicates that no flies showed proboscis extension. n=20-25 flies/genotype, mean±95%CI, Fisher's exact test, *** P<0.001. Acutely silencing neurons in *E605-Gal4; UAS-Shi^{ts}* flies (right) increased spontaneous proboscis extensions (32^{o}C, red bars) compared to genetic controls at permissive temperature (22^{o}C, black bars) or *E605-Gal4* controls. n=24 flies, mean±SEM, student's t-test, **P<0.01.
C. Proboscis extension response to sucrose (10-1000mM) in *E564-Gal4, UAS-Shi^{ts}* (left) and *E564-Gal4* flies (right) at permissive (black) and restrictive temperatures (red). n=30 flies, mean±95% CI, Fisher's exact test, ***P<0.001, **P<0.01, *P<0.05.
D. Proboscis extension response to tarsal (top) or proboscis (bottom) stimulation in *E605-Gal4, UAS-dTRPA1* (left) and *E605-Gal4* control flies (right) at 22^{o}C (black) and

32°C (green). n=30 flies/condition, mean±95% CI, Fisher's exact test, ***P<0.001, **P<0.01.

E. *E564-Gal4; UAS-Shi^{ts}* flies and *E564-Gal4* or *UAS-Shi^{ts}* flies with or without legs (legs vs. stumps) at permissive (22°C, black bars) and restrictive (32°C, red bars) temperatures. Removal of legs in control flies caused an increase in spontaneous proboscis extensions, which was similar at both temperatures. Removal of legs in *E605-Gal4, UAS-Shi^{ts}* flies also increased spontaneous extensions, and was greatly enhanced at restrictive temperature. n=15-19 flies/condition, mean±95% CI, Fisher's exact test, ***P<0.001, **P<0.01.

F. Fly legs were immobilized with wax and proboscis extensions were examined at permissive (black bars) and restrictive (red bars) temperatures. n=16-19 flies, mean±95% CI, Fisher's exact test, **P<0.01, *P<0.05.

G. *UAS-dTRPA1* controls and *E605-Gal4; UAS-dTRPA1* flies with or without legs (legs vs. stumps) at 22ºC (black) and 32ºC (green). Removal of legs in *E564-Gal4, UAS-dTRPA1* flies increased spontaneous extensions, but this effect was abolished at 32ºC upon dTRPA1 activation. n=14-19 flies, mean±95% CI, Fisher's exact test, **P<0.01.

H. Activating E605 neurons abolished the increase in spontaneous proboscis extensions caused by leg immobilization (wax). n=17-20 flies/condition, all data is mean±SEM, ANOVA with Tukey post-hoc test, **P<0.01.

I. *E605-Gal4; UAS-Kir2.1, tub-Gal80^{ts}* flies show severely reduced movement when compared to *E605-Gal4; UAS-Kir2.1, tub-Gal80^{ts}* controls at 22°C or *E605-Gal4* controls. n=20flies/condition, mean±SEM, ANOVA with Tukey post hoc test, ***P<0.001.

Figure 2.1

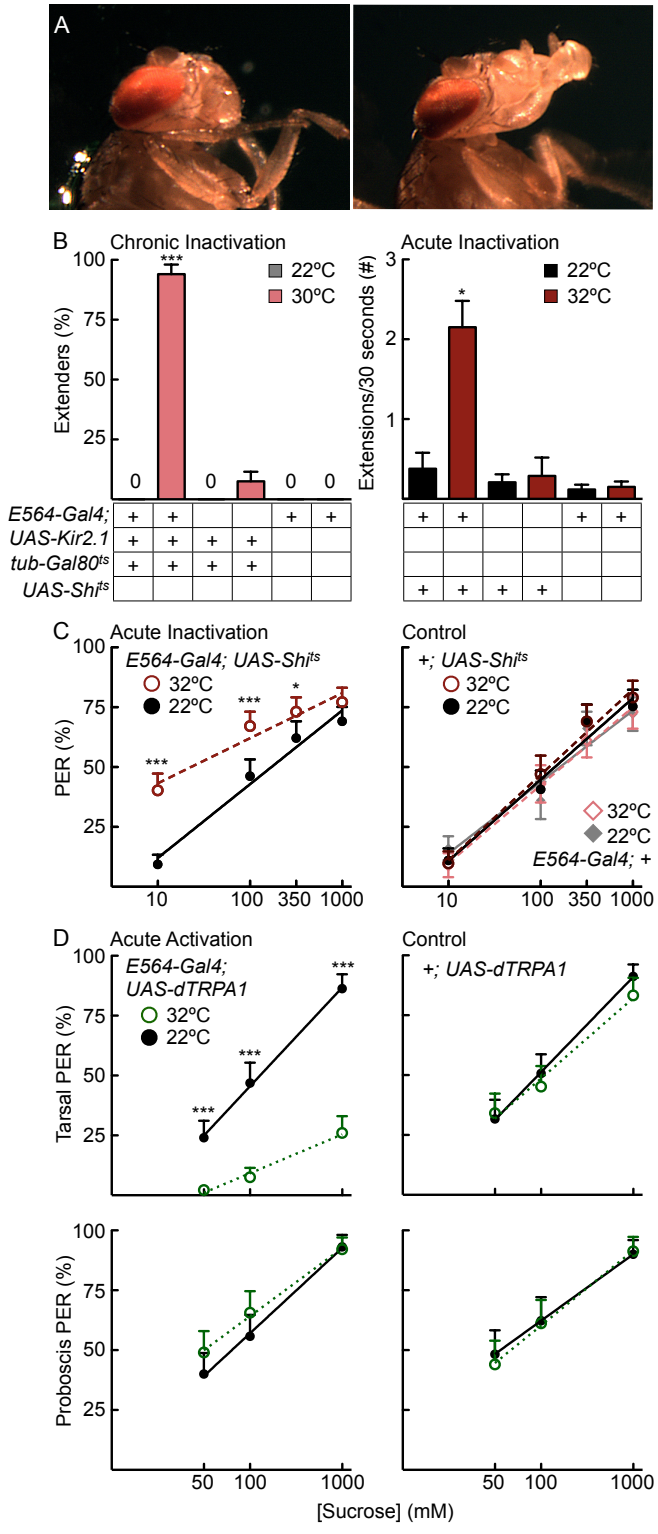

A
B
Chronic Inactivation
Acute Inactivation
Extenders (%)
Extensions/30 seconds (#)
22ºC
30ºC
32ºC
E564-Gal4;
UAS-Kir2.1
tub-Gal80ts
UAS-Shits
C
Acute Inactivation
Control
E564-Gal4; UAS-Shits
+; UAS-Shits
PER (%)
E564-Gal4; +
D
Acute Activation
E564-Gal4; UAS-dTRPA1
+; UAS-dTRPA1
Tarsal PER (%)
Proboscis PER (%)
[Sucrose] (mM)

Figure 2.2

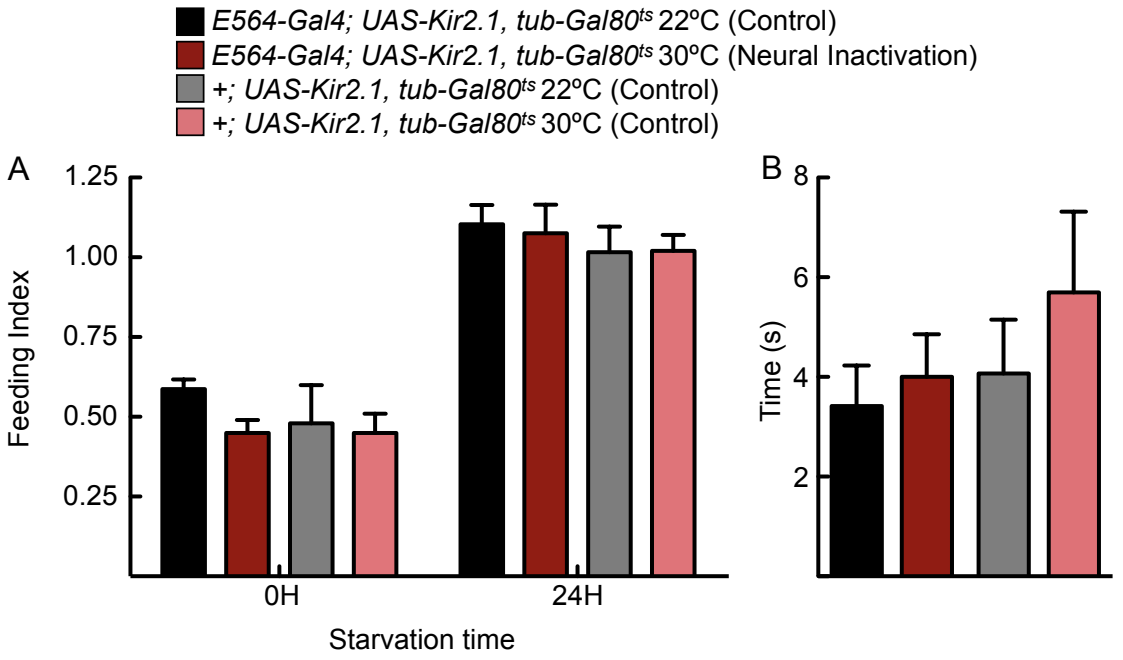

E564-Gal4; UAS-Kir2.1, tub-Gal80ts 22ºC (Control)
E564-Gal4; UAS-Kir2.1, tub-Gal80ts 30ºC (Neural Inactivation)
+; UAS-Kir2.1, tub-Gal80ts 22ºC (Control)
+; UAS-Kir2.1, tub-Gal80ts 30ºC (Control)
A
Feeding Index
1.25
1.00
0.75
0.50
0.25
0H
24H
Starvation time
B
Time (s)
8
6
4
2

Figure 2.3

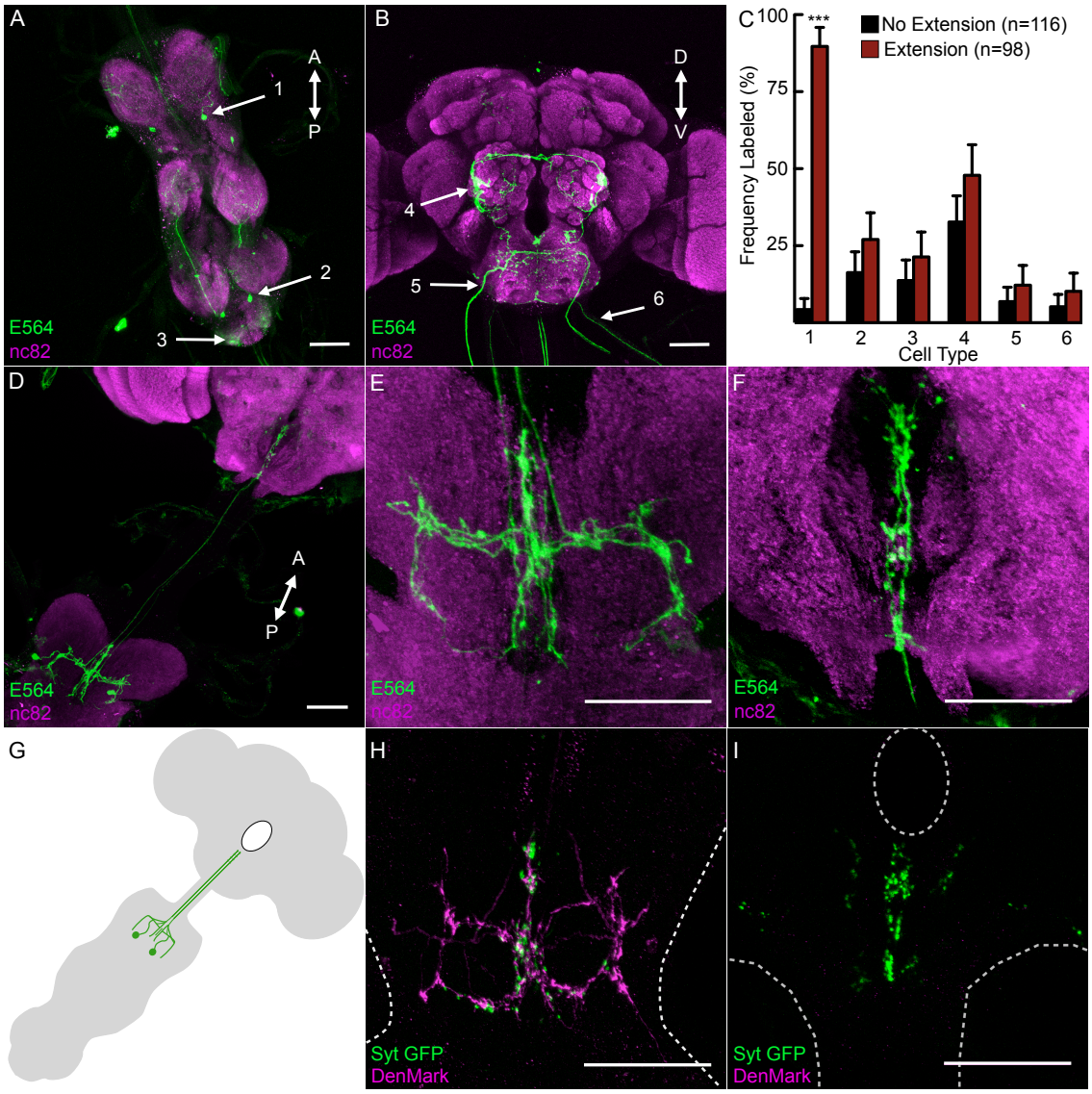

A
A
P
1
2
3
E564
nc82
B
D
V
4
5
6
E564
nc82
C
***
No Extension (n=116)
Extension (n=98)
Frequency Labeled (%)
100
75
50
25
1
2
3
4
5
6
Cell Type
D
A
P
E564
nc82
E
E564
nc82
F
E564
nc82
G
H
Syt GFP
DenMark
I
Syt GFP
DenMark

