A Subset of Brain Neurons Controls a Sexually Dimorphic Proboscis Holding Behavior in Adult *Drosophila Melanogaster*

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ABSTRACT

Taste is essential for humans and animals alike to evaluate food quality and make important decisions about food choice and intake. How complex brains process sensory information to produce behavior is an essential question in the field of sensory neurobiology. Currently, little is known about taste circuits in the brain as compared to other sensory systems. Here, we used the common vinegar fly, *Drosophila melanogaster,* to explore the potential role of brain neurons labeled by a transgenic line *(VT041723-GAL4)* in producing "proboscis holding" behavior (extrusion of the mouthpart without withdrawal). By utilizing the *GAL4/UAS* binary expression system, we expressed a heat-activated cation channel *(UAS-dTrpA1)* in these brain neurons and artificially activated them by elevation of temperature, subsequently examining behavior in the heat-activated proboscis extension reflex (PER) assay. We found that activation of these neurons induced proboscis holding. Interestingly, the proboscis holding phenotype was sexually dimorphic. Male flies rarely showed proboscis holding and those that did had shorter proboscis holding durations. On the other hand, both mated and virgin females showed significantly more proboscis holding and had longer proboscis holding durations than male flies. Overall, we identified a subset of brain neurons labeled by the *VT041723-GAL4* line that controls a sexually dimorphic feeding response (proboscis holding) upon activation.

KEYWORDS: *Drosophila melanogaster;* GAL4/UAS system; taste circuit; feeding behavior; proboscis extension; sexual dimorphism

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Kush Amin is a third-year Biology major. His research explores the link between potential gustatory neurons and feeding behaviors in vinegar flies. He also served as a peer-mentor for Dr. Dahanukar's research program this past summer funded by the National Science Foundation (NSF). Additionally, he is a Quality Management Coordinator for the COPE Health Scholars program at Riverside Community Hospital. In the future, Kush plans to pursue a career in the field of medicine.

INTRODUCTION

One of the fundamental questions in the field of neuroscience is how the human brain responds to different sensory inputs. However, the human brain is too complex to study directly. Therefore, to address this fundamental question, we take advantage of a powerful genetic model organism, *Drosophila melanogaster,* better known as the common vinegar fly. There are several advantages of using the vinegar fly as an experimental model. They have numerically simpler brains, and they are genetically much simpler than mammals with only four pairs of chromosomes, allowing for easier manipulation. They also have fewer neurons compared to their mammalian counterparts, which can be specifically pinpointed with genetic tools. They are easy to rear in a simple cornmeal-based diet and they have a short life cycle of about ten days, allowing researchers to carry out experiments with ease. Notably, vinegar flies have sensory systems similar to those of humans, thus they still exhibit complex behaviors seen in humans, such as mating or feeding (Vosshall and Stocker, 2007). Therefore, the fundamental principles of how neuronal circuits in the fly brain process sensory information have the potential to be applied to human neurobiology as well.

The gustatory system of the fruit fly has emerged as a powerful model to explore the molecular and cellular basis of taste, due to the identification of chemosensory receptor genes and development of methods to assess feeding behaviors. In addition, the molecular genetic approaches available in flies allow us to activate or silence specific neurons. This enables us to explore the functional roles of specific neurons in feeding behaviors. Although there are many studies on the role of peripheral taste neurons in sensing various chemicals (Chen and Dahanukar, 2017; Ling et al., 2014; Raad et al., 2016; Weiss et al., 2011), little is known about how the peripheral sensory information is processed by higher-order neurons in the brain. In this study, we aim to use the *Drosophila* gustatory system to study candidate higher-order brain neurons that might be involved in processing taste information and mediating feeding behaviors.

To address this question, we used the Vienna Tile *GAL4 (VT-GAL4)* transgenic fly lines combined with the *GAL4/UAS* binary expression system to access different subsets of neurons in the adult fly brain (Kvon et al., 2014). We expressed heat-activated ion channels (dTrpA1) in different subsets of neurons labeled by different *VT-GAL4* lines (Kang et al., 2011) and examined the effects of neuronal heat activation on the extrusion of the proboscis, which is the elongated mouthpart of the fly involved in feeding. The extension behavior is commonly known as the proboscis extension reflex (PER) (Shiraiwa and Carlson, 2007). Our data suggests that one of the candidate *VT-GAL4* lines *(VT041723-GAL4),* which activates a unique proboscis holding response in the heat-activated PER assay, may be involved in processing taste information.

