Motor Neurons Controlling Fluid Ingestion in *Drosophila melanogaster*

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A dissertation submitted in partial satisfaction of the requirements for the degree of Doctor of Philosophy in Molecular and Cell Biology in the Graduate Division of the University of California, Berkeley

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Abstract

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How environmental stimuli are detected and processed by the brain to produce a behavioral output remains a central question in neurobiology. The gustatory system in *Drosophila* is an excellent model for studying this problem as flies possess strong innate behaviors to many taste stimuli and can be studied using a variety of molecular and genetic tools. Because of these advantages, it should be possible to assemble a complete anatomical and functional map of the neurons mediating taste perception and behavior.

In *Drosophila*, taste compounds are detected by sensory neurons that send axons into the brain. Second- and higher-order neurons in the gustatory circuit have not yet been identified. In the first part of my thesis, I describe the results of an inducible activation screen to identify neuronal populations whose activity is sufficient to elicit proboscis extension. This screen led to the identification of motor neurons that control pumping behavior.

In the second part of my thesis, I describe my work studying motor neurons that innervate the proboscis musculature of *Drosophila*. These neurons drive a pump that draws fluid into the esophagus during feeding. My results show that these motor neurons are necessary for normal pumping and ingestion to occur, and that their activity is sufficient to elicit pumping. Furthermore, my data suggests that these neurons are not just passive effectors of a pumping circuit but play a role in generating the pumping rhythm.

In the third part of my thesis, I describe a population of neurons whose morphology suggests they may receive input from sensory taste neurons. They project from the taste region of the fly brain to higher order regions that have not been well-described. The results of my experiments showed that these neurons do contact sensory taste neurons, but are not required for normal gustatory learning. Future experiments will help determine if they play a role in the processing of gustatory information.
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CHAPTER 1

Introduction
Introduction

In order to survive, organisms must be able to perceive the world around them and tailor their behavior to the environment. The senses of taste and smell identify chemical cues that signal the presence of nutrients, predators and mates. The neural circuits underlying these senses have long been the subject of research, but the mechanisms underlying chemosensation are only now beginning to be elucidated.

A major advance in chemosensory research came with the discovery of receptors that bind odorant molecules and taste compounds (1–3). These receptors revealed the mechanism for chemical detection and how the olfactory and gustatory systems are organized in the periphery. Olfactory and gustatory systems differ in how they encode chemical information. In the gustatory system, each cell expresses multiple taste receptors that detect molecules of a single taste modality (i.e., sugars or bitter compounds). Distinct non-overlapping neuronal populations respond to different types of gustatory stimuli, forming a ‘labeled line’ for a particular taste. In the olfactory system, each cell expresses only one type of olfactory receptor, but each olfactory receptor can bind to multiple odorants. Different odorants elicit different patterns of activity across the entire olfactory neuron population, producing a combinatorial code. However, behaviorally relevant odors (e.g., pheromones such as cVA in Drosophila) can activate specific neuronal populations that act more like a labeled line for these compounds (4).

Olfaction

Detection of odor cues is accomplished by olfactory sensory neurons (OSNs) located in the nasal epithelium of mammals. These neurons express one of ~1200 olfactory receptors (ORs), G protein coupled receptors (GPCRs) that become activated upon binding odorants (1, 3). Thousands of neurons expressing the same OR project to a few of the glomeruli in the olfactory bulb, anatomically and functionally segregating olfactory information. Also located in the glomeruli are dendrites of second-order olfactory neurons, including juxtaglomerular cells and mitral/tufted cells (5, 6). Juxtaglomerular cells are largely limited to the olfactory bulb and mediate intraglomerular transformations of odor information. Mitral/tufted cells are the main output neurons of the bulb; each mitral cell receives input from a single glomerulus and then projects to higher order brain areas including the anterior olfactory nucleus, the piriform cortex, the olfactory tubercle, the cortical amygdala and the entorhinal cortex (5, 7).

The organization of the mitral/tufted cell projections differ among cortical regions. In the amygdala, the segregation of olfactory information is largely maintained, with neurons from the same glomerulus projecting to similar regions in the amygdala and neurons from different glomeruli projecting to distinct regions (8). In the piriform cortex, mitral/tufted cells from individual glomeruli project broadly and do not exhibit spatial segregation (8). In addition, neighboring neurons display different odor specificities and cells responding to similar odors are broadly distributed (9). From this data, researchers have speculated that the amygdala plays a role in producing innate behavioral responses to odors, whereas the piriform cortex is important for learned associations of odors with other stimuli (8, 10).

The organization of the olfactory system in Drosophila is very similar to that of mammals. Olfactory sensory neurons are located in the antennae and maxillary palps and many express members of the olfactory receptor (OR) family of proteins (11). Each neuron expresses a single ligand-binding OR gene and the co-receptor Orco, which together assemble into a
functional olfactory receptor. Some olfactory neurons do not express ORs but instead express members of the newly discovered ionotropic receptor (IR) family (12).

Neurons expressing the same OR project to the same glomeruli in the antennal lobe, the correlate of the olfactory bulb (11). Local interneurons innervate the glomeruli and participate in intraglomerular computations that shape the responses of the projection neurons (PNs), the output cells of the antennal lobe. Individual PNs innervate a single glomerulus and send their axons to the mushroom bodies and the lateral horn of the protocerebrum. PN projections to these areas are stereotyped, and PNs innervating the same glomerulus tend to target similar regions (13, 14). The lateral horn has been proposed to mediate innate responses to odor stimuli, whereas the mushroom bodies are known to be necessary for learned odor associations; these areas have been proposed to be analogous to the amygdala and the piriform cortex, respectively (15). The output cells of the mushroom body and lateral horn are largely uncharacterized, although recent work has identified mushroom body output neurons necessary for memory retrieval and lateral horn output neurons that are responsive to a fly pheromone (16, 17).

**Gustation in Rodents**

Taste detection in rodents is accomplished by epithelial taste receptor cells (TRCs) located on the tongue and the palate (2, 18, 19). These cells cluster into taste buds, which are housed in small protrusions called papillae. Each TRC responds to a specific type of taste stimulus depending on the receptor expressed by the cell. Sugars, bitter compounds, and amino acids (umami) are detected by members of the T1R and T2R families of GPCRs (2, 18, 19). Sugars are sensed by T1R2 and T1R3 heteromers, amino acids by T1R1 and T1R3 heteromers, and bitter compounds by members of the T2R family. By contrast, salts and acids are detected by ion channels, with sour taste partially mediated by PKD2L1, a TRP channel, and requiring PKD2L1-expressing cells and salt taste mediated by ENACα channels (20–23). Sour-sensing cells also detect carbonation through the carbonic anhydrase Car4 (24). Fatty acids may also be detected by TRCs; recent work has proposed that the molecules GPR40, GPR120, and CD36 may be involved in mediating gustatory perception of lipids (25, 26).

Each taste bud is innervated by cranial ganglia neurons that carry sensory taste information to the nucleus of the solitary tract (NST) in the brain stem (2, 19). From here, taste information is relayed to various brain regions which are multiply interconnected through both feedforward and feedback pathways. From the NST, neurons project to the parabrachial nucleus and from there to the thalamus. These thalamic projections terminate in the gustatory cortex of the insula. In addition to these areas, other brain regions such as the lateral hypothalamus, the central nucleus of the amygdala and the orbitofrontal cortex also receive taste input (2, 19, 27).

While distinct populations of TRCs detect different taste modalities, it is unclear if higher order taste neurons maintain this functional segregation. Taste-responsive neurons in the NST, parabrachial nucleus, and the ventroposterior medial nucleus of the thalamus vary in stimulus selectivity (19, 27). Electrophysiological recordings from these regions found neurons that were both broadly-tuned to multiple types of taste stimuli or were more selective for certain classes of tastants. However in these studies, the connectivity of the recorded neurons to TRC types was unknown. Studies of neuronal responses in the gustatory cortex have also yielded conflicting interpretations. Recordings from individual neurons in this area have revealed both broadly-tuned and narrowly-tuned responses (19, 27). However, recent work has identified four well-defined, non-overlapping regions of the gustatory cortex that contain cells selective for specific
taste substances (sugar, salts, bitters, and amino acids) (28). In this work, researchers first mapped the projections from taste-responsive thalamic cells to the appropriate region of the gustatory cortex and then used calcium imaging to monitor the responses from large numbers of cells; these strategies allowed these investigators to identify these previously undiscovered ‘hot spots’. The contributions of these narrowly-tuned neurons and the broadly-tuned neurons described in previous studies to taste perception have yet to be elucidated.

**Gustation in *Drosophila melanogaster***

Many of the basic principles underlying gustatory perception in mammals holds true in *Drosophila*. Studying gustatory behavior in this model organism brings several advantages. Flies possess strong innate behaviors to many taste stimuli, making it possible to identify causal relationships between neuronal activity and behavior. *Drosophila* is also an especially versatile model organism and can be studied using a variety of molecular and genetic tools. In particular, the availability of enhancer trap collections expressing the exogenous transcriptional activator GAL4 in subsets of cells makes it possible to screen different neurons for their contribution to gustatory behavior. Because of these advantages, it should be possible to assemble a complete anatomical and functional map of the neurons mediating taste perception and behavior.

Gustatory substances are detected by sensory neurons responsive to sweet, bitter, and salty stimuli, as well as carbon dioxide and water (29–31). The primary taste organ is the proboscis, but taste neurons are also found on the wing margins, tarsi, ovipositor, and internal mouthparts (11). These neurons are housed in chemosensory bristles. In the proboscis, each bristle contains two to four gustatory neurons and one mechanosensory neuron. A dendrite from each cell body extends into a pore at the tip of the bristle, where it is able to contact environmental stimuli.

In the periphery, sweet and bitter taste perception is mediated by non-overlapping populations of cells (11, 29). Each population can be distinguished by the expression of particular gustatory receptors (Grs), multi-pass transmembrane receptors that bind taste ligands. Sweet sensing neurons express sugar-sensing Grs such as Gr5a, Gr64a and Gr64f, whereas bitter sensing neurons express Gr66a, the caffeine receptor, along with other bitter-sensitive Grs (29). Ablation and inducible activation studies have shown that Gr5a neurons are necessary and sufficient for sensing multiple sugars, whereas Gr66a neurons are necessary and sufficient for perception of many bitter substances (32, 33).

Recent studies have also identified neurons and genes that mediate perception of water and carbon dioxide (30, 31, 34, 35). Carbon dioxide is a metabolic byproduct of yeast, an important *Drosophila* food source. Detection of water is accomplished by ppk28, a member of the degenerin/epithelial Na+ channels (Deg/ENAC) family of receptors. Detection of carbon dioxide is accomplished by neurons in the taste pegs in the fly’s proboscis (34, 35).

Receptors expressed in leg taste neurons have also been shown to play a role in courtship behaviors. Leg neurons co-expressing Gr32a and Gr33a suppress male-male courtship, whereas leg neurons expressing ppk23 and ppk29, other members of the Deg/ENAC receptor family, promote male-female courtship and suppress male-male courtship (36–38). These receptors are likely sensing cuticular pheromones, non-volatile compounds secreted onto the cuticle that allow chemical communication among flies. In fact, ppk23 and ppk29-expressing neurons respond to these cues (38).
From the periphery, sensory neurons in the proboscis project axons into the thoracic ganglion and the subesophageal ganglion (SOG), the first relay station in the brain (11). There the axons cluster according to taste modality, with bitter-sensing and sugar-sensing neurons terminating in different projection patterns. Whether this functional segregation extends into higher order brain regions is unknown, as the targets of sensory taste neurons have yet to be identified. In this thesis, I present the results of a screen to identify neurons whose activity is sufficient to elicit proboscis extension. This screen identified motor neurons required for proboscis extension but failed to yield any candidate second-order neurons. I also present experiments characterizing neurons whose anatomy suggests they may receive input from sensory taste neurons.