Figure 2.4

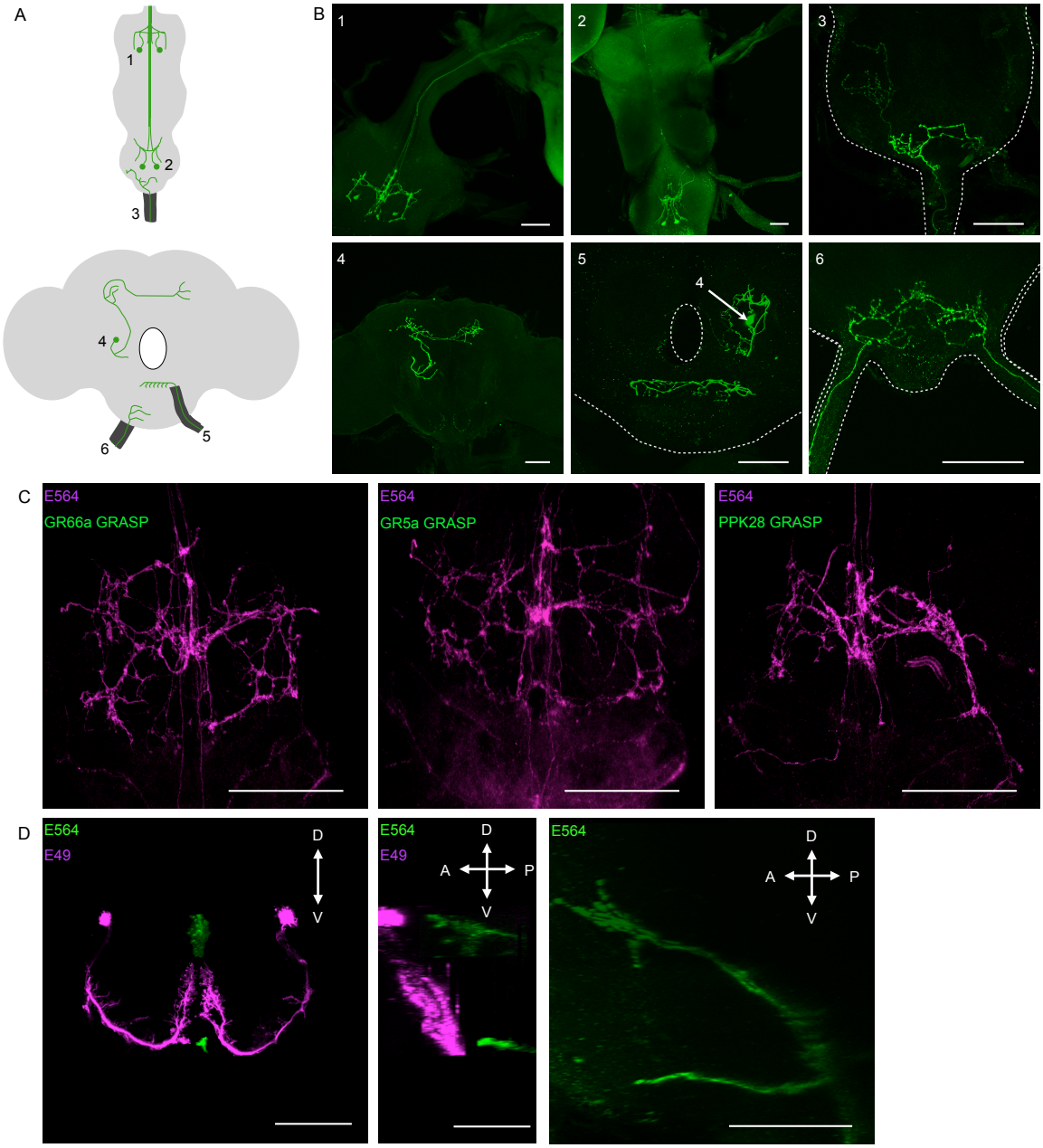

A
1
2
3
4
5
6
B
1
2
3
4
5
4
6
C
E564
GR66a GRASP
E564
GR5a GRASP
E564
PPK28 GRASP
D
E564
E49
D
V
E564
E49
D
A
P
V
E564
D
A
P
V

Figure 2.5

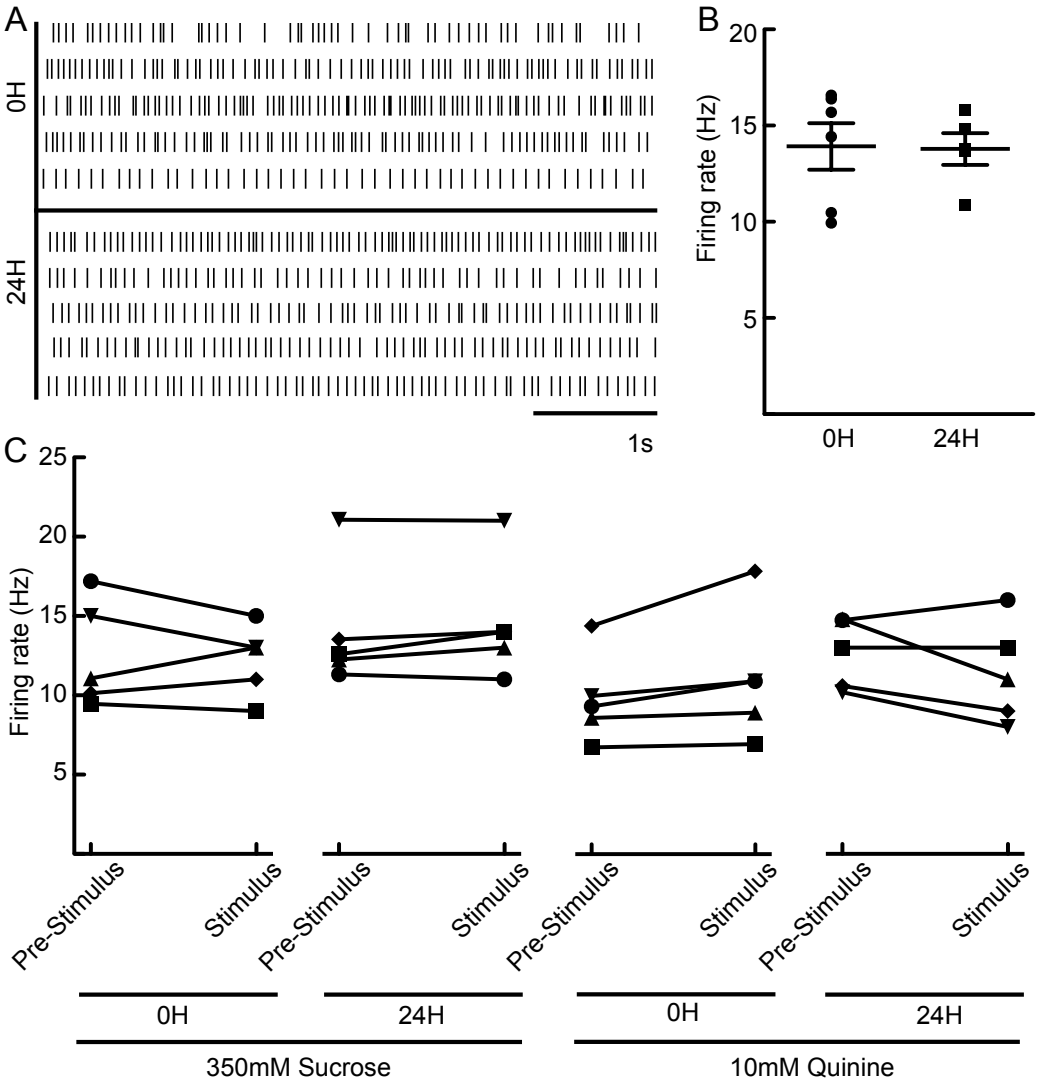

A
0H
24H
1s
B
Firing rate (Hz)
20
15
10
5
0H
24H
C
Firing rate (Hz)
25
20
15
10
5
Pre-Stimulus
Stimulus
Pre-Stimulus
Stimulus
Pre-Stimulus
Stimulus
Pre-Stimulus
Stimulus
0H
24H
0H
24H
350mM Sucrose
10mM Quinine

Figure 2.6

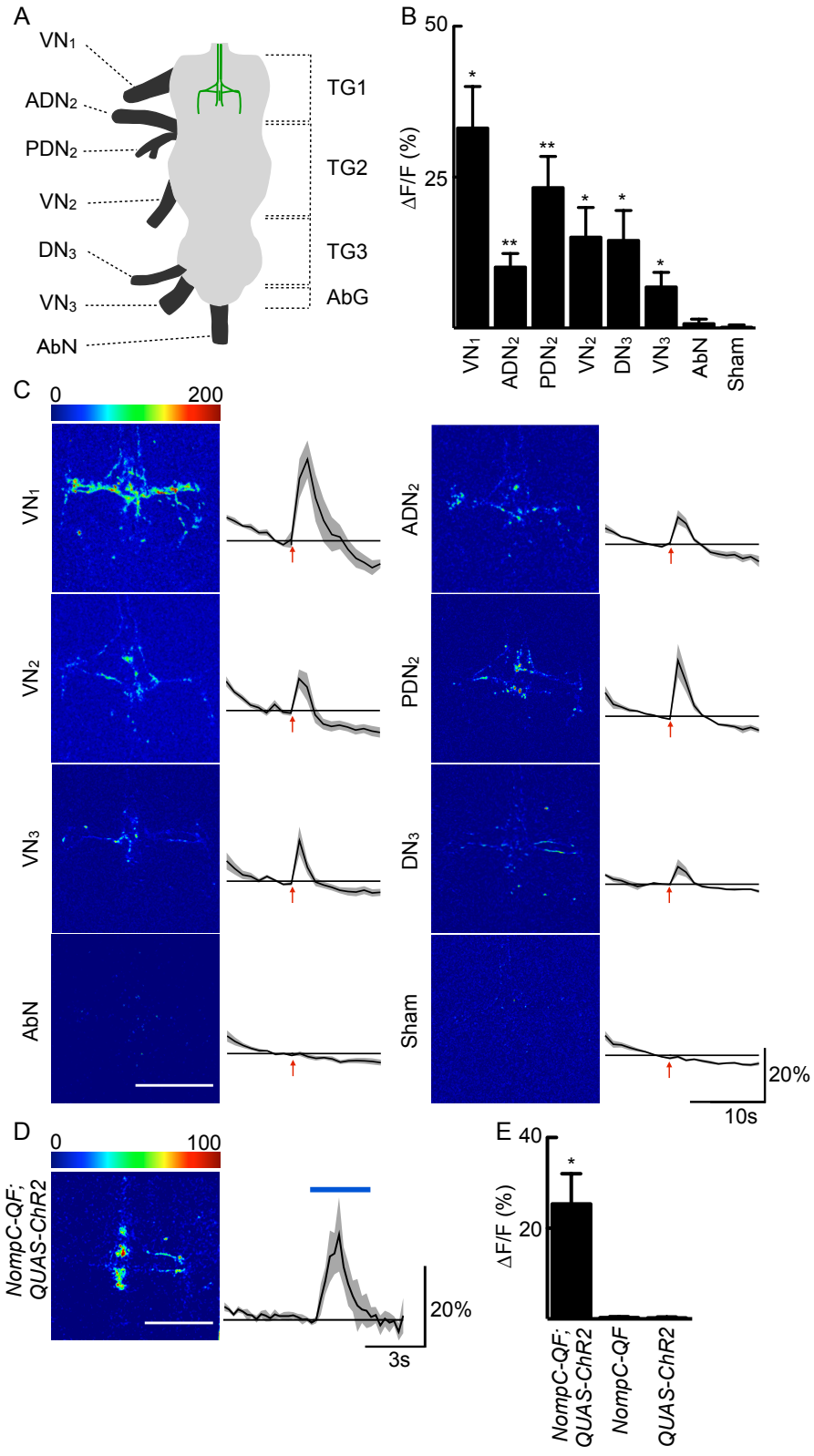

A
VN1
ADN2
PDN2
VN2
DN3
VN3
AbN
TG1
TG2
TG3
AbG
B
50
25
ΔF/F (%)
VN1
ADN2
PDN2
VN2
DN3
VN3
AbN
Sham
C
0
200
VN1
ADN2
VN2
PDN2
VN3
DN3
AbN
Sham
20%
10s
D
0
100
NompC-QF; QUAS-ChR2
20%
3s
E
40
20
ΔF/F (%)
NompC-QF; QUAS-ChR2
NompC-QF
QUAS-ChR2