MATERIALS AND METHODS

Fly strains

Flies were reared on standard cornmeal-dextrose-agar food at 25°C and 60-70% relative humidity under a 12 hour:12 hour dark:light cycle. Three transgenic fly strains were used in this study: *VT041723-GAL4* (Vienna Drosophila Resource Center) (Kvon et al., 2014), *UAS-GFP* (Weiss et al., 2011), and *UAS-dTrpA1* (Bloomington Drosophila Stock Center #26263). The genotype of the experimental fly line was *UAS-dTrpA1/UAS-dTrpA1; VT041723-GAL4/VT041723-GAL4*. We had two control genotypes: the *VT-GAL4* only control (+/+; *VT041723-GAL4/VT041723-GAL4)* and the UAS-dTrpA1 only control *(UAS-dTrpA1/UAS-dTrpA1;* +/+). The +/+ chromosomes indicate wild-type. With only one transgene each, these control genotypes did not have *GAL4/UAS* binary expression and were expected to show no proboscis extensions. Thus, the control flies also ensured that observed proboscis extensions in experimental flies resulted from the binary expression of the *GAL4* and *UAS* transgenes, rather than either of the transgenes alone.

Heat-activated proboscis extension reflex (PER) assay

Individual flies were immobilized on glass coverslips with drops of clear, non-toxic nail polish (Sally Hansen Insta-Dri Top Coat) and then incubated for 1-2 hours in a humidified chamber made by filling a pipette tip box with water and placing damp Kimwipes (Kimberly-Clark Kimtech) on top. One by one, each coverslip containing an individual fly was placed on a 34ºC heat block and proboscis extension behaviors were observed under a light microscope. We recorded the following parameters for

each experimental trial: trial number, sex, participation, and extension duration. In all experiments, we tested male, mated female, and virgin female flies. To eliminate other variables, we kept the ages and sample size of flies for each sex constant.

Statistical analyses

All data are presented as mean ± S.E.M. Statistical tests were conducted using Prism 7 (GraphPad Software). Differences between means of different groups were evaluated for statistical significance with the Kruskal-Wallis Test, followed by the *post hoc* Dunn's multiple comparison test.

RESULTS

To identify higher-order neurons in the brain that process taste information, we took advantage of the transgenic resources in the Vienna Tiles GAL4 *(VT-GAL4)* Library at the Vienna Drosophila Resource Center (VDRC). GAL4 is a yeast transcriptional activator that can bind to *UAS* sequences, thereby inducing gene expression downstream of the *UAS* sequences. In the *VT-GAL4* library, 964 *VT-GAL4* lines with different genomic DNA sequences show different labeling patterns that can be visualized by genetic crosses with *UAS-GFP* transgenic flies. The expression patterns of these *VT-GAL4* lines in the adult Drosophila brain have been well-documented on the VDRC website (Kvon et al., 2014). The Dahanukar lab has done a preliminary image-based screen for neurons that arborize in and around the subesophageal zone (SEZ), the primary taste center in the fly brain, and pinpointed several potential candidate lines. In this study, one candidate was selected for further analysis: *VT041723-GAL4 (VT23-GAL4)*. The *VT23-GAL4* line labels neurons in the anterior SEZ as well as other taste areas of the fly brain, suggesting that these neurons might be involved in processing taste information ***(Figure 1A).***

To determine whether the *VT23*-labeled neurons are involved in feeding behaviors, we artificially activated these neurons and measured proboscis extension. We expressed the Drosophila transient receptor potential channel, subfamily A, member 1 (dTrpA1), a heat-activated cation channel (Kang et al., 2011), in *VT23*-labeled neurons through the *GAL4/UAS* binary expression system (Brand and Perrimon, 1993). By crossing *UAS-dTrpA1* transgenic flies with *VT23-GAL4* transgenic flies, we are able to use the progeny from the crosses and activate the *VT23*-labeled neurons by elevating ambient temperature to 34°C. We used the proboscis extension reflex (PER) as a feeding behavioral readout (Shiraiwa and Carlson, 2007). Proboscis extension has been extensively used as a robust feeding behavior assay where the fly protrudes its mouthpart (proboscis) as an indication of food acceptance when sensing attractive chemicals in the environment. Interestingly, we found that activation of *VT23*-labeled neurons caused a unique PER response in which flies do not retract the proboscis but keep it extended for several minutes or longer. We therefore have termed this behavior "proboscis holding" ***(Figure 1B).***