Although fine mapping of the chemosensory circuits has just begun, technological advances in circuit tracing and neuronal manipulation are furthering our understanding of how these systems are organized. For instance, the development of optogenetic tools has allowed researchers to more easily activate neurons and observe their effects on behavior and information processing (39). In addition, genetically encoded calcium sensors enable investigators to observe the responses of a large population of neurons to chemical stimuli (40). It will be exciting to see how these and other innovations expand our knowledge of how chemosensory processing occurs.

**Neural control of gustatory behaviors**

Gustatory stimuli can evoke stereotyped motor behaviors. For instance, detection of sugars can induce a fly to extend its proboscis and ingest the tastant (41). In many systems, complex motor behaviors such as feeding are controlled by central pattern generators (CPGs), neural circuits capable of producing rhythmic firing patterns in the absence of external stimuli. CPGs can produce rhythmic output through the activity of an intrinsic pacemaker neuron or through network interactions (42). This rhythmic output can be altered by sensory and other modulatory inputs which are often necessary to produce the pattern observed in the behaving animal.

Some of the CPGs controlling feeding in other systems have been described. The crustacean stomatogastric ganglion (STG) contains neurons comprising two CPGs that drive stomach muscles (43). Unlike most CPGs which are comprised of interneurons, the STG is largely made up of motor neurons. One CPG controls the gastric mill, which chews food, while the other CPG controls the pylorus, which filters food out of the stomach and to the gut. The pyloric rhythm is set by an intrinsically oscillating neuron, while the gastric mill rhythm emerges from the synaptic interactions of the CPG components. The output of both CPGs can be affected by sensory input, modulatory neurotransmitters, and neurons from other ganglia (44). These studies have shown that the control of even simple motions can be complex (45).

While a CPG underlying feeding in *Drosophila* has not been fully characterized, some of the motor neurons involved in feeding have been described. Previous work has shown that a pair of motor neurons located in the SOG (e49 neurons) are necessary and sufficient to produce proboscis extension (46). Researchers have also identified neurons innervating a pump in the fly’s proboscis that draws fluid into the esophagus. Inactivation of these neurons leads to a decrease in short-term food consumption (47). In this thesis, I further characterized pump neurons and their role in driving the pump. My results indicate that pump motor neurons are not just passive effectors of a CPG, but can also influence the pumping rhythm.
CHAPTER 2

An inducible activation screen to identify neurons mediating proboscis extension
Summary

Gustatory perception in *Drosophia* begins with the detection of tastants in the environment by sensory neurons. Neurons in the brain that process this sensory information have not yet been described. To uncover other neurons in the gustatory circuit, I performed an inducible activation screen to identify neurons whose activity is sufficient to elicit proboscis extension. Photoactivatable adenylate cyclase (PACα), an enzyme that shows enhanced cAMP production when illuminated with blue light, was used to activate neurons and was expressed in different neuronal subsets using a GAL4 enhancer trap collection. Out of 144 lines tested, 15 showed higher levels of proboscis extension than negative controls. GRASP experiments indicated that neurons in many of these lines contacted sugar-sensing neurons. G-CaMP imaging of one line, 520, showed that multiple neurons labeled in this line responded preferentially to 1 M sucrose. Further analysis of the expression pattern revealed that some of these neurons were motor neurons previously shown to mediate proboscis extension, also accounting for the inducible activation phenotype. Subsequent analysis of the other lines identified in the screen showed that most of them also labeled these neurons. Additional sugar-responsive motor neurons identified in line 520 became the subject of the work described in Chapter 3.
Introduction

While progress has been made in identifying and characterizing the sensory inputs and motor outputs of the gustatory system in *Drosophila*, the neural circuitry bridging these two populations is unknown. Unlike the highly organized antennal lobe, where the projections of olfactory receptor neurons and projection neurons cluster into well-defined glomeruli, the subesophageal ganglion (SOG) is a more homogenous mass of neuropil, making the identification of second- and higher-order taste neurons difficult (11).

The combination of new methods of analyzing neural circuits and the versatility of *Drosophila* as a model organism allows for several approaches to identifying second and higher-order taste neurons. I initially tried to identify second-order neurons by searching for cells that arborized near the sensory neurons, but this strategy was unsuccessful. I then turned to a behavioral screen to uncover neurons that could elicit proboscis extension. Stimulation of sensory neurons with palatable substances such as sucrose elicits a fast and stereotypic proboscis extension reflex, suggesting that second-order neurons may target e49 motor neurons that mediate proboscis extension and feeding (41). From this screen, I hoped to identify other neurons that contribute to proboscis extension.

Advances in optogenetics have generated multiple tools with which to activate neurons, including photoactivatable adenylate cyclase (PACα) and Channelrhodopsin2 (ChR2) (39, 48). PACα is an adenylate cyclase that displays increased cAMP production upon illumination with blue light, thereby activating neurons by opening cyclic nucleotide-gated ion channels. ChR2 is a cation channel that is directly activated by blue light. After performing initial experiments to determine which tool would be best suited for a neuronal activation screen, I screened 160 lines using PACα. This screen identified motor neurons that were responsive to taste substances. Analysis of the function of these motor neurons is the subject of Chapter 3.
Results

An inducible activation screen to identify neurons sufficient to elicit proboscis extension
To identify neurons that can elicit proboscis extension, I screened a GAL4 enhancer trap collection kindly given us by Ulrike Heberlein. In this collection, insertion of a GAL4 transcriptional activator into random locations in the genome leads to expression of GAL4 in different subsets of cells. Using the GAL4-UAS system, different reporters can be expressed in these cells to characterize their anatomy and function (49).

PACα and ChR2 were tested in a preliminary screen for proboscis extension with the help of two rotation students, Allan-Hermann Pool and Chris Rodgers (Fig. 2.1 A). Activating e49 with either PACα or ChR2 elicited high levels of extension, but only ChR2 activation of Gr5a cells elicited extension. Negative controls (UAS-act, either UAS-PACα or UAS-ChR2) did not extend to blue light. We expressed UAS-PACα and UAS-ChR2 in different subsets of neurons using 33 enhancer-trap GAL4 lines from the Heberlein collection that were chosen for their sparse GAL4 expression in the brain. We tested whether flies exhibited proboscis extension when placed under blue light. A large proportion (33%) of flies expressing ChR2 exhibited extension probabilities >0.2; a smaller proportion (13%) of flies expressing PACα exhibited extension. Because PACα produced a lower proportion of responding flies and allowed for more tractable analysis of the responders, it was chosen for use in the larger screen. In addition, PACα did not elicit extension in sugar-sensing neurons; therefore, we expected to uncover neurons involved in proboscis extension that were not sensory neurons.

I then screened 110 more lines for proboscis extension, again selecting for sparse GAL4 expression in the brain (Fig. 2.1 B). Twenty-six (24%) of the lines tested exhibited extension at levels higher than the UAS-PACα negative controls. Further testing showed that only 15 of these lines showed greater extension during bright blue light illumination than during dim white light illumination. Activation of e49 neurons did not produce extension under dim white light (Fig. 2.1 C).

GAL4 lines identified in the screen label neurons that contact sensory taste neurons
Analysis of the GAL4 expression in the top 12 responding lines showed broad labeling in the brain (Fig. 2.2). However, several lines exhibited neuronal projections in the SOG and had the potential to label second order neurons. To determine which lines were likely to label neurons that contacted sugar-sensitive Gr5a sensory neurons, I performed anatomical studies using the GRASP technique. In this experiment, two portions of a membrane-tethered GFP molecule are expressed in separate populations of neurons. Fluorescence is not observed unless the two populations contact each other, reconstituting the GFP molecule (46). A summary of these GRASP experiments is shown in Table 2.1. All lines except 460 showed GRASP signal indicating contact with sugar-sensitive Gr5a cells in the brain. However, five lines also showed GRASP signal in proboscis sensory neurons, suggesting that the GRASP signal in the brain is due to expression in Gr5a cells. The remaining lines (44, 460, 491, 520, 646, 662 and 783) did not show GRASP signal in proboscis sensory neurons.

Multiple neurons in 520-GAL4 show calcium increases in response to sucrose
I chose line 520 for further study because of the strong proboscis extension phenotype observed in the inducible activation screen (probability of extension = 0.97), the relative sparseness of GAL4 expression compared to the other lines, and the observation of GRASP signal in the brain
that was unlikely to arise from Gr5a sensory neurons. I next performed imaging experiments using G-CaMP, a genetically-encoded fluorescent calcium indicator, to determine if neurons in this line are responsive to taste stimuli (Fig. 2.3) (50). Stimulation of flies with 1 M sucrose produced responses in multiple neurons, which would often fire at different time points after stimulation (Fig. 2.3 A). Pooling responses across different neurons showed that they responded preferentially to 1 M sucrose over water, 100 mM sucrose, caffeine and 100 mM NaCl (Fig. 2.3 B).

I further analyzed a G-CaMP imaging movie to reveal the morphology of the responding cells (Fig. 2.3 C). I generated an average baseline image of the neurons before stimulation and an average image consisting of frames during the peak response of the neuron. I then subtracted the baseline image from the peak response image to obtain an image of the responding cells (Fig. 2.3 C, right panel).

**Proboscis extension phenotypes are due to e49 neurons**

To determine the types of cells that are labeled by 520, I performed experiments to visualize subsets of the 520 neurons using a technique in which a ubiquitously-expressed GAL80 transgene (a GAL4 repressor) is stochastically excised from the genome by a heat shock FLPase (46). These experiments show that 520 labels pump motor neurons whose morphologies are similar to some of the sucrose-responsive neurons observed in G-CaMP experiments (Fig. 2.3 C-E). 520 also labels e49 motor neurons (Fig. 2.3 F), which have previously been shown to mediate proboscis extension and respond to sucrose (46). The proboscis extension elicited by PACα activation is most likely due to this neuron.

Similar experiments revealed that 10/12 lines identified in the screen exhibited GAL4 expression in these motor neurons, although many of them did not show levels of proboscis extension as high as that observed with e49. It is possible that GAL4 expression in these neurons was weak or varied from fly to fly. Only two lines (463, 783) were not observed to label e49 neurons, but they were not good candidates for further study due to their dense GAL4 expression.

I further characterized the sucrose-responsive pump motor neurons within the 520 line to determine how they control feeding. These results will be discussed in the following chapter.
Conclusions

To identify other elements of the gustatory circuit, I performed an inducible activation screen to find neurons whose activity is sufficient to produce proboscis extension. I screened a collection of GAL4 enhancer trap lines and used the inducible activator PACα, a photoactivatable adenylate cyclase, to activate neurons labeled by these lines. From the screen, I identified 12 candidate lines and performed expression studies to determine if they labeled second-order taste neurons. I also performed G-CaMP imaging experiments using line 520 and found that multiple neurons in this line responded to 1 M sucrose, a palatable stimulus. Analysis of these images revealed that the responding neurons were likely to be pump motor neurons. Further expression analysis showed that 520, along with most of the other GAL4 lines identified in the screen, labeled e49 motor neurons, accounting for the phenotype in the inducible activation screen.