Figure 2.7

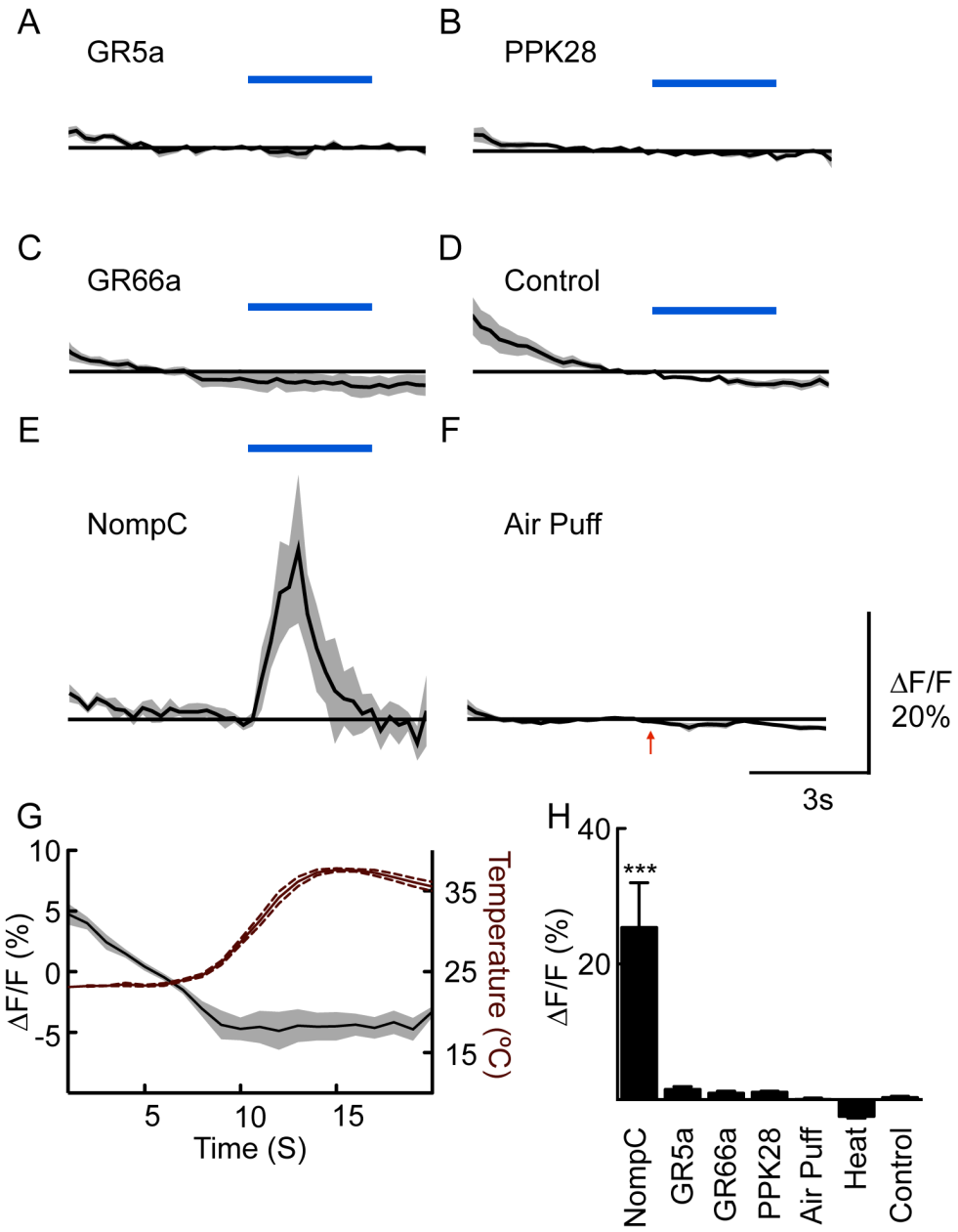

A
GR5a
B
PPK28
C
GR66a
D
Control
E
NompC
F
Air Puff
ΔF/F
20%
3s
G
ΔF/F (%)
Temperature (°C)
Time (S)
H
ΔF/F (%)
***
NompC
GR5a
GR66a
PPK28
Air Puff
Heat
Control

Figure 2.8

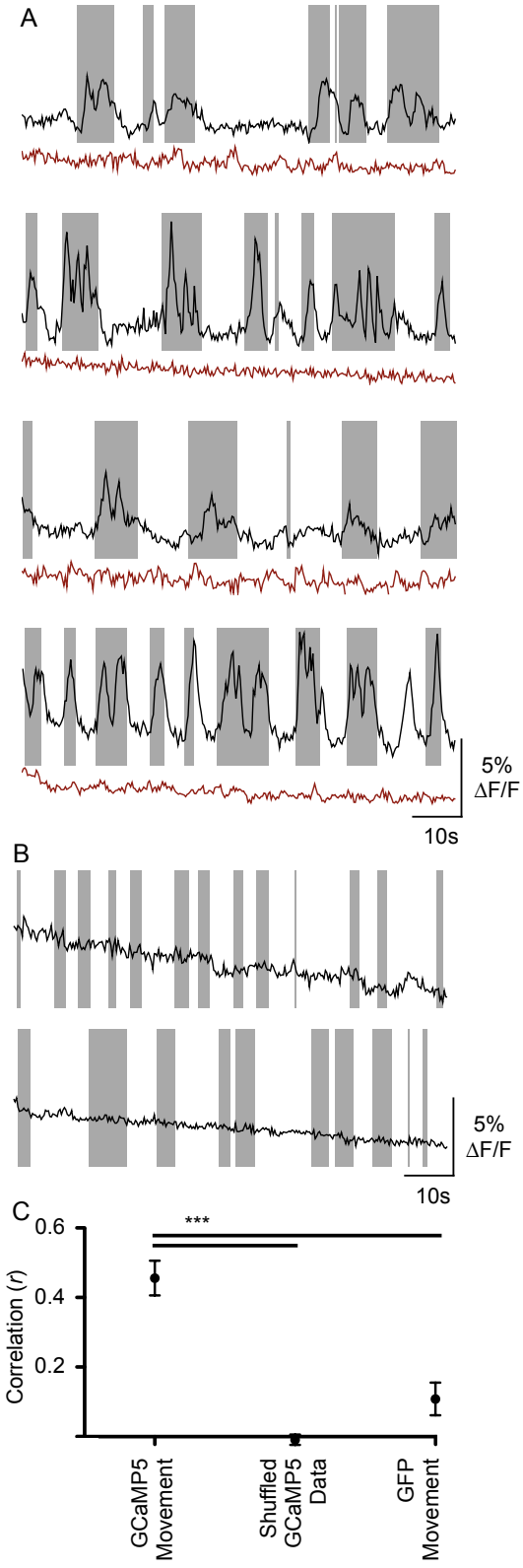

A
5%
ΔF/F
10s
B
5%
ΔF/F
10s
C
0.6
0.4
0.2
Correlation (r)
***
GCaMP5 Movement
Shuffled GCaMP5 Data
GFP Movement

Figure 2.9

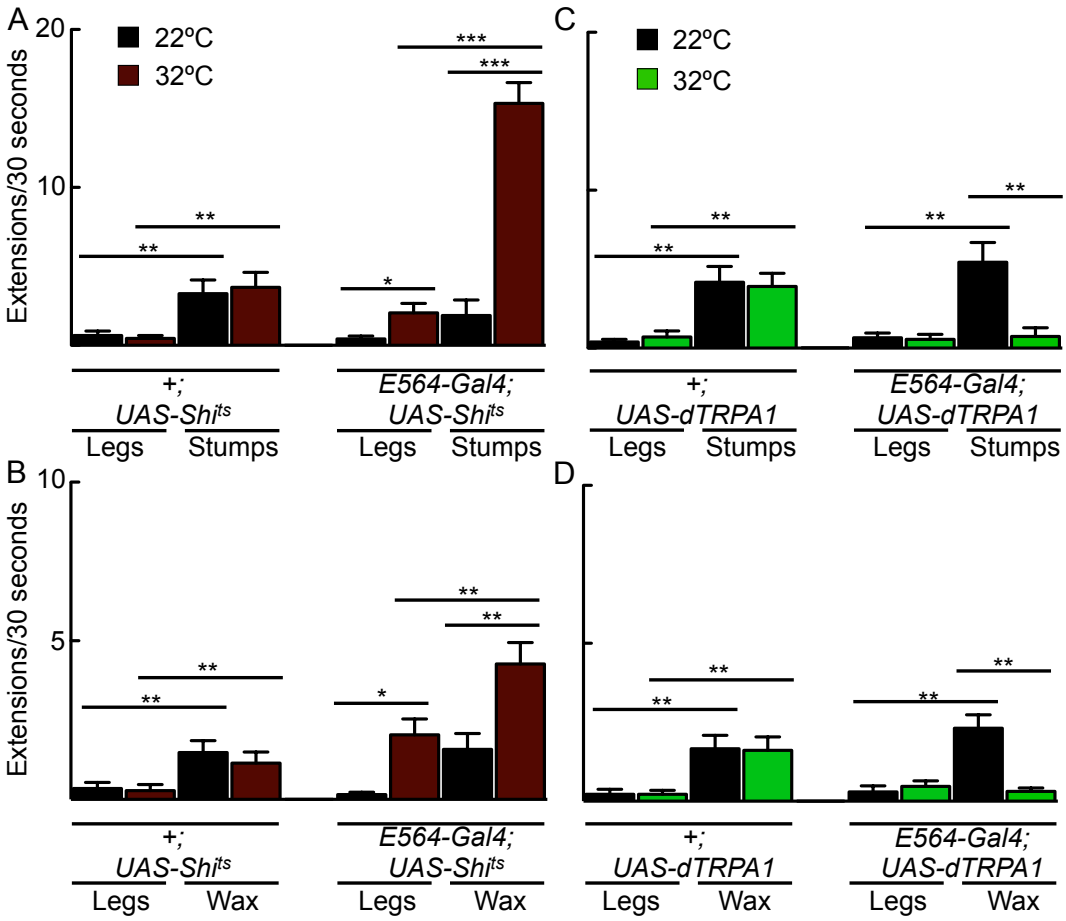

A
C
B
D
22ºC
32ºC
Extensions/30 seconds
20
10
5
+;
UAS-Shits
E564-Gal4;
UAS-Shits
+;
UAS-dTRPA1
E564-Gal4;
UAS-dTRPA1
Legs
Stumps
Wax
*
**
***

Figure 2.10

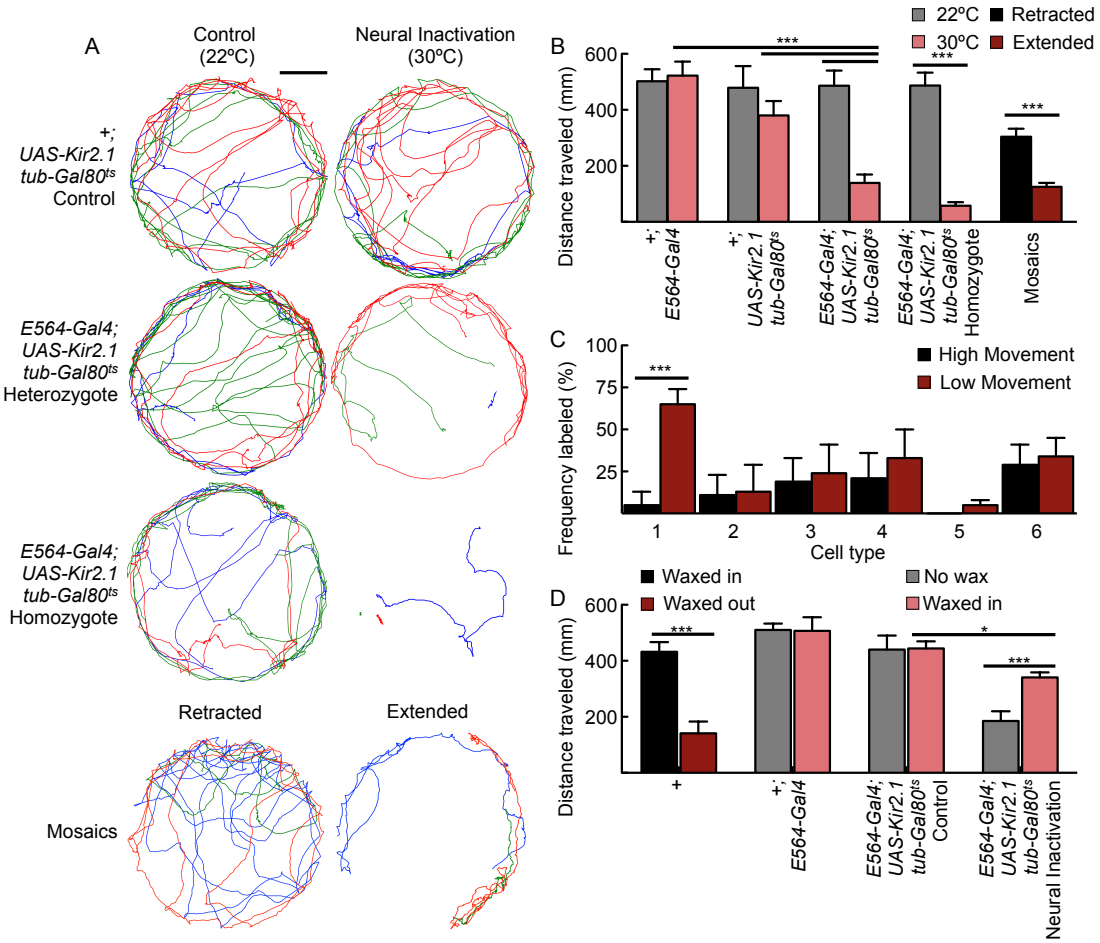
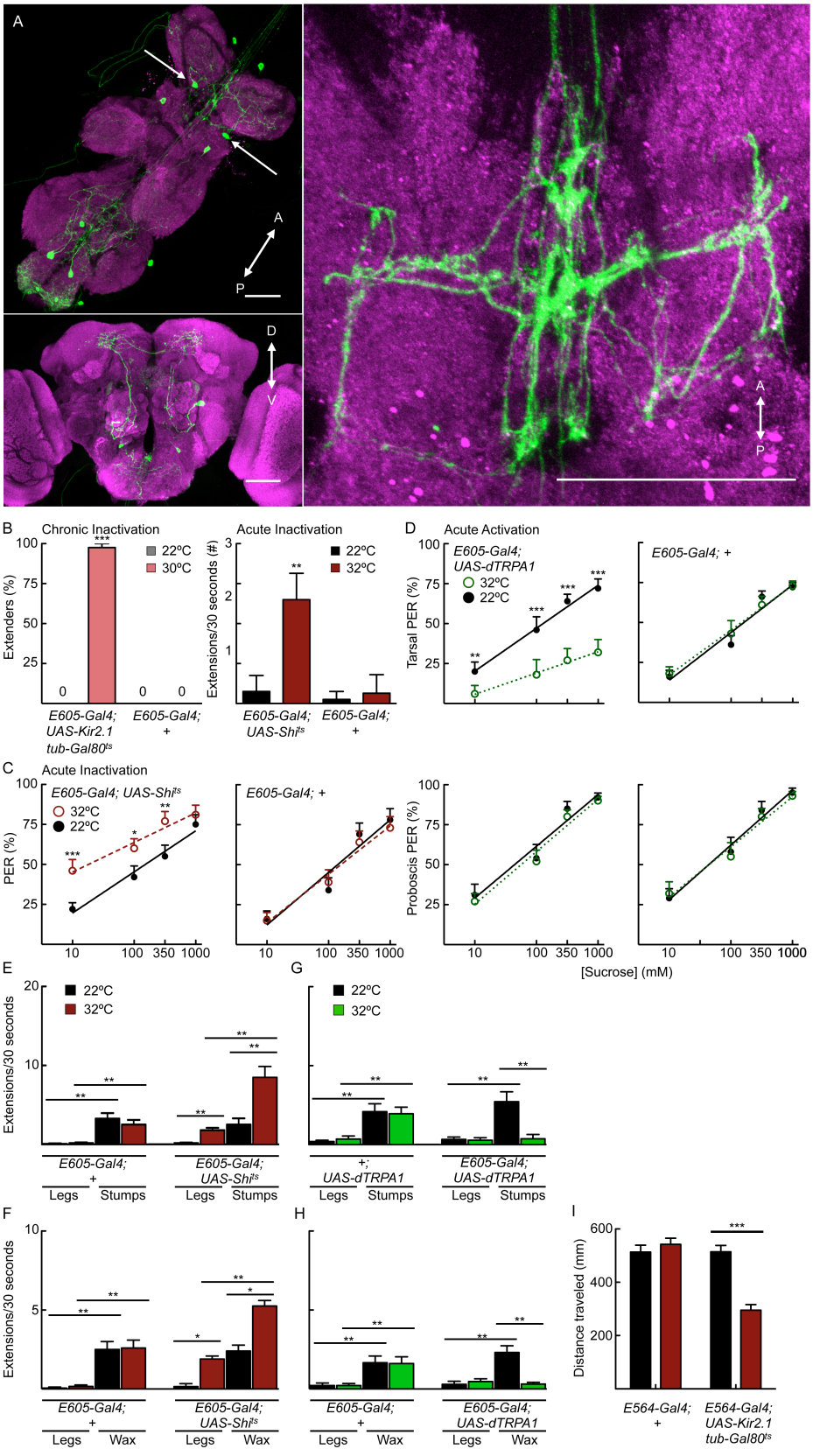