These results give insights into how to design future inducible activation screens. The use of PACα as a neuronal activator most likely limited the types of neurons that could be identified in this screen to e49 neurons. Because this activator is an adenylate cyclase, CNG channels must be present to produce action potentials during transient activation of PACα. It is possible that most neurons do not express these channels; indeed, activation of Gr5a cells with PACα did not elicit extension (Fig. 2.1 A and C). Future screens that make use of inducible ion channels such as ChR2 or dTRPA1 would allow the identification of more neuronal types. However, our preliminary experiments using ChR2 led to a large proportion of GAL4 lines exhibiting extension, possibly because many of the lines label Gr5a cells (Table 2.1). Future screens should use lines with sparse expression in the central nervous system and lacking expression in sensory cells and e49 neurons. Sparse expression would make it easier to functionally characterize causal neurons, to determine if they contact sensory cells and to identify any neurons that may show G-CaMP responses. Lack of expression in sensory and e49 neurons would also help guarantee that the proboscis extension phenotype observed was due to activity in a novel population of neurons. This could be accomplished by including transgenes that express GAL80 in sensory and e49 cells. For instance, a separate bipartite expression system such as the LexA/LexAOp system could be used to express the GAL4 repressor GAL80 in these neuronal populations using specific LexA drivers.

Our imaging results using 520 also show that multiple motor neurons respond at different times after sucrose stimulation. This may occur if e49 motor neurons respond to taste stimuli before pump motor neurons, as extension typically occurs before feeding. Whether this is the case and how e49 is coordinated with other neurons would be interesting points of future study.
Materials and Methods

Experimental Animals
*Drosophila* were reared on standard cornmeal/agar/molasses medium at 25 °C. The screen was performed using GAL4 lines from the Heberlein GAL4 enhancer trap collection. The following lines were also used: *UAS-mCD8::GFP* (51), *UAS-PACα* (48), *UAS-ChR2::YFP* (a gift from Steven Stowers), *UAS-G-CaMP1.3* (52), *Gr5a-LexA::VP16* (46); *UAS-CD4::spGFP1-10* (46); *LexAop-CD4::spGFP11* (46).

Immunohistochemistry
Immunohistochemistry was performed as previously described (32) using the following primary antibodies: rabbit anti-GFP (1:1000; Invitrogen, cat# A11122) and mouse anti-GFP (1:100; Sigma, cat# G6539). Images shown are collapsed confocal stacks.

Generation of FLPout clones
FLPout clones were generated as previously described (46).

Inducible activation screen
Tested GAL4 lines were crossed to *UAS-PACα* and *UAS-ChR2*. For the PACα screen, 2-5 day old female flies were starved for 24 hours prior to the assay. Flies were glued to glass slides using myristic acid and allowed to recover for 2-4 hours in a humid chamber. Illumination with blue light was accomplished by placing the slides on a Zeiss LSM 510 microscope and observing the flies through the FITC filter cube with the mercury lamp. Intensity of the blue light was adjusted to 12 mW/cm². For the retest, flies were observed prior to stimulation under dim illumination with white light. Each fly was stimulated twice for 30 s, with a 30 s break in between stimulations. Flies were considered to respond if they extended during either stimulation, as a certain level of cAMP may have been required to achieve activation and cAMP produced during the first stimulation may have persisted until the next one. n=7-11 flies were tested for each genotype.

For the ChR2 preliminary screen, female flies were collected on the day of eclosion and placed on fly food containing 1 mM retinal for four days. Flies were starved for 24 hours before the assay and on 1 mM retinal in water. Illumination with blue light was accomplished as described above; intensity of the blue light was adjusted to 10 mW/cm². Each fly was stimulated three times for 10 s, with 10 s in between each stimulation; the responses of each fly were averaged. n=8-20 flies were tested for each genotype.

G-CaMP Imaging
G-CaMP imaging was performed as previously described (46). To generate averaged and subtracted images, frames 39 to 48 were averaged to form a baseline image and frames 70-78 were averaged to form a peak fluorescence image. The baseline image was subtracted from the peak fluorescence image, and the resulting picture was adjusted for contrast and brightness. These manipulations were performed using ImageJ.
Figure 2.1. An inducible activation screen to identify neurons whose activity is sufficient to elicit proboscis extension.  
(A) Preliminary screen comparing proboscis extension elicited by neuronal activation using UAS-\( \text{PAC} \alpha \) or UAS-ChR2. 4/30 (13%) lines exhibited extension when neurons were activated using PAC\( \alpha \) and 10/30 (33%) lines exhibited extension when neurons were activated using ChR2.  
(B) PAC\( \alpha \) screen of 110 Gal4 enhancer trap lines. Out of these lines, 26 showed greater extension compared to control UAS-PAC\( \alpha \) flies.  
(C) Retest of extendors from the full PAC\( \alpha \) screen. All lines showed greater extension compared to control UAS-PAC\( \alpha \) flies but only 15/26 lines showed greater extension during blue light stimulation compared to dim white light.  

Figure 2.2. Expression patterns of 12 GAL4 enhancer trap lines showing extension in an inducible activation screen for proboscis extension using PAC\( \alpha \). Neurons are expressing UAS-CD8-GFP. Scale bar, 100 µm.

Figure 2.3. 520 labels e49 neurons as well as other sugar-responsive motor neurons  
(A) Example showing responses of multiple neurons to 1 M sucrose. Colored ROIs in the image (left) correspond to the lines in the graph (right). The arrow points to the approximate time of stimulation. Scale bar, 50 µm.  
(B) Neurons in the 520 line show a higher peak change in fluorescence in response to 1 M sucrose as compared to water, 100 mM sucrose, caffeine and salt.  
(C) Subtraction of an average baseline image (left) from an average image of frames taken during the peak of the fluorescence response (center) produces an image where the morphologies of the responding neurons are distinguishable (right). Scale bar, 50 µm.  
(D–F) Subsets of neurons in 520 labeled by heat-shock mediated excision of a tub-GAL80 transgene, a GAL4 repressor. Neurons express UAS-CD8-GFP. (D) Neurons responding to 1 M sucrose exhibit similar morphologies to motor neurons in the 520 line. (E) A single motor neuron in the 520 line. (F) 520 also labels e49 motor neurons, accounting for the PAC\( \alpha \) phenotype. Scale bars, 50 µm.
Figure 2.2
Table 2.1: Presence of Gr5a GRASP signal in brains and proboscis sensory neurons of GAL4 lines identified in the inducible activation screen

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CHAPTER 3

Motor neurons controlling fluid ingestion in *Drosophila melanogaster*
Summary

Rhythmic motor behaviors such as feeding are driven by neural networks that can be modulated by external stimuli and internal states. In *Drosophila*, ingestion is accomplished by a pump that draws fluid into the esophagus. Here, we examine how pumping is regulated and characterize motor neurons innervating the pump. Frequency of pumping is not affected by sucrose concentration or hunger but is altered by fluid viscosity. Inactivating motor neurons disrupts pumping and ingestion, while activating them elicits arrhythmic pumping. These motor neurons respond to taste stimuli and show prolonged activity to palatable substances. This work describes an important component of the neural circuit for feeding in *Drosophila* and is a first step toward understanding the rhythmic activity producing ingestion.
Introduction

In many systems, complex motion is controlled by central pattern generators (CPGs), neural circuits that can produce oscillatory activity independent of sensory input (42, 53). Feeding behaviors such as chewing and sucking require coordinated contraction of different muscle groups in a rhythmic pattern. In *Drosophila*, ingestion is driven by a pump located in the proboscis (54, 55). While the mechanics of fluid ingestion have been examined in other insects, the neural circuits controlling ingestion have not been extensively characterized (56–58).

The fruit fly *Drosophila melanogaster* is an excellent model system for examining neural control of fluid ingestion because both neurons and behavior can be studied using molecular and genetic approaches. In *Drosophila*, feeding begins with detection of a palatable food source followed by proboscis extension and fluid ingestion. Sensory neurons located in the proboscis, legs, mouthparts, wing margins and ovipositor allow the fly to detect a variety of compounds, including sugars, bitter substances, carbon dioxide and water (29–31). Many of these neurons send projections to the subesophageal ganglion (SOG) of the fly brain (11). Also located in the SOG are motor neurons that innervate muscles involved in feeding behaviors (46, 59). Two muscles (muscles 11 and 12) comprise a pump; the activity of these muscles fills a chamber (the cibarium) with fluid and expels the fluid into the esophagus (41, 54, 55). Previous work has identified motor neurons projecting to muscle 11; when these neurons are inhibited, food consumption on a short timescale decreases (47). While neurons comprising a pump CPG have not been identified, there is evidence for the existence of a larval feeding CPG in *Drosophila* and other insects. In these experiments, nerve recordings of isolated brain preparations displayed rhythmic firing patterns that resemble activity recorded during feeding (60–62).

How do pump motor neurons control ingestion? Motor neurons may be simple passive effectors of a pumping CPG or could contribute to its rhythm. We performed neuronal inhibition experiments to determine the requirement of muscle 11 and muscle 12 motor neurons in pumping behavior. To determine if motor neurons can drive pumping behavior, we performed inducible activation experiments. If motor neurons were passive effectors of a pumping CPG, their activation would lead to prolonged contraction of the target muscles. If motor neuron activity directly contributed to rhythmic pumping, activation could produce pumping.

In this work, we examine the regulation of pumping behavior and characterize the role of motor neurons in producing pumping. These studies describe a key component of the feeding circuit in *Drosophila* and provide insight into the rhythmic activity underlying fluid ingestion.
Results

**Pump frequency is affected by viscosity but not sucrose concentration or hunger**

To characterize the pumping rhythm and its modulation, we examined whether pump frequency in wild type flies is affected by sucrose concentration, hunger state or viscosity. Previous studies have shown that the proboscis extension response (PER), a behavior in which flies extend their proboscis when presented with palatable stimuli, can be modulated by sucrose concentration and hunger (41), but their effect on pumping behavior is unknown. To measure pump frequency, we monitored fluid movement through the cibarium by filming flies as they drank sucrose mixed with dye. Dye intensity in the cibarium was measured over time (Fig. 3.1 A and B) and pump frequency was estimated as the location of the peak in the discrete Fourier transform of this signal (Fig. 3.1 B and C).

Pumping behavior is stereotyped and rhythmic; flies showed reliable pump frequencies of ~6 Hz. The pump frequency of Canton-S flies changed little under different fasting regimes or when given fluids of similar viscosities but differing sucrose concentrations or (Fig. 3.1 B, C and D). By contrast, proboscis extension was influenced both by sucrose concentration and starvation time. These data suggest that regulation of food intake in response to these factors is accomplished by modulating initiation of feeding without altering the mechanics of ingestion.

Fluid viscosity however does affect pump frequency. Pump frequency was reduced when flies were fed sucrose mixed with increasing amounts of methylcellulose (MC), a thickener (Fig. 3.1 B and E) or when given highly viscous sucrose solutions (Fig. 3.1 E). We analyzed the duration of cibarial filling versus emptying and found that the majority of the pump cycle is devoted to filling the proboscis (Fig. 3.1 F), suggesting that this is the rate-limiting step in pumping. In fact, the reduction in pump frequency upon drinking 500 mM sucrose + 2.5% MC is largely due to an increase in cibarial filling time. These results show that fluid viscosity affects pump frequency, with a greater effect observed on the rate of cibarial filling. The observation that viscous fluids reduce pump frequency suggests that the pump rate is influenced by feedback from receptors that monitor cibarial expansion or fluid content.

**Three GAL4 lines label motor neurons projecting to the cibarial pump**

To characterize the neural control of cibarial pumping, we made use of three GAL4 enhancer trap lines that label motor neurons innervating the pump. We named these lines *MN11*+12, *MN11* and *MN12*. Motor neurons in *MN11*+12 innervate both muscles 11 and 12 (Fig 3.2 B), whereas motor neurons in *MN11* innervate only muscle 11 (Fig 3.2 C) and motor neurons in *MN12* innervate only muscle 12 (Fig 3.2 D). The *MN11*+12 and *MN11* lines are very sparse with little or no labeling outside motor neurons in the central brain (Fig 3.2 B and C) and sparse labeling in thoracic ganglia (Fig 3.3). These lines also express GAL4 in 1-4 mouthpart sensory neurons in the dorsal cibarial sense organ (DCSO; Fig 3.4 A). The *MN12* line labels central neurons in addition to motor neurons (Fig 3.2 D).

Line *MN11*+12 labels more motor neurons than the other two lines (Table 3.1). To determine if there is overlap among these neurons, we performed cell counts in flies carrying each pairwise combination of GAL4 drivers. We found that *MN11* labels a subset of *MN11*+12 neurons, whereas *MN12* may label neurons not present in either of the other lines (Table 3.1; *MN11*+12 vs. *MN11*+12 + *MN11*, p-value = 0.605; *MN11*+12 vs. *MN11*+12 + *MN12*, p-value = 0.028; *MN12* vs. *MN11* + *MN12*, p-value = 9.046e-21; t-test). Earlier studies identified motor neurons by injecting horseradish peroxidase into specific proboscis muscles and assaying for
peroxidase activity in the brain (59). Three motor neurons innervating muscle 11 and two innervating muscle 12 were observed. Each of the GAL4 lines identified here labels fewer cells, suggesting each line labels a subset of the neurons projecting to these muscles.

We visualized single neurons in these lines using two methods: (i) by stochastically excising the GAL4 repressor GAL80 from a subset of neurons, and (ii) by taking advantage of natural variability in GAL4 expression (46). Individual motor neurons exhibit bilateral arbors in the dorsal SOG and project to either muscle 11 or 12 (Fig 3.5).

Proper pumping and ingestion requires neurons in these GAL4 lines
To determine how motor neuron activity affects pump frequency and ingestion, we expressed UAS-tetanus toxin (TNT) using these GAL4 lines. Tetanus toxin inhibits synaptic transmission by cleaving n-synaptobrevin and preventing vesicle fusion (63). Inhibiting MN11+12 motor neurons reduced the pump frequency (Fig 3.6 A and B), ingestion rate (Fig 3.3 C) volume of fluid ingested per pump (Fig 3.6 D) and the fluid content within the cibarium (Fig 3.6 E and F). Slower pump frequency is not due to inactivation of DCSO neurons, as this phenotype is still observed when motor neurons but not DCSO neurons are inactivated using a different GAL4 line (Fig 3.4 B and C). Inhibiting MN12 neurons also reduced the frequency, ingestion rate and fluid content (Fig 3.6 B, C and E) but did not affect volume per pump (Fig 3.6 D). Lastly, inhibiting MN11 neurons had no effect on frequency or fluid content (Fig 3.6 B and E) but did reduce ingestion rate and volume per pump (Fig 3.6 C and D). The reduction in pump frequency upon silencing of MN11+12 and MN12 neurons was primarily due to a longer cibarial filling time whereas the cibarial emptying time was less affected (Fig 3.6 G).

These results show that both muscle 11 and muscle 12 contribute to normal fluid consumption. However, silencing muscle 12 motor neurons affects pump frequency, duration of cibarial filling and fluid content, whereas silencing muscle 11 motor neurons does not. These data suggest that the two muscles have different roles in fluid ingestion, with muscle 12 critical for pumping and muscle 11 critical for ingestion downstream of pumping (i.e., swallowing). However it is possible that muscle 12 and muscle 11 do play a role in swallowing and pumping respectively, as not all the motor neurons projecting to each muscle have been inactivated.

Inducible activation of motor neurons leads to pumping or cibarial expansion
We next determined whether activating motor neurons is sufficient to produce pumping. We used the GAL4 lines to express UAS-dTRPA1, a cation channel that opens at temperatures >25ºC, depolarizing cells (64). We also included the tsh-GAL80 transgene to limit expression in the thoracic ganglion (Fig 3.3) (65). Prior to the experiment, flies were fed a small amount of 1 M sucrose containing blue dye to enable visualization of the cibarium. No sensory stimulus was applied during dTRPA1 activation.

Flies expressing dTRPA1 in MN11+12 neurons exhibited pumping behavior when placed at 32ºC (Fig 3.7 A); however, pump frequency was slower and more variable than during drinking (Fig 3.7 B and C). Pumping was not due to activation of DCSO neurons, as it was still observed when motor neurons but not DCSO neurons are activated using a different GAL4 line (Fig 3.4 D). Activation of MN12 neurons alone or MN11 neurons alone did not produce pumping (Fig 3.7 A). Instead, activation of MN12 neurons caused prolonged expansion of the cibarium (Fig 3.7 D and E). No obvious phenotype was observed with activation of MN11 neurons (Fig 3.7 A).
Thus, simultaneous activation of muscle 11 and muscle 12 motor neurons is sufficient to produce pumping, although it is usually slower and more sporadic than during drinking. This shows that tonic activation of muscle 11 and muscle 12 motor neurons is sufficient to generate pumping and suggests that both motor outputs together influence the pumping rhythm. Activation of MN12 neurons elicits cibarial expansion without pumping, while activation of MN11 motor neurons is not sufficient to produce either cibarial expansion or pumping.

**Pump motor neurons respond to taste compounds**

We also monitored taste-induced activity in MN11 and MN12 motor neurons with the genetically encoded calcium indicator GCaMP to characterize their response properties to palatable and aversive stimuli (40).

GCaMP imaging of MN11 and MN12 neurons revealed stronger responses to sucrose as compared to water and caffeine (Fig 3.8). These compounds differ in viscosity and palatability, with sucrose being the most palatable and caffeine being aversive. Oscillatory fluorescence changes corresponding to pumping were not detected, as G-CaMP kinetics are likely limiting (decay $t_{1/2} = 384 \pm 76$ ms, 10 action potentials in mouse cortex; (40)). For MN11 neurons, the peak fluorescence change was unaffected by the identity of the taste compound; however, response duration increased with sucrose as compared to water (Fig 3.8 B-D).

MN12 neurons showed a larger peak fluorescence change and longer response duration to sucrose as compared to water and caffeine (Fig 3.8 and E-F). The peak fluorescence change and response duration were significantly larger with 2 M sucrose compared to water and caffeine and with 1 M sucrose compared to caffeine.

These results demonstrate that MN11 and MN12 neurons can respond to multiple compounds but show prolonged activation to palatable sensory stimuli that elicit pumping.
Conclusion

In this work, we characterized the role of cibarial pump motor neurons in producing pumping behavior. We found that inactivation of these motor neurons can reduce pump frequency and ingestion, whereas activation of these neurons can elicit arrhythmic pumping. Finally, we showed that these neurons respond to tastants, with a longer response observed with more palatable compounds. In addition, we found that pump frequency is not regulated in response to stimulus palatability or hunger, but does change with viscosity.

The role of muscles 11 and 12 in pumping behavior

Our experiments suggest that the two muscles comprising the pump have different roles in fluid ingestion. Inactivation of muscle 12 motor neurons inhibits pumping but does not reduce the volume per pump whereas inactivation of muscle 11 motor neurons does not affect pumping but does affect the volume per pump. Similarly, activation of MN12 neurons produces cibarial expansion, whereas activation of MN11 neurons does not. In addition, anatomical studies have shown that muscle 12 inserts in the roof of the cibarium, whereas muscle 11 is located more posteriorly (54, 55). These data argue that the two muscles have distinct roles in fluid ingestion, and are most consistent with the notion that contraction of muscle 12 produces cibarial expansion whereas contraction of muscle 11 leads to swallowing (Fig 3.9). However, our data do not formally exclude a role for muscle 12 and muscle 11 in swallowing and pumping respectively, as the GAL4 lines used do not label all the motor neurons projecting to each muscle.

The role of motor neurons in generating the pumping rhythm

In many systems, central pattern generators (CPGs) produce rhythmic output that drives motor behaviors such as feeding. CPGs are usually comprised of interneurons that then synapse onto motor neurons. Motor neurons can act as simple passive effectors of the CPG or they can interact with the CPG to influence its output. For example, excitatory motor neurons that receive input from the leech swimming CPG do not influence its output pattern; by contrast, inhibitory motor neurons synapse onto CPG neurons and can affect their activity (66). Motor neurons can also form part of the CPG. In the crustacean stomatogastric system, motor neurons innervate stomach muscles and also synapse with each other to form a CPG that controls the chewing and filtering of food (44).

Our data show that motor neurons innervating the cibarial pump in Drosophila can influence its output. Inactivating MN11+12 and MN12 neurons produces slower pumping, whereas activating MN11+12 neurons can elicit pumping. However, activating either MN11 or MN12 neurons does not elicit rhythmic pumping. Instead, activating MN12 neurons produces prolonged expansion of the cibarium rather than pumping, arguing that cibarial filling is mediated by muscle 12 neurons. As pumping is observed upon MN11+12 neuronal activation, muscle 11 neurons may indirectly cause cibarial emptying (Fig 3.9). Alternatively, muscle 12 motor neurons in the MN12 and MN11+12 lines may not be identical and could have different effects on the pump.

Motor neurons could influence the pumping CPG in several ways. These motor neurons could form part of the CPG (as in the crustacean stomatogastric system), or could influence the CPG through synaptic connections (similar to the leech swimming system). Consistent with these models, expressing the presynaptic terminal marker UAS-n-synaptobrevin-GFP (67) in MN11+12 neurons leads to labeling in the brain as well as in pump muscles (Fig. 3.10),
suggesting they may have postsynaptic partners in the brain. Dissecting the circuit responsible for pumping in *Drosophila* will require a closer examination of the functional connectivity of motor neurons with each other and with unidentified upstream neurons.

**The role of sensory feedback in pumping**

Although CPGs can produce oscillatory activity in isolation, sensory and other modulatory inputs can affect the output pattern (42, 44). Similarly, pumping is likely to be influenced by feedback from receptors that monitor cibarial expansion or fluid content. The observation that viscous fluids reduce pump frequency supports this idea. Moreover, this reduction in pump frequency is largely due to an increase in the duration of cibarial filling rather than emptying. This result suggests that achieving a specific level of cibarial expansion or fluid content may promote emptying of the cibarium, thereby influencing the pump frequency (Fig 3.10). Proprioceptors lining the cibarial walls have been described in the blowfly and if present in *Drosophila* may provide this input (68, 69); chemosensory cells are also present within the cibarium (11).