A
Control (22ºC)
Neural Inactivation (30ºC)
+; UAS-Kir2.1 tub-Gal80ts Control
E564-Gal4; UAS-Kir2.1 tub-Gal80ts Heterozygote
E564-Gal4; UAS-Kir2.1 tub-Gal80ts Homozygote
Retracted
Extended
Mosaics
B
22ºC
30ºC
Retracted
Extended
Distance traveled (mm)
600
400
200
***
+; E564-Gal4
+; UAS-Kir2.1 tub-Gal80ts
E564-Gal4; UAS-Kir2.1 tub-Gal80ts Homozygote
Mosaics
C
Frequency labeled (%)
100
75
50
25
High Movement
Low Movement
1
2
3
4
5
6
Cell type
D
Waxed in
Waxed out
No wax
Waxed in
Distance traveled (mm)
600
400
200
*
+
+; E564-Gal4
E564-Gal4; UAS-Kir2.1 tub-Gal80ts Control
E564-Gal4; UAS-Kir2.1 tub-Gal80ts Neural Inactivation

Figure 2.11


A
A
P
D
V
A
P
B
Chronic Inactivation
22ºC
30ºC
Extenders (%)
E605-Gal4; UAS-Kir2.1 tub-Gal80ts
E605-Gal4; +
Acute Inactivation
22ºC
32ºC
Extensions/30 seconds (#)
E605-Gal4; UASShits
E605-Gal4; +
D
Acute Activation
E605-Gal4; UAS-dTRPA1
32ºC
22ºC
Tarsal PER (%)
E605-Gal4; +
C
Acute Inactivation
E605-Gal4; UAS-Shits
32ºC
22ºC
PER (%)
E605-Gal4; +
Proboscis PER (%)
[Sucrose] (mM)
E
22ºC
32ºC
Extensions/30 seconds
E605-Gal4; +
E605-Gal4; UAS-Shits
Legs
Stumps
G
22ºC
32ºC
+; UAS-dTRPA1
E605-Gal4; UAS-dTRPA1
F
Extensions/30 seconds
E605-Gal4; +
E605-Gal4; UAS-Shits
Legs
Wax
H
E605-Gal4; +
E605-Gal4; UAS-dTRPA1
I
Distance traveled (mm)
E564-Gal4; +
E564-Gal4; UAS-Kir2.1 tub-Gal80ts

CHAPTER 3

Identification of putative synaptic partners of PER_{in} neurons

Summary

How modulatory neurons alter the activity in circuits to influence the probability of feeding behavior is unknown . The proboscis extension modulator PER_{in} identified in chapter 2 inhibits proboscis extension, providing the opportunity to examine how this neuron interacts with taste circuits to influence behavior. An anatomical screen of known modulators of feeding behavior in *Drosophila* identified the neurons expressing the neuropeptide Hugin as making possible synaptic contact with PER_{in} neurons, both with the dendrites in the nerve cord, as well as the axons in the suboesophegeal ganglion. Simultaneous Hugin stimulation while recording from PER_{in} neurons did not activate PER_{in}, suggesting that PER_{in} is not downstream of Hugin neurons. In contrast, stimulation of PER_{in} neurons produced strong calcium increases in Hugin neurons, showing that a subset of Hugin neurons are activated by PER_{in}. Silencing or activating Hugin neurons, however, has no impact on proboscis extension, suggesting that this interaction is not specifically relevant to feeding initiation.

Additionally, a behavioral suppressor screen using the InSITE Gal4 enhancer trap collection revealed lines that could suppress the constitutive phenotype seen in flies with PER_{in} chronically silenced. This screen identified a limited number of lines that likely influence proboscis extension and might be involved in PER_{in}-mediated modulation of proboscis extension.

Introduction

Currently, our knowledge of the neural substrates of feeding behavior in *Drosophila* is limited. Much is understood about peripheral detection of taste compounds, as well as the motor neurons which drive feeding behavior (Gordon and Scott, 2009; Manzo et al., 2012; Rajashekhar and Singh, 1994; Scott et al., 2001; Wang et al., 2004). A small number of interneurons in the SOG that influence feeding have also been described (Flood et al., 2013; Marella et al., 2012). Still, almost nothing is known about how these disparate elements come together to produce a functional circuit for feeding behaviors.

PER_{in} neurons do not appear to make synaptic contact with either the sensory or motor neurons of the feeding circuit (Figure 2.4CD). Several Gal4 lines that modulate feeding behavior have been identified in previous studies, and might be candidates to interact with PER_{in} neurons. *Drosophila* insulin-like peptide (DILP) is released when the animal is fed, and overexpression can suppress the fly's hunger response in the larval state (Wu et al., 2005). Adipokinetic hormone (AKH) is released when the fly is hungry and is essential for glucose homeostasis (Kim and Rulifson, 2004). Hugin, a neuromedin U homologue, shows high levels of expression in fed flies and suppresses the initiation of feeding (Melcher and Pankratz, 2005). NeuropeptideF (NPF) expression is high in starved flies and promotes food intake in fasted animals (Wu et al., 2003). Dopamine release increases in starved flies, and it promotes feeding initiation (Marella et al., 2012). How these different classes of neurons fit into the feeding circuit is still unknown. It is possible that one or more of them interact with PER_{in} neurons. In this chapter, I examined the projection patterns of these neurons to reveal neurons that make potential synaptic contacts with PER_{in}.

As other, unidentified neurons might be synaptic partners with PER_{in}, an alternative strategy to identify potential partners is a behavioral screen for neurons that suppress the behavioral phenotype of PER_{in} inactivation. I used this behavioral screening strategy to potentially find neurons downstream of PER_{in} in the feeding circuit, as well as other neurons that influence proboscis extension.

The anatomical screen of neuromodulators revealed Hugin neurons as potential synaptic partners with PER_{in}, and the behavioral screen for suppression of proboscis extension revealed a small number of Gal4 lines that express in neurons required for the constitutive proboscis extension phenotype seen when PER_{in} neurons are silenced. These lines provide a starting point for the continued analysis of PER_{in} neurons and their role in modulating proboscis extension.

Results

Hugin neurons are potential synaptic partners with PER_{in} neurons

To determine if known modulators of feeding behavior might be synaptic partners with PER_{in} neurons, I examined the nervous system of flies expressing GFP in both PER_{in} neurons and neurons expressing DILP2, AKH, NPF, Hugin, or dopamine. DILP2, AKH, and NPF neurons did not project near PER_{in} dendrites or axons; dopamine neurons were too dense to clearly determine connectivity; and Hugin neurons appeared to contact both PER_{in} dendrites as well as their axons.

Double labeling of Hugin and PER_{in} neurons revealed significant overlap of projections in both the VNC and SOG (Figure 3.1AB). Hugin neurons send a descending projection down the ventral nerve cord, which comes into close contact with the posterior projections of PER_{in} dendrites (Figure3.1A). Hugin neurons also send dense projections to the dorsal medial region of the SOG where PER_{in} axons terminate (Figure3.1B). This close overlap in projections suggests synaptic contact.

To further test this possibility, I utilized the GRASP method to verify that Hugin and PER_{in} neurons come in close contact with each other (Gordon and Scott, 2009). This technique utilizes a membrane-bound split GFP, with one portion of GFP expressed in once cell type, and the other in a different type. Neurons that come in close contact reconstitute GFP, and therefore are potential synaptic partners. As suggested by the anatomy, GRASP signal is seen in both the dendrites and axons of PER_{in} neurons (Figure3.1CD). Therefore it is possible that a subset of Hugin neurons is upstream of PER_{in} neurons, and a subset is downstream. It is also possible that the same Hugin neurons form reciprocal connections with PER_{in} neurons.

Activating PER_{in} neurons drives calcium responses in Hugin neurons.

To test whether there is functional connectivity between Hugin and PER_{in} neurons, I expressed the heat-activated ion channel dTRPA1 in Hugin neurons to test if they could modulate activity in PER_{in} neurons. Hugin neurons can then be activated by heating the fly brain using an infrared laser while recording from PER_{in}, any acute influences on PER_{in} activity by Hugin neurons could be determined. The application of heat drives activity in Hugin neurons expressing dTRPA1, but not GFP alone, indicating that infrared light drives activity in Hugin neurons in a dTRPA1-dependent manner (Figure 3.2AB). Recording from PER_{in} neurons showed no change in activity during stimulation of Hugin neurons (Figure3.2AB). It is possible, then, that Hugin neurons do not form functional synapses with PER_{in} dendrites, or that Hugin modulates PER_{in} neurons on a time scale not visible in these brief activation experiments (2 seconds).

To test whether PER_{in} neurons are upstream of Hugin neurons, I activated PER_{in} neurons with dTRPA1 and simultaneously monitored GCaMP responses in Hugin neurons. This method allowed the simultaneous monitoring of all ~20 Hugin-expressing neurons during PER_{in} activation. Interstingly, a subset of Hugin neurons responded to PER_{in} activation, but not to heat alone (Figure 3.2C). While it cannot be determined if it is the same subset between animals, it does appear that only 2-4 cells per line responded to PER_{in} activation.

Manipulating Hugin neurons does not alter PER behavior

If Hugin neurons are part of a functional circuit with PER_{in}, it seems likely that they would play a role in the modulation of proboscis extension in general or in the PER_{in}-mediated modulation of proboscis extension. Acutely silencing or activating Hugin neurons using Shi^{ts} or dTRPA1, respectively, shows no consistent affect on proboscis extension (Figure 3.3A). This contrasts with PER_{in} neurons, whose activity suppresses proboscis extension (Figure 2.1D). While Hugin manipulations alone may not be sufficient to alter proboscis extension, they may be required for the expression of PER_{in}-mediated suppression. To test this possibility, I suppressed synaptic transmission in Hugin neurons during dTRPA1-mediated activation of PER_{in}, (Sweeney et al., 1995). Blocking synaptic transmission in Hugin neurons did not alter PER_{in} suppression of proboscis extension (Figure 3.3B). Simultaneous activation of both Hugin and PER_{in} in neurons with dTRPA1 also failed to alter PER_{in} suppression of proboscis extension (Figure 3.3B). These data suggest that Hugin neurons, while apparently activated by PER_{in}, are not involved in PER_{in} modulation of proboscis extension. It is also possible that different subsets of Hugin neurons alter proboscis extension in opposing ways, and that activating or silencing the whole population results in no behavioral effect.

A behavioral screen for modulators of proboscis extension

The constitutive proboscis extension phenotype observed when PER_{in} neurons are chronically silenced provides an opportunity to screen for neurons whose activity is required to express this behavior. This method should identify any neurons involved in generating proboscis extension (like motor neurons), even if they may not have a strong phenotype when silenced on their own.

Flies expressing Kir2.1 in PER_{in} neurons simultaneously with neurons in 110 Gal4 lines in the InSITE collection, 15 neuromodulator Gal4 lines, and PER_{in} alone were assayed for any effect on the constitutive proboscis extension phenotype observed with PER_{in} silencing. Control lines and lines not affecting proboscis extension should show the full proboscis extension phenotype seen when PER_{in} neurons were silenced alone (Figure 2.1B), while those affecting proboscis extension should exhibit a reduction in constitutive extension observed.

Most of the 125 lines tested showed high probability of constitutive extension with a limited number showing a reduction (Figure3.4AB). Using two standard deviations below the mean probability of extension (0.88) as a cutoff for lines of interest (0.6) yielded 7 InSITE lines and 6 neuromodulator lines that express in neurons required for constitutive proboscis extension (Figure 3.4AB, red line). Some of these neuromodulator lines such as *DILP2-Gal4* and *AKH-Gal4* have been implicated in feeding behavior, suggesting that the screen correctly identified neurons that might be involved in proboscis extension. Other lines known to play a role in proboscis extension modulation such as *TH-Gal4* showed no effect, indicating that this method will not be able to identify all of the neurons involved in proboscis extension. Further characterization of the expression pattern of these lines is required before any conclusions can be drawn regarding the identity of neurons responsible for the observed phenotypes.