The pumping phenotypes observed upon motor neuron inactivation may also be partially due to sensory feedback. Our data show that silencing *MN12* neurons does lead to slower cibarial expansion. However, sensory cues signaling cibarial expansion may not be necessary or sufficient to produce cibarial contraction and pumping. Flies with inactivated muscle 12 neurons can pump but show reduced fluid content within the cibarium, suggesting cibarial contraction can occur even if a normal level of cibarial expansion or fluid content is not reached. In addition, activation of *MN12* neurons elicits prolonged expansion rather than pumping. However as activation experiments were performed in the absence of fluid intake, it is possible that activation of sensory receptors monitoring fluid content could produce cibarial contraction.

**Regulation of feeding in *Drosophila***

Feeding behavior must adapt to varying external stimuli and internal states; this can occur by changing the probability of initiation or termination, or by altering feeding mechanics. As shown here and in other work, initiation of feeding as measured by PER increases with higher sucrose concentrations and longer fasting periods (41). By contrast, pump frequency is not affected by these factors. Previous work in the blowfly has shown that feeding duration increases with longer fasting periods (70). Thus, it appears that regulation of feeding in response to these factors acts on initiation and termination rather than on the mechanics of feeding.

**Motor neurons and the feeding circuit**

How cibarial pump motor neurons interact with the rest of the feeding circuit in *Drosophila* is an open question. Fluid ingestion must be coordinated with other aspects of the feeding motor program such as proboscis extension and retraction. Furthermore, PER and pumping may be initiated by different chemosensory inputs, as taste neurons are present within the cibarium itself and stimulation of tarsal sensory neurons can elicit PER but not pumping (11, 41).

Our imaging data show that pump neurons can be activated in response to multiple substances, although sucrose elicits greater activity compared to water or caffeine. This result is consistent with the fact that flies prefer sucrose over water or caffeine (29, 41). Both *MN11* and *MN12* neurons show longer response durations to sucrose, while *MN12* neurons also show an increase in peak fluorescence changes. These results suggest that increased activation of acceptance sensory neurons is propagated to the motor neurons through the pump circuit. The
different viscosities of the tested compounds may also contribute to variations in the responses. Electrophysiological experiments that simultaneously monitor sensory neurons, motor neurons and muscle activity will aid in understanding the sensorimotor transformation that leads to pumping.

Our work paves the way for future studies of the function of pump motor neurons and their role in the feeding circuit. A more precise understanding of these neurons will provide insight into the architecture of neural circuits underlying behavior as well as elucidate mechanisms by which rhythmic motor activity is generated.
Materials and Methods

Experimental Animals

_Drosophila_ were reared on standard cornmeal/agar/molasses medium. For _UAS-TNT_ experiments, flies were raised at 18°C; for _UAS-dTRPA1_ and Canton-S experiments, flies were raised at room temperature. All other flies were raised at 25°C. Lines _MN11+12-GAL4_ and _MN11-GAL4_ are NP lines from the _Drosophila_ Genetic Resource Center (NP1363 and NP0534, respectively). Line _MN12-GAL4_ is from Dr. Thomas Clandinin's collection (# 423). The following lines were also used: Canton-S (Wuerzburg), _UAS-mCD8::GFP_ (51), _UAS-TNT_ (63), _tsh-GAL80_ (65), _UAS-dTRPA1_ (64), _UAS-G-CaMP3_ (gift from Charles Zuker and (40)), _721-GAL4_ (Heberlein collection), _UAS-n-syb-GFP_ (Bloomington), _UAS-DenMark_ (Bloomington), _tub > GAL80 > (46); and _hs-FLP, MKRS_ (Bloomington).

Immunohistochemistry

Immunohistochemistry was performed as previously described (32) using the following primary antibodies: rabbit anti-GFP (1:1000; Invitrogen, cat# A11122), nc82 (1:500, Developmental Studies Hybridoma Bank; concentrate), Alexa Fluor 647 phalloidin (1:400; Invitrogen, cat# A22287), rabbit anti-DsRed (1:500; Clontech; cat# 632496), rabbit anti-DsRed (1:1000; Biovision; cat# 3993-100) and mouse anti-GFP (1:1000; Invitrogen; cat# A11120). Secondary antibodies used were Alexa Fluor 488 goat anti-rabbit IgG (1:100; Invitrogen, cat # A11008), Alexa Fluor 568 goat anti-mouse IgG (1:100; Invitrogen, cat# A11004), Alexa Fluor 568 goat anti-rabbit IgG (1:100; Invitrogen; cat# A11036) and Alexa Fluor 488 goat anti-mouse IgG (1:100; Invitrogen; cat# A11029). Images shown are collapsed confocal stacks.

Labeling of single neurons

Visualization of single neurons was performed as previously described (46).

Pumping Assay

We used 2-10 day old female flies for all experiments except for _MN12 TNT_ experiments, when we used 15-18 day old flies. Flies were food-deprived before the start of the assay (~24 hours unless stated otherwise) on a wet Kimwipe. Flies were glued onto a glass slide using nail polish and parafilm was placed over the lower thorax to limit leg movements. Flies were then placed in a humid chamber for 2-4 hours.

Flies were fed sucrose (1 M unless stated otherwise) mixed with 5 mg/mL erioglaucine, a blue food dye (Sigma, cat# 861146-5G). For Canton-S experiments, flies were also fed sucrose mixed with methylcellulose, a thickener (Sigma, cat# M7140-100G). Flies were observed with an Olympus SZX16 stereomicroscope and filmed at 60 fps using a Firefly MV camera (FMVU-13S2C-CS; Point Grey Research). Wiretrol capillaries (Drummond Scientific, cat# 5-000-1003, inner diameter 0.494 mm) were used to present taste stimuli. The capillary was photographed before and after feeding to determine the amount of fluid ingested.

For _721 TNT_ experiments, flies were filmed using a QuickCam Communicate STX web camera (Logitech). The flies carried the _Gr5a-LexAVP16_ and _LexAOp-GAL80_ transgenes to eliminate TNT expression in sugar-sensitive taste neurons.

For each movie, we drew a region of interest (ROI) around the cibarium and measured the average pixel intensity within the ROI over a three-second pumping epoch. For Canton-S
flies presented with 50 mM sucrose, we used a pumping epoch of one second as they rarely drank longer. We then calculated the discrete Fourier transform of this average ROI intensity trace and estimated the pump frequency as the location of the peak. If prolonged extension but no pumping occurred, we assigned a frequency of 0.

To determine the duration of cibarial filling and emptying, we first identified the local maxima and minima of the average intensity trace. A minimum corresponds to a full cibarium whereas a maximum corresponds to an empty cibarium. The duration of filling was calculated as the average time between each maximum and the subsequent minimum, and the duration of emptying was calculated as the average time between each minimum and the subsequent maximum.

The rate of fluid ingestion was calculated as volume ingested / feeding duration. We measured the length of fluid in the capillary tube before ($l_1$) and after ($l_2$) feeding; volume of fluid ingested was calculated as $(l_2 - l_1) * \pi * (\text{inner diameter of capillary} = 0.494 \text{ mm})^2$. Duration of feeding was determined from the number of frames in each movie in which the fly was actively drinking. We calculated the volume of fluid ingested per pump as the (volume of fluid ingested / duration of feeding) / (pump frequency).

To estimate the fluid content within the cibarium, we measured the area of an ROI drawn around the region of the cibarium filled with blue dye. We chose three frames with the maximal cibarial expansion (i.e., largest dye area) within their respective pump cycles. We normalized the area by the width of the proboscis to account for differences in size among the flies. The normalized values were then averaged for each fly.

**Proboscis extension response**

This assay was conducted simultaneously with the pumping assay using the same flies and taste compounds. Flies were first given water mixed with 5 mg/mL erioglaucine until no proboscis extension occurred. We then applied taste stimuli to the legs and proboscis and scored the occurrence of proboscis extension during the first presentation.

**Inducible Activation using UAS-dTRPA1**

We tested 2-5 day old female flies. To restrain flies, we used a chamber in which two glass coverslips formed a slit; four flies were inserted into the slit by the cervix. The proboscis was secured in an extended position using a drop of glass-fill candle wax (Yaley, Joann.com). Wax was used to fix the legs to the coverslip to prevent obstruction of the cibarium. Flies were then placed in a humid enclosure for 2-4 hours.

Flies were initially fed a small amount of 1 M sucrose mixed with 10 mg/mL erioglaucine to visualize the cibarium. We filmed each fly at 22-24°C for 30 seconds at 30 fps using a Firefly MV camera; the chamber was then transferred to a heat block for 2 minutes before filming at 30-32°C.

Pumps were counted manually. For 721 dTRPA1 experiments, pumps were counted over 5 seconds. Intensity traces were generated using the method described for measuring pump frequency. To determine the pump frequency over time, we identified the local minima in each intensity trace, each corresponding to a full cibarium, and recorded a 1 if a minimum occurred and a 0 otherwise. We then estimated the rate using a one-second moving average.

To measure the change in cibarial expansion, we drew an ROI around the cibarium in three frames of each movie at ~23, 27, and 30 seconds and averaged the areas of these ROIs. We
then subtracted the mean cibarial area at 24°C from the mean cibarial area at 32°C to find the change in cibarial area.

**G-CaMP Imaging**
For these experiments, 2-12 day old female flies were food-deprived for ~24 hours on a wet Kimwipe. Five flies were mounted onto a specialized imaging chamber in which slits hold the flies by the cervix. The heads were fixed in place using nail polish and the proboscis was secured with a drop of candle wax. The antennae and the cuticle above the SOG was removed and the exposed brain was bathed in AHL (52).

G-CaMP imaging was performed on a 3I spinning disk system with a 40x water objective using a 488 nm laser. Images were acquired at 19.9 fps, 256 x 256 pixels, 85 x 85 µm². Some samples were acquired at 13.3 fps; no significant difference in the analyzed metrics was found between samples acquired at 19.9 fps and 13.3 fps.

A capillary filled with a taste compound plus 2.5 mg/mL erioglaucine was presented to the proboscis 8 seconds after the start of filming; total imaging time was 40 seconds. Each fly was stimulated once with a single tastant on the proboscis. After imaging we photographed each fly using a Firefly MV camera.

To analyze the movies, we first used DipImage (‘http://www.diplib.org’), a software toolbox for Matlab, to compensate for X-Y translational motion. We then drew an ROI around each neuron and measured the average pixel intensity over the ROI for each frame. We calculated %ΔF/F for each frame as (F - Fbaseline)/Fbaseline * 100. Fbaseline was found by averaging the pixel intensities during the 3 seconds before stimulation (5 to 8 seconds after the start of the movie). We then applied a one-second moving average to the %ΔF/F trace and used this new trace to find the peak %ΔF/F and the response duration, calculated as the length of time that the response was >15% to minimize confounds due to movement or imperfect baseline subtraction.

To create the %ΔF/F pseudo-colored image, we first generated a baseline image by averaging the frames 3 seconds before stimulation (from 5 to 8 seconds after the start of the movie). Using the peak %ΔF/F frame, we applied the following equation to each pixel: (pixelpeak – pixelbaseline) / pixelbaseline * 100.

**Data Analysis**
We used ImageJ to select ROIs and measure pixel intensities, areas and capillary fluid lengths. Further analysis was performed using custom scripts written in Python. Matlab was used to generate the pseudo-colored image and to perform the Tukey-Kramer test for multiple comparisons. Fisher’s exact tests were performed by inputting data into the website (‘http://www.graphpad.com/quickcalcs/contingency1.cfm’). T-tests were performed using Python.

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Figure 3.1. Pump frequency is affected by viscosity but not hunger or sucrose concentration.

(A) A Canton-S fly drinking 100 mM sucrose mixed with blue dye. An ROI (dotted circle) is drawn around the cibarium. Scale bar, 0.1 mm.
(B) (Left) Sample traces showing the average intensity over time in a cibarial ROI for different fasting conditions and sucrose concentrations. When the cibarium is full of dye, intensity is low and when the cibarium is empty, intensity is high. (Right) Discrete Fourier transforms of these traces.
(C) Average pump frequency and PER response after 24-hour fasting upon feeding with multiple sucrose concentrations. The approximate viscosities of pure sucrose solutions at 20°C are as follows: 50 mM, 1.055 cP; 100 mM, 1.08 cP; 500 mM, 1.72 cP (71).
(D) Average pump frequency and PER response for different fasting periods upon feeding with 100 mM sucrose.
(E) Average pump frequency upon feeding with 500 mM sucrose mixed with methylcellulose, a thickening agent, and viscous sucrose solutions. The approximate viscosities of pure sucrose solutions at 20°C are as follows: 1 M, 4.05 cP; 2 M, 370.1 cP (71).
(F) Average cibarial filling and emptying time during a single pump upon feeding with 500 mM sucrose mixed with methylcellulose and viscous sucrose solutions. n=14-21 flies for pump frequency and n=47-101 flies for PER measurements. Error bars are SEM for pump data and 95% binomial confidence intervals for PER data. *P<0.5; **P<0.01; ***P<0.001; ANOVA followed by Tukey-Kramer test for pump data and Fisher’s exact test for PER data. For (E) and (F), comparisons are to 0% MC and 0.5 M sucrose.

Figure 3.2. Anatomy of cibarial pump motor neurons and muscle innervation in the GAL4 lines.

(A) (Left) A fly drinking sucrose mixed with blue dye; arrowhead points to open cibarium. Scale bar, 0.1 mm. (Right) Schematic of fly head showing muscles 11 and 12.
(B) MN11+12 motor neuron cell bodies are located in the brain (left panel) and project to muscles 11 (center panel, lateral view) and 12 (right panel, frontal view). The center panel shows the dotted region in (A); arrows point to neuronal projections. (C) MN11 motor neurons project to muscle 11.
(D) MN12 motor neurons project to muscle 12; arrowheads point to motor neurons. Scale bar, 100 µm. A, anterior; P, posterior; D, dorsal; V, ventral. Motor neurons express CD8-GFP (green), neuropil is labeled with nc82 (magenta), and muscles are labeled with phalloidin (blue).

Figure 3.3 The tsh-GAL80 transgene limits GAL4 expression in the thoracic ganglion.

(A-C) (Left panels) Thoracic ganglion expression in flies containing the MN11+12, MN11, and MN12 GAL4 drivers along with UAS-CD8-GFP. Flies carrying the tsh-GAL80 transgene show reduced expression in the thoracic ganglion (middle panels) but normal brain expression (right panels). Scale bars, 100 µm.

Figure 3.4. Neurons in the dorsal cibarial sense organ (DCSO) are not responsible for pumping phenotypes.

(A) MN11+12 (left) and MN12 (center) express GAL4 in DCSO neurons, but 721 (right) does not.
(B) 721 expresses GAL4 in motor neuron cell bodies in the brain (left, arrowheads) that project to muscle 11 (center, arrows) and muscle 12 (right).

(C) Reducing synaptic transmission from 721 neurons using TNT leads to slower pumping.

(D) Activation of 721 neurons using dTRPA1 produces pumping. Genetic (721-GAL4 and UAS-dTRPA1) controls show significantly less pumping than 721 x dTRPA1 flies at 32 °C, as do temperature (721 x dTRPA1 (25)) controls which were measured at 25 °C. n=8 flies for TNT experiments; n=11-19 for dTRPA1 experiments except for 721 x dTRPA1 where n=5. Error bars are SEM. ***P<0.001, *P<0.05; t-test. Scale bars, 100 µm. A, anterior; P, posterior; D, dorsal; V, ventral.

Figure 3.5. Individual motor neurons project to either muscle 11 or 12. Individual MN11+12 neurons were visualized by heat-shock-FLPase-mediated stochastic excision of a GAL80 transgene flanked by FRT sites (46). Individual MN12 neurons were observed due to natural variability in the GAL4 line.
(A) A single motor neuron in the MN11+12 line projects to muscle 11 (center panel, lateral view; right panel, frontal view).
(B) A single motor neuron in the MN12 line projects to muscle 12. Scale bars, 100 µm. A, anterior; P, posterior; D, dorsal; V, ventral.

Figure 3.6. Inhibiting neurons using UAS-tetanus toxin (UAS-TNT) reduces pump frequency and fluid ingestion.
(A) (Left) Sample traces showing the average intensity over time for an ROI drawn around the cibarium. (Right) Discrete Fourier transforms of intensity traces.
(B) Average pump frequency, (C) rate of fluid ingestion, (D) volume of fluid ingested per pump and (E) cibarial fluid content.
(F) Cibarial fluid content was estimated by calculating the average area of an ROI drawn around the cibarial region filled with blue dye during frames when the cibarium was maximally open (i.e., when the dye area was largest). ROI area was normalized by the width of the proboscis to normalize for size differences among flies. Scale bar, 0.1 mm.
(G) Average cibarial filling and emptying time during a single pump. n=18-33 flies for each genotype, except for cibarial fluid content measurements, where n=10-11 flies. Error bars are SEM. *P<0.5; **P<0.01; ***P<0.001; ANOVA followed by Tukey-Kramer test; comparisons are to genetic controls.

Figure 3.7. Activating neurons using UAS-dTRPA1 can elicit pumping or cibarial expansion.
(A) Average number of pumps over a 30-second time window. Pumps were counted before (24°C) and during (32°C) activation.
(B) Sample traces showing the average intensity over time for an ROI drawn around the cibarium. Flies were initially fed a small amount of 1 M sucrose mixed with blue dye before filming. (i) Sample trace of a UAS-dTRPA1 fly drinking 1 M sucrose mixed with blue dye at 32°C. (ii), (iii), (iv) Sample traces of flies observed at 32°C without tastant presentation.
(C) Pump frequency over a one-second moving time window for the traces shown in (B).
(D) An MN12 x dTRPA1 fly before (top) and during (bottom) dTRPA1 activation. The dotted line surrounds the blue region and was used to estimate cibarial area. Scale bar, 0.1 mm.
(E) Change in cibarial area upon shifting flies to 32°C from 24°C. n=18-36 flies for each genotype. Error bars are SEM. ***P<0.001; one-way ANOVA (cibarial area) and two-way ANOVA (pump count) followed by Tukey-Kramer test; comparisons are to genetic (cibarial area) or both genetic and temperature controls (pump count).

**Figure 3.8. Motor neurons show calcium responses to taste stimuli, with stronger responses to sucrose.**
(A) Heat maps showing the peak %(ΔF/F) of MN11 and MN12 neurons in response to various compounds. Scale bar, 10 µm.
(B) Sample %(ΔF/F) traces from MN11 and MN12 motor neurons in response to various compounds.
(C-D) Data from MN11 neurons. (C) Peak %(ΔF/F), and (D) response duration, calculated as time when the response >15%.
(E-F) Graphs as in (C-D) for MN12 motor neurons. n=10-13 flies per data point. Error bars are SEM. *P<0.5; **P<0.01; ANOVA followed by Tukey-Kramer test.

**Figure 3.9. A model for how pump motor neurons and muscles produce pumping behavior.**
Muscle 12 activation produces cibarial opening, whereas muscle 11 activation leads to fluid ingestion (i.e., swallowing). Cibarial opening activates sensory receptors (SR) that detect cibarial expansion or fluid content, which then promotes inhibition of muscle 12 motor neurons (MN12) and cibarial emptying. Muscle 11 motor neurons (MN11) and muscle 12 motor neurons may reciprocally inhibit each other, producing the pumping seen during inducible activation of MN11+12 neurons. These interactions shown may be indirect and influence a pumping CPG upstream of the motor neurons.

**Figure 3.10. MN11+12 motor neurons express both the dendritic marker DenMark (72) and the presynaptic terminal marker n-synaptobrevin-GFP (nsyb-GFP (67)) in the brain, but express only nsyb-GFP in the pump muscles (center panel, lateral view; right panel, frontal view).**
(A-B) Expression of DenMark in MN11+12 motor neurons; CD8-GFP is included for visualization of the neurons.
(C-D) Expression of nsyb-GFP in MN11+12 motor neurons; CD8-RFP is included for visualization of the neurons. Scale bars, 50 µm. A, anterior; P, posterior; D, dorsal; V, ventral.
Figure 3.1

A

B

(i) 10 h/100 mM suc
(ii) 24 h/100 mM suc
(iii) 24 h/500 mM suc
(iv) 24 h/500 mM suc + 2.5% MC

Time (s)

Pump frequency (Hz)

Normalized magnitude

C

D

E

F

Time (s)

Normalized magnitude

Normalized magnitude

Normalized magnitude

Normalized magnitude

Normalized magnitude

Normalized magnitude
Figure 3.2
Figure 3.3

Thoracic Ganglion
- tsh-GAL80

Thoracic Ganglion
+ tsh-GAL80

Brain
+ tsh-GAL80

A

MN11+12

B

MN11

C

MN12

35
Figure 3.4
Figure 3.7

A

Number of pumps

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<th>24</th>
<th>32</th>
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<th>32</th>
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<th>32</th>
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<td>MN11+12 x dTRPA1</td>
<td>MN11+12-GAL4</td>
<td>UAS-dTRPA1</td>
<td>MN11 x dTRPA1</td>
<td>MN11+12-GAL4</td>
<td>UAS-dTRPA1</td>
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***

B

(i) UAS-dTRPA1 - drinking

(ii) UAS-dTRPA1

(iii) MN11+12 x dTRPA1 - fly 1

(iv) MN11+12 x dTRPA1 - fly 2

C

(i) Pump frequency (Hz)

(ii) Pump frequency (Hz)

(iii) Pump frequency (Hz)

(iv) Pump frequency (Hz)

D

UAS-dTRPA1

24°C  | 32°C

MN12 x dTRPA1

24°C  | 32°C

E

Δcubital area (µm²)

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<th>MN11+12 x dTRPA1</th>
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***
Figure 3.8

A

B

C

D

E

F

Figure 3.8

A

B

C

D

E

F
Figure 3.9

- MN12
- MN11

- Muscle 12
- Muscle 11

- SR

- cibarial opening
- fluid ingestion
Figure 3.10
Table 3.1: Cell counts/hemisphere of motor neurons in GAL4 lines
(mean ± SEM; n=15-25 brains)