Conclusions

These experiments have identified putative synaptic partners with PER_{in} neurons, as well as Gal4 lines expressing in neurons required for constitutive proboscis extension. Utilizing sparse Gal4 lines, it is possible to identify potential partners by proximity of projections and then to verify this observation with GRASP. Identified lines can then be tested for connectivity through electrophysiology and calcium imaging.

Hugin appears to be a good candidate for a post-synaptic partner of PER_{in}, and possibly a pre-synaptic partner as well. Imaging of Hugin neurons indicates that a subset of these neurons respond to activation of PER_{in} neurons. Whether the neurons activated are consistent across animals is unclear, but the development of techniques such as photoactivatable GFP will allow anatomical comparisons of activated neurons across animals. Hugin neurons are not morphologically identical, with at least 4 distinct types identified in larvae (Bader et al., 2007). Additionally, 3 of the 4 types project to the dorsal medial SOG, the termination site of PER_{in} axons in the SOG, making it difficult to determine the identity of the Hugin neurons activated by PER_{in} neurons. These experiments were also limited by the lack of more sensitive calcium indicators. Further inspection of the PER_{in}-Hugin circuit would benefit from the use of the improved GCaMPs, which have a significantly higher signal-to-noise ratio as well as faster kinetics.

The anatomy of Hugin and PER_{in} strongly suggests reciprocal connections, although the physiology is only consistent with Hugin neurons as post-synaptic to PER_{in}. It is very possible that Hugin neurons modulate PER_{in} over a longer time scale than can be observed in the TRP-activation paradigm used in these experiments. Similarly, if there were only sub-threshold effects on PER_{in} neurons from Hugin stimulation, the method I employed of cell-attached recording would be unable to identify them.

Behaviorally, the interaction between PER_{in} and Hugin is unclear. Manipulating PER_{in} neurons results in rather drastic proboscis extension phenotypes, while similar manipulations of Hugin neurons have no effect. Behavioral experiments where one cell type is activated and the other is inhibited or co-activated also failed to show any affect of Hugin manipulation. The heterogeneity of the Hugin line may play a role in these findings, as different Hugin cell types may influence proboscis extension in opposing ways. Restriction of the *Hugin-Gal4* line to fewer neurons using Gal80 or similar techniques could resolve this problem. It might also be possible that the PER_{in}-Hugin interaction has no effect on proboscis extension.

Utilizing the constitutive proboscis phenotype in PER_{in}-silenced animals has proved a useful tool in the screening for neurons required for proboscis extension. This method is attractive as it does not require any taste-stimulationallowing large numbers of lines can be screened in very little time. The lack of dependency on taste stimulation might also reveal modulators of proboscis extension that play no role in gustatory processing. For example, silencing neurons in the *DILP2-Gal4* line has no affect on proboscis extension to sucrose, but this screen identified it as one of the strongest repressors of constitutive extension. This method also identified lines

from the InSITE collection that warrant further investigation. As I only selected sparsely-expressing lines for this screen, it is likely possible to figure out which neurons in these lines repress constitutive proboscis extension and how they fit into the overall feeding circuit.

Using anatomical, behavioral, and functional data it is possible to connect the different elements of a circuit into a more coherent whole. The gustatory circuitry in the fly lacks the modular organization of the olfactory system, making the identification of higher-order neurons a significantly more cumbersome endeavor. By using the identified modulators of feeding behavior, such as PER_{in}, it is possible to start assembling a more complete picture of the neural circuits for feeding behavior.

Materials and methods.

Fly strains

The following fly lines were used: *E564-Gal4* (from the Gal4 collection kindly proved by Ulrike Heberlein), *Hugin-Gal4, Hugin-YFP* (Melcher and Pankratz, 2005), *UAS-Kir2.1* (Baines et al., 2001), *tub-Gal80ts* (McGuire et al., 2004), *UAS- CD4::spGFP1-10, LexAop-CD4::spGFP11,* , *UAS-mCD8::GFP* (Lee and Luo, 1999), *UAS-dTRPA1* (Hamada et al., 2008), *UAS- UAS-Shi^{ts}* (Kitamoto, 2001), *UAS-mCD8:: dsRed* (Ye et al., 2007), *LexAop-GCaMP1.3, E605-Gal4, IsoD1,* and enhancer trap Gal4 lines from the InSITE collection (Gohl et al., 2011). Flies were grown on standard fly food.

Immunohistochemistry

Antibody staining and imaging was carried out as previously described (Wang et al., 2004). The following antibodies were used: rabbit anti-GFP (Invitrogen, 1:1,000), mouse anti-GFP (Invitrogen, 1:1000), rat anti-CD8 (Cederlane labs, 1:500). Brightness or contrast of single channels was adjusted for the entire image using ImageJ software.

Transgenic flies

Hugin-LexA flies were generated by PCR amplification of 1.9kb of the Hugin promoter sequence from wild type flies (w^b) and cloning into the pLOT vector. Primers for amplification were 5' CTTCAGGGCCTTGGCT and 3' GGGACAACTGATGCCA.

Proboscis extension

PER was performed as previously described (Marella et al., 2012), except that each individual stimulation of each animal was treated as an independent data point. For the Shibire^{ts} inducible silencing experiments and the dTRPA1 inducible activation experiments, flies were transferred to a heating block at 32^o C for X minutes and then assayed for behavior. The Shibire^{ts} flies were raised at 19^{o}C. Constitutive extension was determined as a complete extension of the proboscis (with both the rostrum and haustellum fully extended) maintained over several seconds in the absences of stimulus. For the *UAS-Kir2.1, tub-Gal80^{ts}* experiments, Kir2.1 was induced by a 3-day temperature shift to 30^{o}C then returned to 22^{o}C for one day prior to testing.

Electrophysiology

Hugin recordings were performed as previously described (Marella et al., 2012). Hugin neurons were identified by GFP fluorescence. For PER$_{in}$ recordings, flies were immobilized ventral side up, with cover glass separating the front tarsi and head of the fly from the recording chamber. E564 neurons were labeled with GFP and PER$_{in}$ neurons identified for recordings based on their fluorescence and anatomical position. For Hugin recordings, data was band-passed filtered between 10 and 300Hz using a butterworth-type filter. Pre-stimulus spike rates were calculated using 15s of recording prior to stimulation; post-stimulus spike rates were calculated using 1s of recording after stimulation.

Laser activation of dTRPA1

The laser used in electrophysiology experiments was custom-built with an IR laser module (808 nm, 3.2-V dc, 150 mW; AixiZ Service and International LLC)(Masek and Scott, 2010). A voltage regulator was used to adjust the power output, with 2-3 V as the output sufficient to heat the fly's brain.

Calcium imaging

G-CaMP3 responses were monitored as previously described (Marella et al., 2006), Hugin neurons were imaged at 13 Hz on a 3i spinning disk confocal system, using a 20x water objective. For the heat stimulus, a custom heat probe was placed directly under the fly, and temperature was ramped to 30^{o}C while imaging.

Figure 3.1. Hugin neurons form putative synapses with PER_{in} neurons in the VNC and SOG.

A. Expression of *UAS-CD8::dsRed* in *E564-Gal4* neurons (green) and *Hugin-YFP* (magenta) in the VNC. Panels show both channels (left), *E564* alone (middle), and *Hugin* (right). The region shown is the same as in Figure 2.3EH
B. Expression of *UAS-CD8::dsRed* in *E564-Gal4* neurons (green) and *Hugin-YFP* (magenta) in the SOG. Panels show both channels (left), *E564* alone (middle), and *Hugin* (right). The dorsal medial portion of the SOG containing PER_{in} axons is outlined with a dotted circle. The region shown is the same as in Figure 2.3FI
C. Contacts between Hugin and PER_{in} neurons in the VNC as examined by the GRASP method. Flies were of the genotype *E564-Gal4/LexAop-CD4::GFP11; Hugin-LexA/UAS-GFP1-10, UAS-CD8::dsRed*. PER_{in} dendrites were visualized with an anti-CD8 antibody (magenta), and GRASP signal with anti-GFP antibody. Panels show both channels (left), GRASP alone (middle), and PER_{in} dendrites alone (right). The region shown is the same as in Figure 2.3EH
D. Contacts between Hugin and PER_{in} neurons in the SOG as examined by the GRASP method. Flies were of the genotype *E564-Gal4/LexAop-CD4::GFP11; Hugin-LexA/UAS-GFP1-10, UAS-CD8::dsRed*. PER_{in} dendrites were visualized with an anti-CD8 antibody (magenta), and GRASP signal with anti-GFP antibody. Panels show both channels (left), GRASP alone (middle), and PER_{in} dendrites alone (right). The region shown is the same as in Figure 2.3FI. All scale bars represent 50μM

Figure 3.2. Activation of PER_{in} neurons stimulates Hugin neurons, but activation of Hugin neurons does not alter PER_{in} activity.

A. Raster plots of 5 seconds of recordings Hugin neurons (top and middle panels) and PER_{in} neurons (bottom panel) during heating of the fly brain (pink boxes). Hugin neurons expressing dTRPA1 (top panel) show reliable activation to heating of the brain, while those expressing only GFP (middle panel) show no alteration in activity. PER_{in} neurons show no modulation of activity when Hugin neurons expressing dTRPA1 are stimulated with heat (bottom panel).
B. Summary of data shown in A. Spike rates were calculated in the two seconds before heating (pre-stimulus), and the two seconds during heating (stimulus). PER_{in} neurons are not modulated by dTRPA1-mediated activation of Hugin neurons. Stimulus duration was 2 seconds. n=5 flies for each condition, paired t-test
**P<0.01.
C. Hugin neurons show calcium responses to activating PER_{in} neurons (left), but not in control flies (right). Black line is mean ΔF/F value, with gray areas representing ±SEM. Red line is mean temperature with dashed lines representing ±SEM.

Figure 3.3. Hugin silencing and activation does not alter proboscis extension response, or alter PER_{in}-mediated suppression of proboscis extension.

A. Acutely silencing neurons in *Hugin-Gal4*; *UAS-Shi^{ts}* flies (left) does not alter PER. Only the lowest concentration tested shows a modest increase in PER. Acutely

activating neurons in *Hugin-Gal4; UAS-dTRPA1* flies (right) does not alter PER. n=26-48 flies/condition, mean±95% CI, Fisher's exact test, *P<0.05.
B. Silencing Hugin neurons in *E564-Gal4/Hugin-LexA; UAS-dTRPA1/LexAop-TNT* does not alter *E564-Gal4; UAS-dTRPA1* mediated suppression of PER (Figure 2.1D). Activating Hugin neurons in *E564-Gal4/Hugin-Gal4; UAS-dTRPA1* does not alter *E564-Gal4; UAS-dTRPA1* mediated suppression of PER (Figure2.1D). n=24-44 flies/condition, mean±95% CI, Fisher's exact test, ***P<0.001.

Figure 3.4. A screen for suppression of constitutive proboscis extension.

A. Probability of constitutive extension in the 110 InSITE lines tested, including control flies (IsoD1). Lines were sorted by their probability score. A score of 1 indicates that all flies tested showed constitutive proboscis extension, a score of 0 indicates none of the flies tested showed constitutive extension. Two standard deviations below the mean (0.6) is represented by the dashed red line. Flies were of the genotype *E605-Gal4; Kir2.1, Gal80^{ts}/LineXXX-Gal4*
B. Probability of constitutive extension of the 16 neuromodulator lines tested. The same cutoff as in A is shown here as a dashed red line.

Figure 3.1

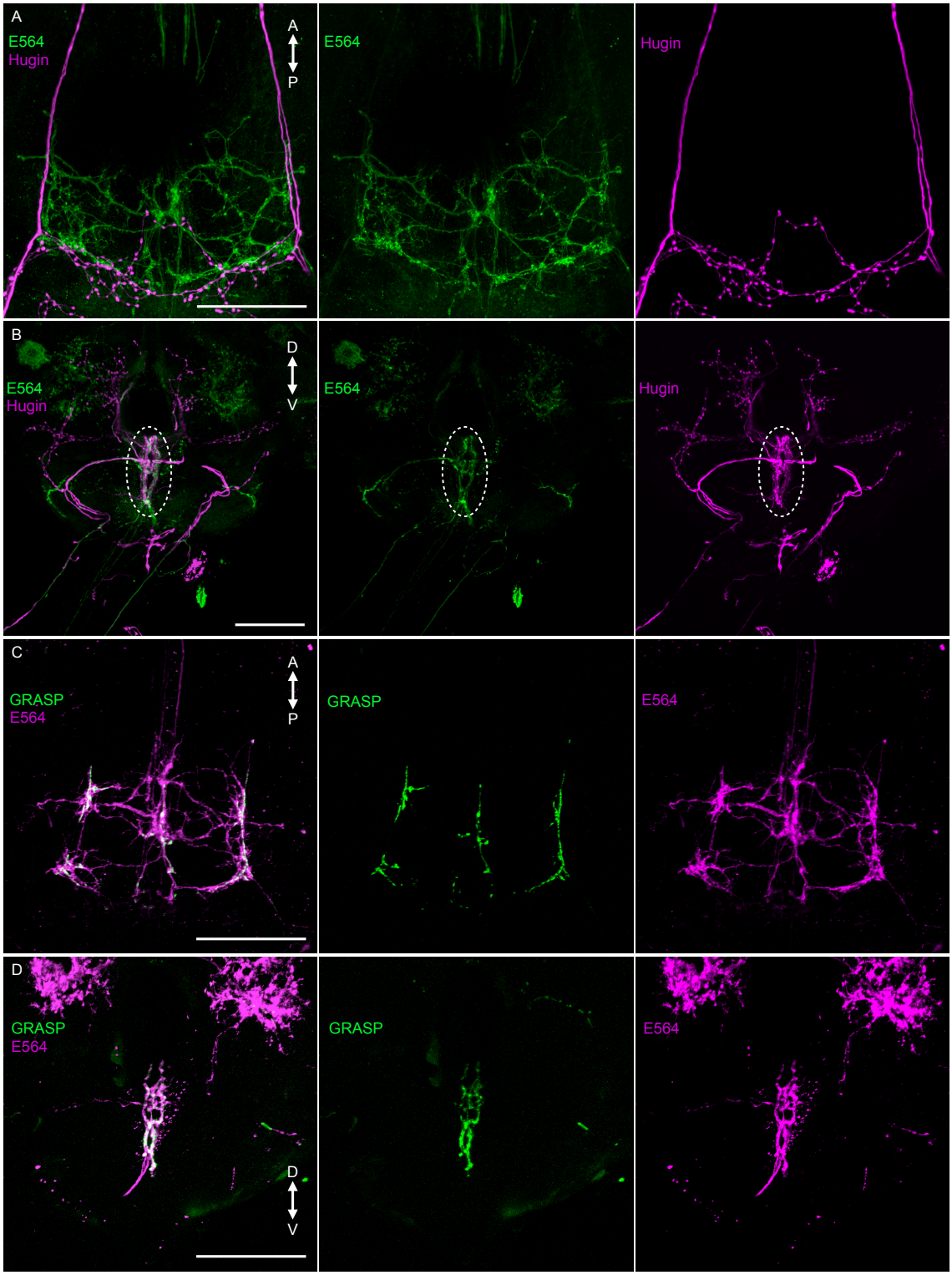

A
E564
Hugin
A
P
E564
Hugin
B
E564
Hugin
D
V
E564
Hugin
C
GRASP
E564
A
P
GRASP
E564
D
GRASP
E564
D
V
GRASP
E564