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<th>GAL4 drivers</th>
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<td>MN11+12</td>
<td>2.8 ± 0.7</td>
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<tr>
<td>MN11</td>
<td>1.5 ± 0.9</td>
</tr>
<tr>
<td>MN12</td>
<td>1.0 ± 0.4</td>
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<tr>
<td>MN11+12 and MN11</td>
<td>2.8 ± 0.7</td>
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<tr>
<td>MN11+12 and MN12</td>
<td>3.2 ± 0.6</td>
</tr>
<tr>
<td>MN11 and MN12</td>
<td>2.7 ± 0.7</td>
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CHAPTER 4

Characterization of potential taste projection neurons
Summary

Integration of information from multiple senses allows organisms to engage their environment more effectively. In *Drosophila*, the association of gustatory and olfactory stimuli has been most extensively studied using a classical conditioning paradigm in which flies learn to prefer odors that have previously been paired with sugar. The neural basis for this association is beginning to be understood. Here I characterize two GAL4 enhancer trap lines, 112697-GAL4 and 605-GAL4, that label neurons with projections to regions of the brain important for taste and odor detection as well as learning. My results show that these neurons contact sensory taste neurons, but their targets in the brain are unclear. Output from these neurons is not necessary for proper learning in a gustatory-based assay. Future experiments must be conducted to determine if these neurons are synaptically connected to sensory taste neurons and what their functional role may be.
Introduction

The brain integrates information from multiple sensory modalities to produce a unified percept. In *Drosophila*, studies using a classical conditioning paradigm that pairs gustatory and olfactory cues have provided insights into this process. In this paradigm, flies are fed sucrose (the unconditioned stimulus) in the presence of an odor (the conditioned stimulus). When later tested using a two-choice olfactory assay, flies prefer the sucrose-paired odor over an unpaired odor (73). Previous research has shown that output from the mushroom bodies, a target of olfactory projection neurons, is necessary for proper learning to occur. In addition, the neurotransmitter octopamine is also necessary for proper learning in this assay, and activation of octopaminergic neurons can substitute for sucrose as the unconditioned stimulus (73). However, how sensory taste information is transmitted to octopaminergic neurons is unknown.

Honeybees also exhibit associative olfactory learning, and past work has identified a sucrose-responsive octopaminergic neuron, VUMmx1, that likely plays a role in this process (74, 75). This neuron exhibits projections in the SOG, the antennal lobe, and the mushroom bodies, and activation of this neuron is sufficient to act as an appetitive stimulus in olfactory learning assays. This data suggests that VUMmx1 likely transmits information about the presence of sucrose in the environment to higher brain centers.

Flies can also display associative learning in a taste-based assay. When presented with sugar stimuli, flies normally extend their proboscis; however, if flies are subjected to a punishing stimulus upon extension, subsequent presentations of sugar will not elicit extension (76). Proper learning in this assay also requires the mushroom bodies (76 and unpublished data). How sensory taste information is transmitted to learning centers is unknown.

Here I present experiments characterizing two enhancer trap lines, 112697-GAL4 and 605-GAL4, that label neurons whose anatomy suggested they may be potential second-order taste projection neurons (TPNs) similar to VUMmx1 neurons (Fig. 4.1).
Results

112697-GAL4 and 605-GAL4 project to the SOG, antennal lobe, and dorsomedial brain
Initial examination of the expression patterns of lines 112697 and 605 show neurons with projections to the SOG, the antennal lobe and the dorsomedial region of the brain near where the mushroom bodies are located (Fig. 4.1 A-B). Unlike VUMmx1 neurons which are located in the ventral SOG, the cell bodies of these neurons are located lateral to the antennal lobe, similar to the location of olfactory projection neurons (75, 77). To more clearly visualize their morphology, I generated flies in which only a subset of the neurons in these lines were labeled by stochastically excising a GAL80 transgene (a GAL4 repressor) using a heat shock FLPase. These experiments revealed two types of neurons, one with SOG projections (Fig. 4.1 C) and one without (Fig. 4.1 D). Both types appeared to arborize in the antennal lobe and the dorsomedial brain.

Potential TPNs contact sensory taste neurons
Next I performed experiments using the GRASP technique to determine if the SOG projections of these neurons contacted the axons of sensory taste neurons. To more easily visualize SOG projections, GRASP experiments were performed in conjunction with stochastic GAL80 excision so that only some of the 112697 and 605 neurons were labeled. These experiments showed that SOG projections from these neurons do contact sensory taste neurons (Fig. 4.2 A-C). Overall, neurons contacted bitter-sensitive Gr66a cells in 5/6 brains, sugar-sensitive Gr5a cells in 2/8 brains, and water-sensitive ppk28-cells in 1 brain. Because the projections of potential TPNs terminate in the dorsal SOG, it is possible that they contact sensory taste neurons from the internal mouthparts, which express Gr66a and ppk28 but not Gr5a. (Other neurons in the mouthparts express the receptor Gr64f, which are required for responses to multiple sugars (78)). GRASP experiments are indicative only of contact, not synaptic connections; further experiments are needed to determine whether these neurons receive input from specific populations of sensory taste neurons.

Potential TPNs do not contact glomeruli or mushroom body cells
Next, I more closely examined the projections of potential TPNs in the antennal lobe and the dorsomedial brain (Fig. 4.3 A). Surprisingly, the projections of the potential TPNs in the antennal lobe appear to terminate posterior to the glomeruli, the target of olfactory sensory neurons (Fig. 4.3 A). The postsynaptic partners of these potential TPNs are unclear. Similarly, closer inspection of the dorsomedial projections also revealed that they do not target the mushroom bodies. Instead, they terminate in a region anterior to the mushroom body dendrites (i.e., the calyces) and posterior to the mushroom body axons (i.e., the lobes; Fig. 4.3 B). Again, the potential targets of projections in this region are unknown. Expression of UAS-n-synaptobrevin-GFP, which localizes to presynaptic terminals, suggests that the projections in the SOG, antennal lobe, and dorsomedial brain may be presynaptic (Fig. 4.3 C) (67).

Potential TPNs are not required for gustatory learning
Lastly, I sought to examine if these neurons are necessary for gustatory learning in a taste association paradigm. In this assay, flies are touched on the legs with sucrose, an appetitive stimulus that elicits proboscis extension. Upon extension, the proboscis is touched with quinine, a bitter substance, which causes retraction. Subsequent presentation of sucrose leads to reduced
extension. Proboscis extension resumes after multiple trials in which the flies are given sucrose without quinine. Previous experiments in our lab have shown that the mushroom bodies are required for proper learning in this assay (unpublished data). To determine if potential TPNs might play a role in this learning paradigm, these neurons were transiently inactivated using \textit{UAS-ts-shibere}. This gene encodes a temperature-sensitive dynamin that disrupts synaptic vesicle recycling and transmission when flies are placed at the restrictive (32 °C) temperature (79).

Results of this experiment show that synaptic output from neurons in the 112697 line is not required for proper learning in this assay (Fig. 4.4 A). Flies tested at restrictive and permissive temperatures behaved similarly in the assay. Without quinine, flies extend to sucrose on almost every trial, showing that the reduction in proboscis extension is not due to fatigue or desensitization (Fig. 4.4 B). Finally, flies expressing \textit{UAS-ts-shibire} in sweet-sensitive Gr5a neurons exhibit a reduction in proboscis extension at the restrictive temperature, signifying that the \textit{UAS-ts-shibere} transgene is capable of reducing neuronal output (Fig. 4.4 C).
Conclusions

Two enhancer trap lines, 112697 and 605 express GAL4 in neurons that send projections to the SOG and higher brain regions. GRASP experiments suggest that they contact sensory taste neurons, but anatomical analysis showed that they do not project to the glomeruli or the mushroom bodies. Behavioral experiments show that they are not necessary for proper performance in a gustatory associative learning assay.

My anatomical experiments suggest that these neurons contact sensory taste neurons. Because GRASP does not reveal synaptic connections, future experiments are needed to determine if the potential TPNs identified here are synaptically connected to sensory neurons. G-CaMP imaging or electrophysiological monitoring of the neurons during gustatory stimulation will reveal if they are part of the taste circuit. While technically challenging, electrical stimulation of mouthpart neurons while recording from potential TPNs would determine if they were monosynaptically connected.

Our results do not support a role for these neurons in gustatory learning, suggesting that information about the two taste stimuli (sucrose on the leg and bitter on the proboscis) are processed independently of these neurons. However, it is possible that 112697 labels only a subset of TPNs and other neurons compensate for their inactivation. Whether these neurons play a role in olfactory learning is unknown. Although their projections do not contact glomeruli or mushroom body neurons, they may connect to interneurons that receive or transmit information from these areas. Future experiments to test whether they are required for olfactory learning or respond to odors may reveal such a role for these neurons.
Materials and Methods

Experimental Animals
*Drosophila* were reared on standard cornmeal/agar/molasses medium at 25 °C, except for flies used in the learning assay which were reared at room temperature (~23 °C). Line 112697 is from the NP collection at the *Drosophila* Genetic Resource Center (NP1559) and line 605 is from Dr. Thomas Clandinin’s collection. The following lines were used: *UAS-mCD8::GFP* (51), *Gr5a-LexA::VP16* (46); *Gr66a-LexA::VP16* (generated in the lab); *ppk28-LexA::VP16* (generated in the lab); *UAS-CD4::spGFP1-10* (46); *LexAop-CD4::spGFP11* (46); *MB-DsRED* (80); *UAS-n-synaptobrevin* (67); *UAS-ts-shibere* (76); and *Gr5a-GAL4* (32).

Immunohistochemistry
Immunohistochemistry was performed as previously described (32) using the following primary antibodies: rabbit anti-GFP (1:1000; Invitrogen, cat# A11122), mouse anti-GFP (1:100; Sigma, cat# G6539), rabbit anti-dsred (1:500, BioVision), rat anti-CD8. Images shown are collapsed confocal stacks.

Generation of FLPout clones
FLPout clones were generated as previously described (46).

Gustatory learning paradigm
Flies were starved on a wet Kimwipe for 12-16 hours before the start of the assay. Flies were glued onto a glass slide using nail polish and left to recover for 5-8 hours in a humidified chamber. Prior to the start of the assay, flies were water-satiated and then stimulated with 1 M sucrose three times with water satiation in between each stimulation. Flies that failed to extend to any of the stimulations were discarded. For the five training sessions, the flies were given 50 mM sucrose on the legs; if they extended, they were touched on the labellum with 100 mM quinine. Extension during each session was scored. For test sessions, flies were given sucrose without quinine. Flies were water satiated in between each training and test session. Two separate populations of flies were tested at the permissive (20–23 °C) and restrictive (30-32 °C) temperatures.
Figure 4.1. 112697 and 605 express GAL4 in neurons with projections to the SOG, antennal lobe and dorsal brain regions. (A-B) Full expression pattern of 112697 (A) and (B) 605. White arrowheads point to cell bodies of potential TPNs. (C-D) Anatomy of single cells in the 112697 line with (C) and without (D) projections to the SOG. Red arrow points to SOG projections and red arrowheads point to projections in antennal lobe area and dorsal brain. Neurons express UAS-CD8-GFP and the neuropil is labeled with nc82. Scale bars, 100 µm.

Figure 4.2. Potential TPNs contact Gr66a and ppk28 cells but not Gr5a cells as observed with GRASP. Only a subset of the neurons in the full 112697 and 605 lines express UAS-CD8-RFP and GRASP molecules to more easily visualize the projections. (A-B) Potential TPNs in the 112697 line exhibit GRASP signal between the SOG projections and Gr66a axons (A), but not Gr5a axons (B). (C) A potential TPN in the 605 line exhibits GRASP signal between its SOG projections and ppk28 axons. Arrowheads point to SOG projections and GRASP signal (yellow punctae) if present. Scale bar, 50 µm.

Figure 4.3. Potential TPNs do not send projections to glomeruli or to the mushroom bodies. (A) Projections in the antennal lobe area of a potential TPN in the 112697 line terminate posterior to the glomeruli, which can be visualized as globular regions of nc82 staining in the first three sections. Each image shows a collapsed stack spanning 6.7 µm along the anterior-posterior axis. (B) Projections to the dorsal region of the brain do not contact mushroom body neurons. Dorsal brain projections of a potential TPN in the 605 line terminate anterior to the mushroom body dendrites (i.e., calyx) and posterior to the mushroom body axons (i.e., lobes). Projections in the fourth image do not arise from potential TPNs. Mushroom bodies are labeled using the MB-DsRED transgene. Each image shows collapsed stacks spanning 5 µm along the anterior-posterior axis. Not shown is a 35 µm stack in between the third and fourth images. (C) In potential TPNs, n-synaptobrevin-GFP, a presynaptic terminal marker, is expressed in the SOG, antennal lobe area and dorsal brain projections (arrows). UAS-CD8-RFP is included to visualize the neurons. Scale bars, 50 µm.

Figure 4.4. Inactivating neurons in the 112697 line using ts-shibere does not affect taste learning. (A) Only flies that extended three times to 1 M sucrose prior to training were used in the assay. For training sessions 1-5 (TR1-5), 12-16-hour starved flies were touched on the leg with 50 mM sucrose to elicit proboscis extension. If extension occurred, flies were touched on the proboscis with 100 mM quinine, leading to a reduction in extension to sucrose during subsequent stimulations. For the test sessions (Test1-3), 50 mM sucrose was presented on the legs but no quinine was applied if extension occurred, leading to extinction of the memory. Flies tested at permissive (23 °C) and restrictive (32 °C) temperatures performed similarly in the learning assay. (B) In the absence of punishment with quinine, flies exhibit high levels of PER to sucrose at both permissive (23 °C) and restrictive (32 °C) temperatures.
(C) Flies expressing ts-shibere in sugar-sensitive neurons show reduced PER at the restrictive (32 °C) temperature. N=36-58 flies for (A), 18-20 flies for (B) and 7 flies (3 stimulations each) for (C). Error bars are 95% binomial confidence intervals.
Figure 4.1
Figure 4.2
Figure 4.3
Figure 4.4

(A) 112697 x ts-shi, with quinine

(B) 112697 x ts-shi, no quinine

(C) Gr5a x ts-shi

PER probability

TR1 TR2 TR3 TR4 TR5 Test1 Test2 Test3

TR1 TR2 TR3 TR4 TR5 Test1 Test2 Test3

50 mM sucrose 100 mM sucrose

23 °C 32 °C

23 °C 32 °C
CHAPTER 5

Discussion
Discussion

How sensory information is processed by the brain to give rise to motor behaviors is a long-standing question in neuroscience. The gustatory system in Drosophila affords a unique opportunity to study this question because of the relatively small size of the nervous system, the strong behavioral responses to taste stimuli and the myriad tools available to manipulate and monitor neurons. Recent work has begun to clarify the mechanisms underlying detection of sensory stimuli, but the rest of the gustatory circuit remains largely unmapped.

Strategies to identify and characterize higher-order taste neurons

For my thesis, I aimed to identify and characterize other neurons involved in taste processing. To do so, I performed an inducible activation screen using photoactivatable adenylate cyclase (PACα) to find neurons whose activity was sufficient to elicit proboscis extension. I was unable to identify novel neuronal populations mediating proboscis extension but the screen did uncover motor neurons that were responsive to taste substances. In addition, I performed experiments to characterize two enhancer trap lines that labeled neurons whose morphology suggested they might integrate olfactory and gustatory information and project to mushroom bodies, a higher brain area important for associative learning. Preliminary experiments suggest that they may synapse with sensory taste neurons, but their anatomical targets and function role remain uncertain. These experiments highlight the difficulty of mapping circuits even in as versatile an organism as Drosophila. However, new tools such as transsynaptic tracers and GAL4 lines with sparser neural expression are currently being developed and should be useful for identifying other elements of the gustatory system. In addition, recently developed inducible activators, such as the heat-activated dTRPA ion channel, may be useful for future proboscis extension screens.

Characterizing motor neurons involved in feeding

While the central brain neurons that process taste information remain a mystery, the motor neurons that mediate taste-related behaviors are more amenable for study. For my thesis, I focused on a population of motor neurons that innervate two muscles comprising the cibarial pump, which draws fluid into the esophagus during feeding. I used several enhancer trap lines that express GAL4 in these neurons to examine how their activity contributes to pumping. From this work, I determined that motor neurons innervating muscle 12 of the cibarial pump mediate cibarial expansion, while muscle 11 motor neurons likely mediate swallowing. I also found that sensory information can affect pump frequency and that inducible activation of both types of motor neurons produces pumping, suggesting that they play a role in generating the pumping rhythm.

Are motor neurons part of a pumping CPG?

In many systems, motor behaviors are controlled by central pattern generators (CPGs), neural networks that can produce a rhythmic output in the absence of external stimuli. In Chapter 3, I presented a schematic illustrating the interactions between motor neurons and sensory receptors that could accommodate the observed data. These interactions likely occur by affecting elements of a pumping CPG, as these circuits have been shown to underlie many forms of rhythmic motor activity.

Are muscle 11 and 12 motor neurons components of a pumping CPG? Evidence that support a role for neurons being part of a CPG are as follows: (1) firing in coordination with
rhythmic motor output, (2) disruption of the motor pattern upon inactivation, (3) initiation of the motor pattern upon activation and (4) ability to reset an already-active motor pattern upon activation.

Criterion 1 is fulfilled, as these are motor neurons that directly produce the motor output. Inactivation of the muscle 12 motor neurons does lead to a reduction in pump frequency, although this may be due to reduced activation of sensory receptors. Activation of both muscle 11 and muscle 12 motor neurons together can elicit pumping, fulfilling the third criterion.

Whether the activation of these motor neurons can reset the CPG (i.e., delay or advance the pattern) could be tested by transiently activating these neurons while the fly is drinking. For example, motor neurons expressing dTRPA1 could be rapidly activated with an infrared laser pulse; similarly, motor neurons expressing a light-activated cation channel such as Channelrhodopsin2 could allow for precise activation during known phases of the drinking cycle. If activation of these neurons caused a shift in the timing of the motor pattern, then they could be considered part of the CPG.

A model for pumping
An example for a model that combines a proposed pumping CPG with the results found in Chapter 3 is presented below. A simple model for a CPG that can produce alternating activity in different neurons is the half-center oscillator (42). In this mechanism, two neurons (or populations of neurons) excite downstream neurons while indirectly inhibiting each other. The intrinsic properties of the neurons allows for the eventual cessation of the inhibition. For instance, one neuron could have a slowly inactivating current that eventually stops the neuron from spiking, ‘releasing’ the second neuron from inhibition; alternatively, the second neuron could produce a slowly depolarizing current that eventually allows it to ‘escape’ from the inhibition (81).

A pumping CPG could be comprised of a half-center oscillator (Fig. 5.1, red components). The activity of one of the neurons in the oscillator (O1) could lead to both excitation of muscle 12 motor neurons and inhibition of muscle 11 motor neurons (allowing for cibarial filling) while the activity of the other neuron (O2) could lead to both excitation of muscle 11 motor neurons and inhibition of muscle 12 motor neurons (allowing for cibarial emptying and swallowing). Initiation of activity within this CPG would occur upon stimulation of sensory taste neurons.

Activation of MN11+12 neurons elicits pumping while activation of MN12 neurons alone elicits prolonged cibarial expansion. These results could be observed if muscle 11 motor neurons activated I_{12} neurons and muscle 12 motor neurons activated I_{11} neurons, producing another half-center oscillator (Fig. 5.1, blue components). Tonic activation of both muscle 12 and muscle 11 neurons could produce alternating patterns of activity as the two neuronal populations inhibited each other, whereas tonic activation of either muscle 11 or 12 motor neurons alone would not elicit pumping. In this model, transient activation of the neurons during drinking would likely not reset the pumping rhythm, as the motor neurons do not directly affect the oscillator. Our inactivation experiments suggest that inhibitory inputs deriving from motor neurons are sufficient but not necessary for inhibition of motor neurons, as inactivation of MN11 neurons does not affect pump frequency and inactivation of MN12 neurons does not affect the volume of fluid ingested per pump.

Our data also show that sensory input plays a role in setting the pump frequency. When flies drink viscous compounds, pump frequency is reduced. This could occur if sensory receptors
provided input into the oscillator. Cibarial expansion or filling caused by activation of muscle 12 motor neurons could be sensed by these receptors, which could then excite the O2 neuron, contributing to its escape from inhibition (Fig. 5.1, green components). Viscous substances flow more slowly into the cibarium, reducing the activation of the cibarial receptors and the excitatory drive to O2. Similarly, reducing synaptic transmission from muscle 12 motor neurons could lead to slower expansion of the cibarium and weaker cibarial receptor activation. Consistent with this hypothesis, reduction in pump frequency under these conditions is due primarily to a lengthening in the duration of cibarial filling (and therefore cibarial expansion). However, these flies also show a reduction in the fluid content within the cibarium, suggesting that pumping can occur even if a normal level of fluid content is not achieved. In addition, our inducible activation experiments suggest that sensory input monitoring cibarial expansion is not sufficient to inhibit muscle 12 motor neuron activity, as MN12 neuron activation produces prolonged expansion of the cibarium. Future work to identify the cibarial stretch receptors and manipulate their activity will help elucidate their contribution to pumping. Inactivating both cibarial stretch receptors and muscle 11 motor neurons at the same time would also reveal how much of the inhibitory input to muscle 12 motor neurons could be from these two populations.

Future directions
The work presented here provides a springboard for future studies of gustatory processing in Drosophila. The characterization of the pump motor neurons is a first step towards understanding the neural mechanisms underlying pumping. Experiments like those described above will help elucidate how these motor neurons contribute to the pumping rhythm. In addition, it would be possible to map the gustatory circuit from these neurons using GRASP. One portion of the GFP molecule could be expressed in motor neurons, while the second portion of GFP could be expressed random subsets of neurons using a GAL4 enhancer trap collection. Lines that labeled cells which contacted the motor neurons could then be further examined.

The field of Drosophila neuroscience has been rapidly expanding in recent years. Technological advances including the ability to perform electrophysiology on central neurons, the development of more sophisticated ways to create GAL4 enhancer trap collections, and other methods of identifying neurons that mediate specific behaviors and computations have broadened the field. In the years to come, it should be possible to assemble an anatomical and functional map of the neurons mediating taste perception and behavior, getting us closer to understanding how brains work.
**Figure 5.1. Model for a pumping CPG.** A pumping CPG comprised of a single half-center oscillator. Activation of muscle 12 and inhibition of muscle 11 are controlled by one oscillator neuron whereas inhibition of muscle 12 and activation of muscle 11 are controlled by the other oscillator neuron. Components drawn in red are oscillator neurons; components drawn in blue and green are connections surmised from the work presented in Chapter 3.
Figure 5.1
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