Figure 3.2

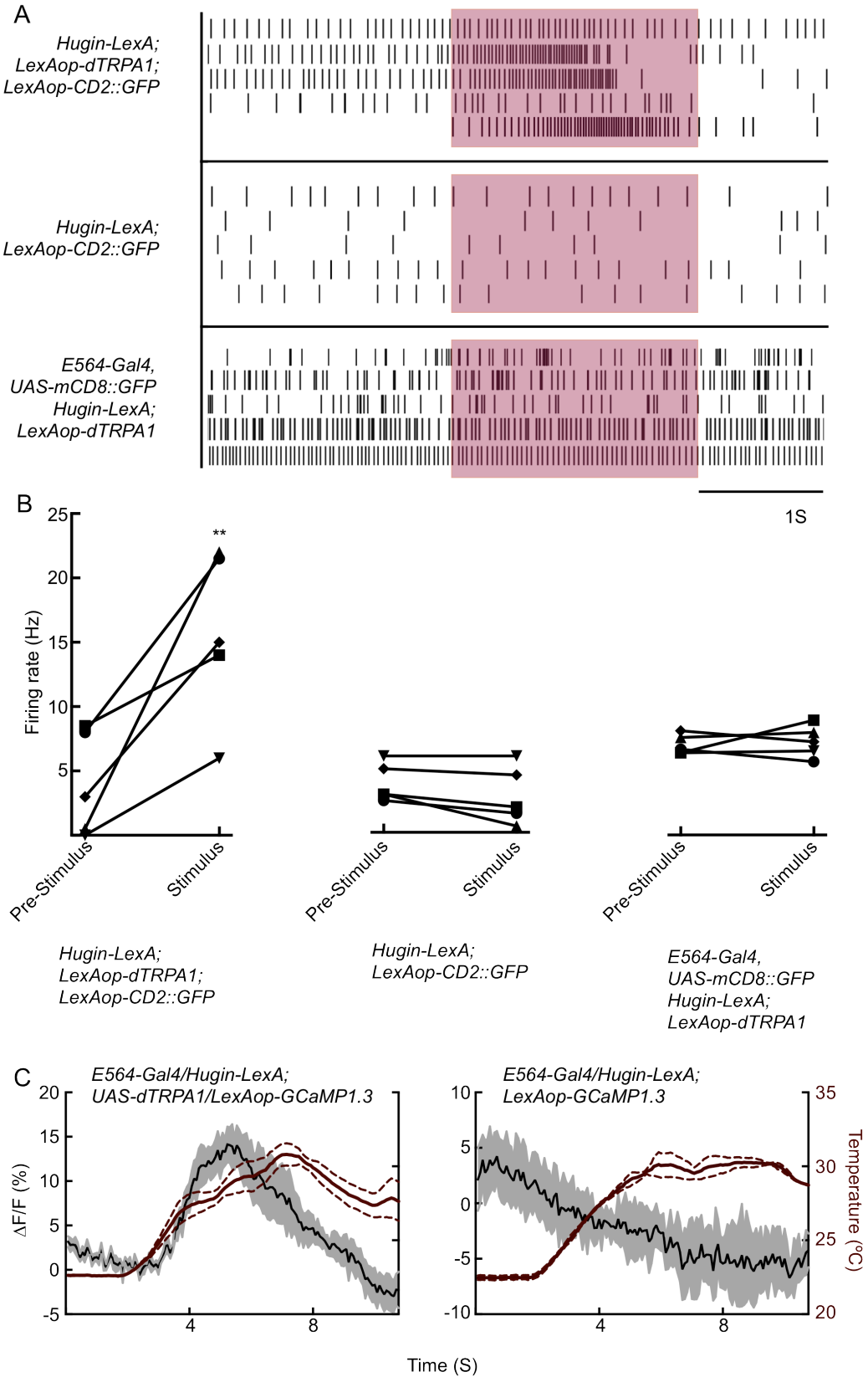

A
Hugin-LexA;
LexAop-dTRPA1;
LexAop-CD2::GFP
Hugin-LexA;
LexAop-CD2::GFP
E564-Gal4,
UAS-mCD8::GFP
Hugin-LexA;
LexAop-dTRPA1
1S
B
Firing rate (Hz)
**
Pre-Stimulus
Stimulus
Hugin-LexA;
LexAop-dTRPA1;
LexAop-CD2::GFP
Hugin-LexA;
LexAop-CD2::GFP
E564-Gal4,
UAS-mCD8::GFP
Hugin-LexA;
LexAop-dTRPA1
C
E564-Gal4/Hugin-LexA;
UAS-dTRPA1/LexAop-GCaMP1.3
E564-Gal4/Hugin-LexA;
LexAop-GCaMP1.3
ΔF/F (%)
Temperature (°C)
Time (S)

Figure 3.3

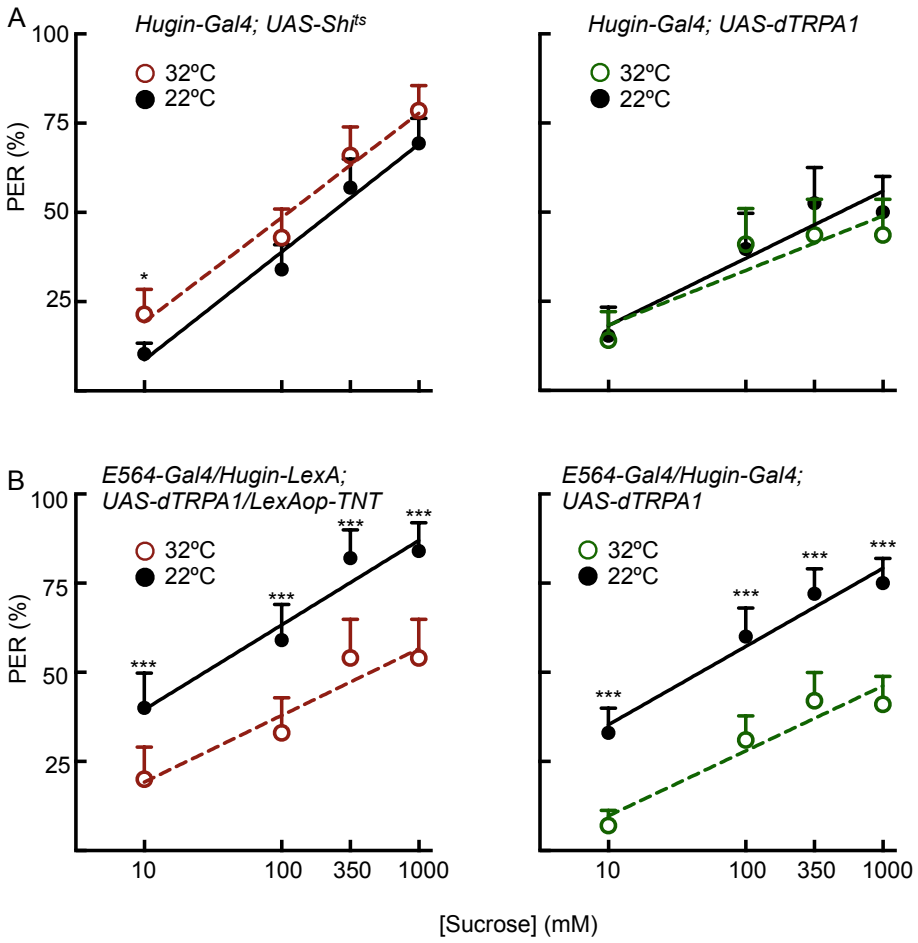
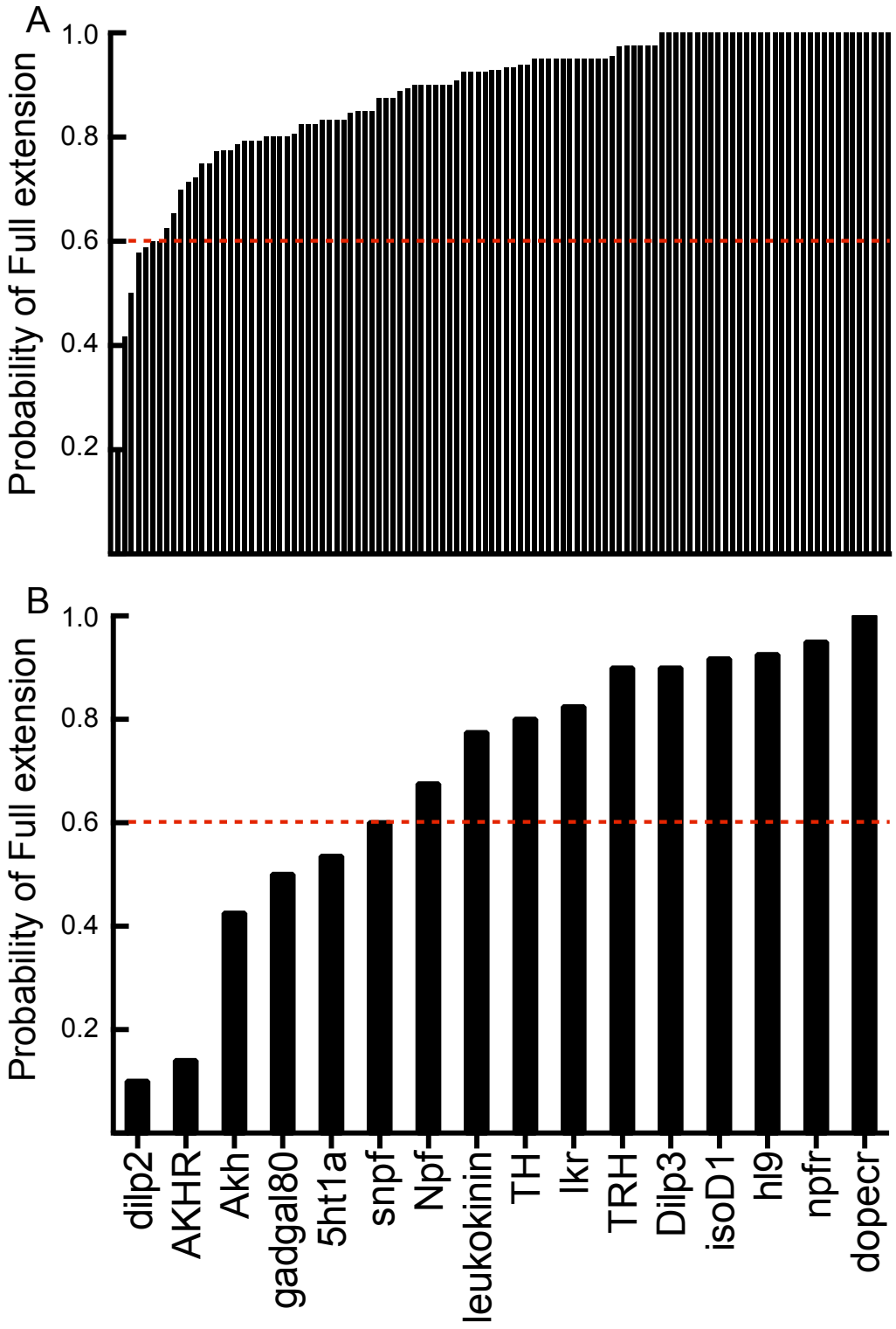

A
Hugin-Gal4; UAS-Shits
32ºC
22ºC
PER (%)
100
75
50
25
*
Hugin-Gal4; UAS-dTRPA1
32ºC
22ºC
B
E564-Gal4/Hugin-LexA;
UAS-dTRPA1/LexAop-TNT
32ºC
22ºC
***
***
***
***
10
100
350
1000
E564-Gal4/Hugin-Gal4;
UAS-dTRPA1
32ºC
22ºC
***
***
***
10
100
350
1000
[Sucrose] (mM)

Figure 3.4


A
Probability of Full extension
1.0
0.8
0.6
0.4
0.2
B
Probability of Full extension
1.0
0.8
0.6
0.4
0.2
dilp2
AKHR
Akh
gadgal80
5ht1a
snpf
Npf
leukokinin
TH
Ikr
TRH
Dilp3
isoD1
hl9
npfr
dopecr

Chapter 4

Discussion

These data represent the first description of mutual inhibition between incompatible behaviors in *Drosophila* and identify a pair of interneurons that mediate this interaction. We have demonstrated that, as with other invertebrates, feeding behavior suppresses and is suppressed by at least one other behavior; locomotion. In addition, we have discovered a mechanism by which mechanosensory input can suppress feeding initiation via activation of the PER_{in} neurons.

The identification of PER_{in} demonstrates the capacity of large-scale behavioral screens to identify novel modulators of proboscis extension. As PER_{in} neurons modulate feeding behaviors through engagement in locomotion, screening for neurons that are taste-responsive would never identify this critical modulator of feeding initiation. Therefore, the screening of large pools of Gal4 neurons with neuronal silencers or activators can identify neurons that modulate behaviors in unexpected ways. The interaction between Hugin and PER_{in} also shows the potential of the GRASP method to screen for functionally connected classes of neurons in *Drosophila*.

This work is also significant to the larger field of invertebrate behavioral modulation. The vast majority of experiments in other invertebrate organisms utilize wholly or partially dissected preparations and behavioral outputs are read by motor neuron activity rather than actual behavior. In contrast with previous studies, our experiments involve manipulating specific populations of neurons in awake, behaving animals. This demonstrates the potential of genetic manipulations available in *Drosophila* to study the role that specific neurons play in modulating behavior in intact animals.

The characterization of PER_{in} neurons also contributes to the debate between command neurons and network reconfiguration as methods of decision-making. Our data is consistent with the command neuron hypothesis, as activation of PER_{in} neurons suppresses a behavior (proboscis extension), and inhibition of them promotes that behavior. It is also unlikely that network reconfiguration plays a role in the interaction between feeding and locomotion, as the two behaviors (locomotion and feeding initiation) utilize non-overlapping pools of motor neurons. The reconfiguration model may only apply to behaviors such as the leech swimming and crawling networks, or the *Aplysia* ingestion and egestion network, where the two behaviors share an overlapping pool of motor neurons (Briggman and Kristan, 2006; Jing and Weiss, 2001).

The interaction between the swimming and feeding networks in *Pleurobranchaea* is an appropriate analogy to the function of PER_{in}. As is the case in locomotion and feeding in the fly, swimming and feeding in the sea slug are mutually exclusive and draw upon non-overlapping motor pools and CPGs. Work in this system revealed that the swimming CPG itself activates a neuron (A-ci1), which in turn activates an inhibitory neuron in the feeding CPG (I1) (Jing and Gillette, 1995). Therefore the engagement in swimming directly inhibits feeding. As movement of the legs of the fly activate PER_{in} which in turn inhibits feeding, it may very well function in a similar manner to A-ci1 and activate inhibitory elements of the feeding circuit in the fly.

Another possibility is that PER_{in} neurons function in a manner similar to a different set of interneurons in *Pleurobranchaea,* the corollary discharge neurons (CD) (Kovac and Davis, 1980). CD neuronsare stimulated by feeding behaviors, are necessary for the feeding-mediated suppression of touch-induced withdrawal, and are sufficient to suppress withdrawal even in the absence of food stimulus. CD neurons are proposed to function not as command neurons themselves, but rather inhibitors of them. The stimulation of CD neurons abolishes activity seen in withdrawal motor neurons when a withdrawal command neuron is activated. PER_{in} may well act in a manner similar to this, and inhibit elements of the proboscis extension circuitry that promote feeding initiation.

While there appear to be as many mechanisms for decision-making as there are behaviors, they all share some key features. In nearly every case investigated thus far it has been observed that in order to make a decision not only must one outcome be favored, but that alternatives must also be suppressed. Our data indicate that a similar process likely governs decision-making in flies, where one behavior actively inhibits others in order to maintain a stable decision.

Future Directions

The identification of PER_{in} advances our understanding of decision-making in the fly and opens the door to examine how PER_{in} activity alters the neural circuitry for feeding behavior. Our attempts to place PER_{in} in the context of well-established components of the feeding circuit such as sensory and motor neurons revealed that PER_{in} likely does not directly interact with either of these classes of neurons, suggesting that PER_{in} influences other components of feeding circuits.

The development of more sensitive genetically encoded calcium indicators and more advanced microscopy methods now allow the simultaneous monitoring of activity in entire brain regions in *Drosophila*. Manipulating PER_{in} neurons with activators or silencers during taste stimulation should reveal how PER_{in} influences the whole proboscis extension circuit. As removing mechanosensory input from the legs also affects proboscis extension, similar experiments in which mechanosensory input is altered will reveal how these manipulations alter the proboscis extension circuit. This method could also be used in conjunction with Gal4 lines labeling known elements of the feeding circuit. For example, PER_{in} might modulate the recently identified feeding command neuron found in the NP883-Gal4 line (Flood et al., 2013). Imaging the Gal4 lines identified in chapter 3 as suppressors of constitutive proboscis extension should also reveal neurons involved in PER_{in} modulation of feeding behaviors.

The direct inputs to PER_{in} are also unknown. While mechanosensory stimulation activates PER_{in} neurons, it is not clear whether this is direct or indirect. Generating or identifying a LexA line that expresses in PER_{in} neurons will allow us to screen Gal4 collections using the GRASP method. Sparse Gal4 lines that show positive GRASP with PER_{in} dendrites should identify putative inputs to PER_{in} neurons. They can then be tested for functional connectivity through calcium imaging of PER_{in} dendrites during activation of identified lines. A line from the Gal4 collection generated in the Rubin lab, R27H08, expresses in PER_{in} neurons. Cloning

the enhancer fragment used to generate this Gal4 to create a LexA line could provide us with this necessary tool.

While it is clear that PER_{in} activity modulates the suppression of proboscis extension by locomotion, the converse effect likely occurs through different neurons, as manual manipulation of the proboscis into the extended position is sufficient to suppress locomotion. A neuronal silencer screen similar to the one that identified PER_{in} could be performed on flies with manually extended proboscises, looking for lines that show no suppression of locomotion. A neuronal silencer screen of lines with normal locomotion when PER_{in} neurons have also been silenced, as was performed in chapter three, should provide similar results.

The identification of PER_{in} also allows us to investigate the interaction between feeding initiation and other behaviors. While this work focused on the suppression of locomotion through feeding initiation, it is likely that interactions between feeding and other behaviors occur as well. Utilizing flies engaged in constitutive proboscis extension should make it possible to test whether feeding initiation has the capacity to suppress other behaviors, such as courtship or grooming.

These experiments should provide us with a more complete picture of how PER_{in} influences feeding decisions, how mechanosensory inputs modulate PER_{in} activity, and yield a more complete description of the behavioral hierarchies in *Drosophila*.

References

Adams, C.M., Anderson, M.G., Motto, D.G., Price, M.P., Johnson, W.A., and Welsh, M.J. (1998). Ripped pocket and pickpocket, novel Drosophila DEG/ENaC subunits expressed in early development and in mechanosensory neurons. J Cell Biol *140*, 143–152.

Al-Anzi, B., Armand, E., Nagamei, P., Olszewski, M., Sapin, V., Waters, C., Zinn, K., Wyman, R.J., and Benzer, S. (2010). The leucokinin pathway and its neurons regulate meal size in Drosophila. Curr Biol *20*, 969–978

Akerboom, J., Chen, T.-W., Wardill, T.J., Tian, L., Marvin, J.S., Mutlu, S., Calderón, N.C., Esposti, F., Borghuis, B.G., Sun, X.R., et al. (2012). Optimization of a GCaMP calcium indicator for neural activity imaging. J Neurosci *32*, 13819–13840.

Bader, R., Colomb, J., Pankratz, B., Schröck, A., Stocker, R.F., and Pankratz, M.J. (2007). Genetic dissection of neural circuit anatomy underlying feeding behavior in Drosophila: distinct classes of hugin-expressing neurons. J Comp Neurol *502*, 848–856.

Baines, R.A., Uhler, J.P., Thompson, A., Sweeney, S.T., and Bate, M. (2001). Altered electrical properties in Drosophila neurons developing without synaptic transmission. J Neurosci *21*, 1523–1531.

Bellman, K.L., and Krasne, F.B. (1983). Adaptive complexity of interactions between feeding and escape in crayfish. Science *221*, 779–781.

Benton, R., Vannice, K.S., Gomez-Diaz, C., and Vosshall, L.B. (2009). Variant ionotropic glutamate receptors as chemosensory receptors in Drosophila. Cell *136*, 149–162.

Bharucha, K.N., Tarr, P., and Zipursky, S.L. (2008). A glucagon-like endocrine pathway in Drosophila modulates both lipid and carbohydrate homeostasis. J. Exp. Biol. *211*, 3103–3110.

Brand, A.H., and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development *118*, 401–415.

Branson, K., Robie, A.A., Bender, J., Perona, P., and Dickinson, M.H. (2009). High-throughput ethomics in large groups of Drosophila. Nat Methods *6*, 451–457.

Briggman, K.L., Abarbanel, H.D.I., and Kristan, W.B. (2005). Optical imaging of neuronal populations during decision-making. Science *307*, 896–901.

Briggman, K.L., and Kristan, W.B. (2006). Imaging dedicated and multifunctional

neural circuits generating distinct behaviors. J Neurosci *26*, 10925–10933.

Briggman, K.L., and Kristan, W.B. (2008). Multifunctional pattern-generating circuits. Annual Review of Neuroscience *31*, 271–294.

Brodfuehrer, P.D., Debski, E.A., O'Gara, B.A., and Friesen, W.O. (1995). Neuronal control of leech swimming. J Neurobiol *27*, 403–418.

Cameron, P., Hiroi, M., Ngai, J., and Scott, K. (2010). The molecular basis for water taste in Drosophila. Nature *465*, 91–95.

Chandrashekar, J., Hoon, M., Ryba, N., and Zuker, C. (2006). The receptors and cells for mammalian taste. Nature *444*, 288-294

Chatterjee, A., Tanoue, S., Houl, J.H., and Hardin, P.E. (2010). Regulation of Gustatory Physiology and Appetitive Behavior by the Drosophila Circadian Clock. Curr Biol. *20*, 300-309.

Chen, Z., Wang, Q., and Wang, Z. (2010). The amiloride-sensitive epithelial Na+ channel PPK28 is essential for drosophila gustatory water reception. J Neurosci *30*, 6247–6252.

Cheng, L.E., Song, W., Looger, L.L., Jan, L.Y., and Jan, Y.-N. (2010). The role of the TRP channel NompC in Drosophila larval and adult locomotion. Neuron *67*, 373–380.

Chyb, S., Dahanukar, A., Wickens, A., and Carlson, J.R. (2003). Drosophila Gr5a encodes a taste receptor tuned to trehalose. Proc Natl Acad Sci USA *100 Suppl 2*, 14526–14530.

Clyne, P.J., Warr, C.G., and Carlson, J.R. (2000). Candidate taste receptors in Drosophila. Science *287*, 1830–1834.

Dahanukar, A., Lei, Y.-T., Kwon, J.Y., and Carlson, J.R. (2007). Two Gr genes underlie sugar reception in Drosophila. Neuron *56*, 503–516.

Davis, W.J., and Gillette, R. (1978). Neural correlate of behavioral plasticity in command neurons of Pleurobranchaea. Science *199*, 801–804

Davis, W.J., Mpitsos, G.J., and Pinneo, J.M. (1974). The behavioral hierarchy of the molluskPleurobranchaea. J. Comp. Physiol. *90*, 207–224.

Demerec, M. (1994). Biology of Drosophila (Cold Spring Harbor Laboratory Pr).

Dethier, V.G. (1976). The hungry fly (Harvard Univ Pr).

Edgecomb, R.S., Harth, C.E., and Schneiderman, A.M. (1994). Regulation of feeding behavior in adult Drosophila melanogaster varies with feeding regime and nutritional state. J. Exp. Biol. *197*, 215–235.

Falk, R., Bleiser-Avivi, N., and Atidia, J. (1976). Labellar taste organs of Drosophila melanogaster. Journal of Morphology *150*, 327–341.

Fischler, W., Kong, P., Marella, S., and Scott, K. (2007). The detection of carbonation by the Drosophila gustatory system. Nature *448*, 1054–1057.

Flood, T.F., Iguchi, S., Gorczyca, M., White, B., Ito, K., and Yoshihara, M. (2013). A single pair of interneurons commands the Drosophila feeding motor program. Nature *499*, 83–87.

Fujishiro, N., Kijima, H., and Morita, H. (1984). Impulse frequency and action potential amplitude in labellar chemosensory neurones of Drosophila melanogaster. J Insect Physiol *30*, 317–325.

Gaudry, Q., and Kristan, W.B. (2010). Feeding-Mediated Distention Inhibits Swimming in the Medicinal Leech. J Neurosci *30*, 9753–9761.

Gaudry, Q., and Kristan, W.B. (2009). Behavioral choice by presynaptic inhibition of tactile sensory terminals. Nat Neurosci *12*, 1450–1457.

Gohl, D.M., Silies, M.A., Gao, X.J., Bhalerao, S., Luongo, F.J., Lin, C.-C., Potter, C.J., and Clandinin, T.R. (2011). A versatile in vivo system for directed dissection of gene expression patterns. Nat Methods *8*, 231–237.

Gordon, M., and Scott, K. (2009). Motor Control in a Drosophila Taste Circuit. Neuron *61*, 373–384.

Hamada, F.N., Rosenzweig, M., Kang, K., Pulver, S.R., Ghezzi, A., Jegla, T.J., and Garrity, P.A. (2008). An internal thermal sensor controlling temperature preference in Drosophila. Nature *454*, 217–220.

Hegemann, P., and Bamberg, E. (2003). Channelrhodopsin-2, a directly light-gated cation-selective membrane channel. Proc Natl Acad Sci USA *100*, 13940–13945.

Heisenberg, M. (2003). Mushroom body memoir: from maps to models. Nat Rev Neurosci *4*, 266–275.

Hergarden, A.C., Tayler, T.D., and Anderson, D.J. (2012). Allatostatin-A neurons inhibit feeding behavior in adult Drosophila. Proc Natl Acad Sci USA *109*, 3967–3972.

Hiroi, M., Meunier, N., Marion Poll, F., and Tanimura, T. (2004). Two antagonistic gustatory receptor neurons responding to sweet-salty and bitter taste in Drosophila. J Neurobiol *61*, 333–342.

Ikeya, T., Galic, M., Belawat, P., Nairz, K., and Hafen, E. (2002). Nutrient-dependent expression of insulin-like peptides from neuroendocrine cells in the CNS contributes

to growth regulation in Drosophila. Current Biology *12*, 1293–1300.

Inagaki, H.K., Ben-Tabou de-Leon, S., Wong, A.M., Jagadish, S., Ishimoto, H., Barnea, G., Kitamoto, T., Axel, R., and Anderson, D.J. (2012). Visualizing neuromodulation in vivo: TANGO-mapping of dopamine signaling reveals appetite control of sugar sensing. Cell *148*, 583–595.

Jiao, Y., Moon, S.J., and Montell, C. (2007). A Drosophila gustatory receptor required for the responses to sucrose, glucose, and maltose identified by mRNA tagging. Proc Natl Acad Sci USA *104*, 14110–14115.

Jiao, Y., Moon, S.J., Wang, X., Ren, Q., and Montell, C. (2008). Gr64f is required in combination with other gustatory receptors for sugar detection in Drosophila. Current Biology *18*, 1797–1801.

Jing, J., and Gillette, R. (1995). Neuronal elements that mediate escape swimming and suppress feeding behavior in the predatory sea slug Pleurobranchaea. J Neurophysiol *74*, 1900–1910.

Jing, J., and Gillette, R. (1999). Central pattern generator for escape swimming in the notaspid sea slug Pleurobranchaea californica. J Neurophysiol *81*, 654–667.

Jing, J., and Weiss, K.R. (2001). Neural mechanisms of motor program switching in Aplysia. J Neurosci *21*, 7349–7362.

Kim, S.K., and Rulifson, E.J. (2004). Conserved mechanisms of glucose sensing and regulation by Drosophila corpora cardiaca cells. Nature *431*, 316–320.

Kitamoto, T. (2001). Conditional modification of behavior in Drosophila by targeted expression of a temperature-sensitive shibire allele in defined neurons. J Neurobiol *47*, 81–92.

Kovac, M., and Davis, W. (1977). Behavioral choice: neural mechanisms in Pleurobranchaea. Science *198*, 632–634.

Kovac, M.P., and Davis, W.J. (1980). Neural mechanism underlying behavioral choice in Pleurobranchaea. J Neurophysiol *43*, 469–487.

Krasne, F.B., and Lee, S.C. (1988). Response-dedicated trigger neurons as control points for behavioral actions: selective inhibition of lateral giant command neurons during feeding in crayfish. Journal of Neuroscience *8*, 3703–3712.

Kristan, W., and Gillette, R. (2007). 20 Behavioral Choice. Cold Spring Harbor Monograph Archive *49*, 533–553.

Lai, S.L., and Lee, T. (2006). Genetic mosaic with dual binary transcriptional systems in : Drosophila : Article : Nature Neuroscience *9*, 703-709

Lee, G., and Park, J.H. (2004). Hemolymph sugar homeostasis and starvation-induced hyperactivity affected by genetic manipulations of the adipokinetic hormone-encoding gene in Drosophila melanogaster. Genetics *167*, 311–323.

Lee, T., and Luo, L. (1999). Mosaic analysis with a repressible cell marker for studies of gene function in neuronal morphogenesis. Neuron *22*, 451–461.

Lee, Y., Moon, S.J., and Montell, C. (2009). Multiple gustatory receptors required for the caffeine response in Drosophila. Proc Natl Acad Sci USA *106*, 4495–4500.

Liu, T., Starostina, E., Vijayan, V., and Pikielny, C.W. (2012). Two Drosophila DEG/ENaC channel subunits have distinct functions in gustatory neurons that activate male courtship. J Neurosci *32*, 11879–11889.

Lu, B., LaMora, A., Sun, Y., Welsh, M.J., and Ben-Shahar, Y. (2012). ppk23-Dependent chemosensory functions contribute to courtship behavior in Drosophila melanogaster. PLoS Genet. *8*, e1002587.

Manzo, A., Silies, M., Gohl, D.M., and Scott, K. (2012). Motor neurons controlling fluid ingestion in Drosophila. Proc Natl Acad Sci USA *109*, 6307–6312.

Marella, S., Fischler, W., Kong, P., Asgarian, S., Rueckert, E., and Scott, K. (2006). Imaging taste responses in the fly brain reveals a functional map of taste category and behavior. Neuron *49*, 285–295.

Marella, S., Mann, K., and Scott, K. (2012). Dopaminergic modulation of sucrose acceptance behavior in Drosophila. Neuron *73*, 941–950.

Masek, P., and Keene, A.C. (2013). Drosophila fatty acid taste signals through the PLC pathway in sugar-sensing neurons. PLoS Genet. *9*, e1003710.

Masek, P., and Scott, K. (2010). Limited taste discrimination in Drosophila. Proc Natl Acad Sci USA *107*, 14833–14838.

McGuire, S.E., Mao, Z., and Davis, R.L. (2004). Spatiotemporal gene expression targeting with the TARGET and gene-switch systems in Drosophila. Science's STKE *2004*, pl6.

Melcher, C., and Pankratz, M. (2005). Candidate gustatory interneurons modulating feeding behavior in the Drosophila brain. PLoS Biology *3*, e305.

Meunier, N., Marion Poll, F., Rospars, J.P., and Tanimura, T. (2003). Peripheral coding of bitter taste in Drosophila. J Neurobiol *56*, 139–152.

Misell, L.M., Shaw, B.K., and Kristan, W.B. (1998). Behavioral hierarchy in the medicinal leech, Hirudo medicinalis: feeding as a dominant behavior. Behav. Brain Res. *90*, 13–21.

Nagel, G., Szellas, T., Huhn, W., Kateriya, S., Adeishvili, N., Berthold, P., Ollig, D., Hegemann, P., and Bamberg, E. (2003). Channelrhodopsin-2, a directly light-gated cation-selective membrane channel. Proc Natl Acad Sci USA *100*, 13940–13945.

Neumann, E.K., Wheeler, D.A., Bernstein, A.S., Burnside, J.W., and Hall, J.C. (1992). Artificial neural network classification of Drosophila courtship song mutants. Biol Cybern *66*, 485–496.

Nicolai, L.J.J., Ramaekers, A., Raemaekers, T., Drozdzecki, A., Mauss, A.S., Yan, J., Landgraf, M., Annaert, W., and Hassan, B.A. (2010). Genetically encoded dendritic marker sheds light on neuronal connectivity in Drosophila. Proc Natl Acad Sci USA *107*, 20553–20558.

Noyes, B.E., Katz, F.N., and Schaffer, M.H. (1995). Identification and expression of the Drosophila adipokinetic hormone gene. Mol. Cell. Endocrinol. *109*, 133–141.

Petersen, L.K., and Stowers, R.S. (2011). A Gateway MultiSite recombination cloning toolkit. PLoS ONE *6*, e24531.

Pfeiffer, B.D., Ngo, T.-T.B., Hibbard, K.L., Murphy, C., Jenett, A., Truman, J.W., and Rubin, G.M. (2010). Refinement of tools for targeted gene expression in Drosophila. Genetics *186*, 735–755.

Potter, C.J., Tasic, B., Russler, E.V., Liang, L., and Luo, L. (2010). The Q system: a repressible binary system for transgene expression, lineage tracing, and mosaic analysis. Cell *141*, 536–548.

Rajashekhar, K.P., and Singh, R.N. (1994a). Neuroarchitecture of the tritocerebrum of Drosophila melanogaster. J Comp Neurol *349*, 633–645.

Rajashekhar, K.P., and Singh, R.N. (1994b). Organization of motor neurons innervating the proboscis musculature in Drosophila melanogaster meigen (diptera: Drosophilidae). International Journal of Insect Morphology and Embryology *23*, 225–242.

Robertson, H.M., Warr, C.G., and Carlson, J.R. (2003). Molecular evolution of the insect chemoreceptor gene superfamily in Drosophila melanogaster. Proc Natl Acad Sci USA *100 Suppl 2*, 14537–14542.

Root, C.M., Ko, K.I., Jafari, A., and Wang, J.W. (2011). Presynaptic facilitation by neuropeptide signaling mediates odor-driven food search. Cell *145*, 133–144.

Root, C.M., Masuyama, K., Green, D.S., Enell, L.E., Nässel, D.R., Lee, C.-H., and Wang, J.W. (2008). A presynaptic gain control mechanism fine-tunes olfactory behavior. Neuron *59*, 311–321.

Rulifson, E.J., Kim, S.K., and Nusse, R. (2002). Ablation of insulin-producing neurons

in flies: growth and diabetic phenotypes. Science *296*, 1118–1120.

Ruta, V., Datta, S.R., Vasconcelos, M.L., Freeland, J., Looger, L.L., and Axel, R. (2010). A dimorphic pheromone circuit in Drosophila from sensory input to descending output. Nature *468*, 686–690.

Scott, K., Brady, R., Cravchik, A., Morozov, P., Rzhetsky, A., Zuker, C., and Axel, R. (2001). A chemosensory gene family encoding candidate gustatory and olfactory receptors in Drosophila. Cell *104*, 661–673.

Scott, R.C., Schuldiner, O., and Neufeld, T.P. (2004). Role and regulation of starvation-induced autophagy in the Drosophila fat body. Dev. Cell *7*, 167–178.

Shaw, B.K., and Kristan, W.B. (1997). The neuronal basis of the behavioral choice between swimming and shortening in the leech: control is not selectively exercised at higher circuit levels. Journal of Neuroscience *17*, 786–795.

Slone, J., Daniels, J., and Amrein, H. (2007). Sugar receptors in Drosophila. Curr Biol *17*, 1809–1816.

Stocker, R., and Schorderet, M. (1981). Cobalt filling of sensory projections from internal and external mouthparts in Drosophila. Cell and Tissue Research *216*, 513-523

Stocker, R.F. (1994). The organization of the chemosensory system in Drosophila melanogaster: a review. Cell and Tissue Research *275*, 3–26.

Sweeney, S.T., Broadie, K., Keane, J., Niemann, H., and O'Kane, C.J. (1995). Targeted expression of tetanus toxin light chain in Drosophila specifically eliminates synaptic transmission and causes behavioral defects. Neuron *14*, 341–351.

Thistle, R., Cameron, P., Ghorayshi, A., Dennison, L., and Scott, K. (2012). Contact chemoreceptors mediate male-male repulsion and male-female attraction during Drosophila courtship. Cell *149*, 1140–1151.

Thorne, N., Chromey, C., Bray, S., and Amrein, H. (2004). Taste perception and coding in Drosophila. Curr Biol *14*, 1065–1079.

Tian, L., Hires, S.A., Mao, T., Huber, D., Chiappe, M.E., Chalasani, S.H., Petreanu, L., Akerboom, J., McKinney, S.A., Schreiter, E.R., et al. (2009). Imaging neural activity in worms, flies and mice with improved GCaMP calcium indicators. Nat Methods *6*, 875–881.

Toda, H., Zhao, X., and Dickson, B.J. (2012). The Drosophila female aphrodisiac pheromone activates ppk23(+) sensory neurons to elicit male courtship behavior. Cell Rep *1*, 599–607.

Tracey, W.D., Wilson, R.I., Laurent, G., and Benzer, S. (2003). painless, a Drosophila gene essential for nociception. Cell *113*, 261–273.

Wang, J.W., Wong, A.M., Flores, J., Vosshall, L.B., and Axel, R. (2003). Two-photon calcium imaging reveals an odor-evoked map of activity in the fly brain. Cell *112*, 271–282.

Wang, Z., Singhvi, A., Kong, P., and Scott, K. (2004). Taste representations in the Drosophila brain. Cell *117*, 981–991.

Weeks, J.C., and Kristan, W.B., Jr (1978). Initiation, maintenance and modulation of swimming in the medicinal leech by the activity of a single neurone. J. Exp. Biol. *77*, 71–88.

Weiss, L.A., Dahanukar, A., Kwon, J.Y., Banerjee, D., and Carlson, J.R. (2011). The molecular and cellular basis of bitter taste in Drosophila. Neuron *69*, 258–272.

Wiersma, C.A. (1947). Giant nerve fiber system of the crayfish; a contribution to comparative physiology of synapse. J Neurophysiol *10*, 23–38.

Wiersma, C.A., and Ikeda, K. (1964). Interneurons commanding swimmeret movements in the crayfish Procambarus clarki. Comp. Biochem. Physiol. *12*, 509–525.

Wu, Q., Wen, T., Lee, G., Park, J.H., Cai, H.N., and Shen, P. (2003). Developmental control of foraging and social behavior by the Drosophila neuropeptide Y-like system. Neuron *39*, 147–161.

Wu, Q., Zhang, Y., Xu, J., and Shen, P. (2005). Regulation of hunger-driven behaviors by neural ribosomal S6 kinase in Drosophila. Proc Natl Acad Sci USA *102*, 13289–13294.

Xu, K., Zheng, X., and Sehgal, A. (2008). Regulation of feeding and metabolism by neuronal and peripheral clocks in Drosophila. Cell Metabolism *8*, 289–300.

Yan, Z., Zhang, W., He, Y., Gorczyca, D., Xiang, Y., Cheng, L.E., Meltzer, S., Jan, L.Y., and Jan, Y.-N. (2013). Drosophila NOMPC is a mechanotransduction channel subunit for gentle-touch sensation. Nature *493*, 221–225.

Yarmolinsky, D.A., Zuker, C.S., and Ryba, N.J.P. (2009). Common sense about taste: from mammals to insects. Cell *139*, 234–244.

Ye, B., Zhang, Y., Song, W., Younger, S.H., Jan, L.Y., and Jan, Y.-N. (2007). Growing dendrites and axons differ in their reliance on the secretory pathway. Cell *130*, 717–729.

Zelle, K.M., Lu, B., Pyfrom, S.C., and Ben-Shahar, Y. (2013). The Genetic Architecture

of Degenerin/Epithelial Sodium Channels in Drosophila. Genes|Genomes|Genetics *3*, 441–450.

Zhang, Y.V., Ni, J., and Montell, C. (2013). The molecular basis for attractive salt-taste coding in Drosophila. Science *340*, 1334–1338.

Zhang, Y.Q., Rodesch, C.K., and Broadie, K. (2002). Living synaptic vesicle marker: synaptotagmin-GFP. Genesis *34*, 142–145.