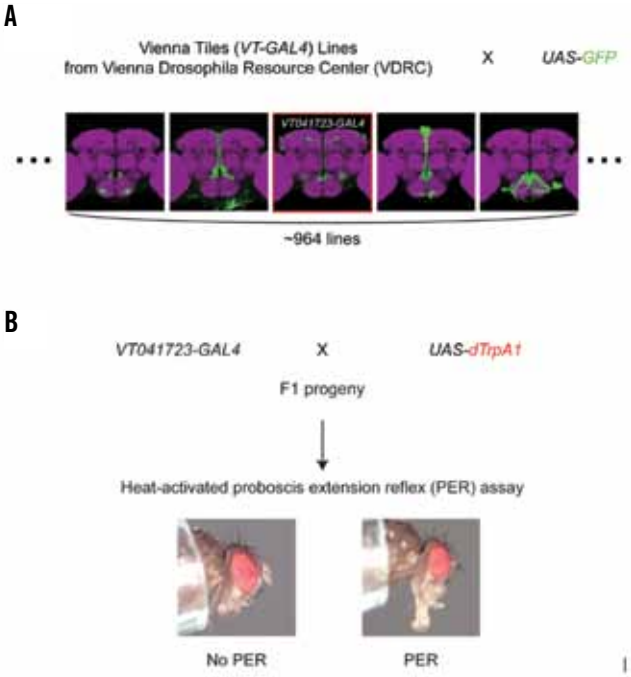

Figure 1. A feeding behavior screen of the VT-GAL4 transgenic library from the VDRC identifies VT041723-GAL4 labeled neurons as a potential candidate for higher-order taste neurons.

A. *Green Fluorescent Protein (GFP) expression patterns in the Drosophila brain driven by the 964 Vienna Tiles GAL4 (VT-GAL4) lines are documented in the Vienna Drosophila Resource Center (VDRC). Five different sets of brain neurons labeled by VT-GAL4 lines are shown as examples. The red box in the middle indicates the VT-GAL4 line (VT041723-GAL4) that was further analyzed based on a previous screen done in the lab.*

B. *Activation of VT041723-GAL4 labeled neurons is achieved by expression of dTrpA1, a heat-activated ion channel. Extensions of the fly mouthpart (proboscis) are scored by the heat-activated proboscis extension reflex (PER) assay.*

To determine if both males and females exhibited the same proboscis holding upon activating *VT23*-labeled neurons, we performed the heat-activated PER assay with male, mated female, and virgin female flies for both experimental and control genotypes. The proboscis holding phenotype was recorded on an all-or-nothing basis. If a fly had an extension time for one minute or longer upon heat activation, it was counted as proboscis holding with a value of 1. If there was no extension or the extension was shorter than one minute, it was given a value of 0. As expected, the *VT23-GAL4* and *UAS-dTrpA1* controls of all three test conditions did not show any proboscis holding. The experimental *VT23>dTrpA1* flies demonstrate varying levels of proboscis holding across sex and mating status. We found that 12.7% of male flies, 60.5% of mated female flies, and 33.3% of virgin female flies showed the proboscis holding response (N = 150; N = 172; N = 45, ***Figure 2A***). Mated female flies had greater participation than both virgin female and male flies, while virgin females had greater participation than the male flies. In summary, we found that activation of *VT23*-labeled neurons in the adult fly brain induces proboscis holding and such behavior may be sexually dimorphic, since female flies show significantly greater participation than male flies.

To further investigate the nature of proboscis holding in *VT23>dTrpA1* flies, we also recorded the duration of proboscis holding. We analyzed the average time of proboscis holding only for *VT23>dTrpA1* flies, since the controls flies did not show this behavior. We timed the flies for a maximum of 420 seconds due to time restraints. We found that the average proboscis holding durations were 119.7±27.01 seconds for male flies, 244.1±15.54 seconds for mated female flies, and 393.4±26.58 seconds for virgin female flies (N = 19; N = 104; N = 10, ***Figure 2B***). The virgin female flies had a significantly longer time of proboscis holding than mated females and males. In addition, female flies had a significantly longer time of proboscis holding than male flies.

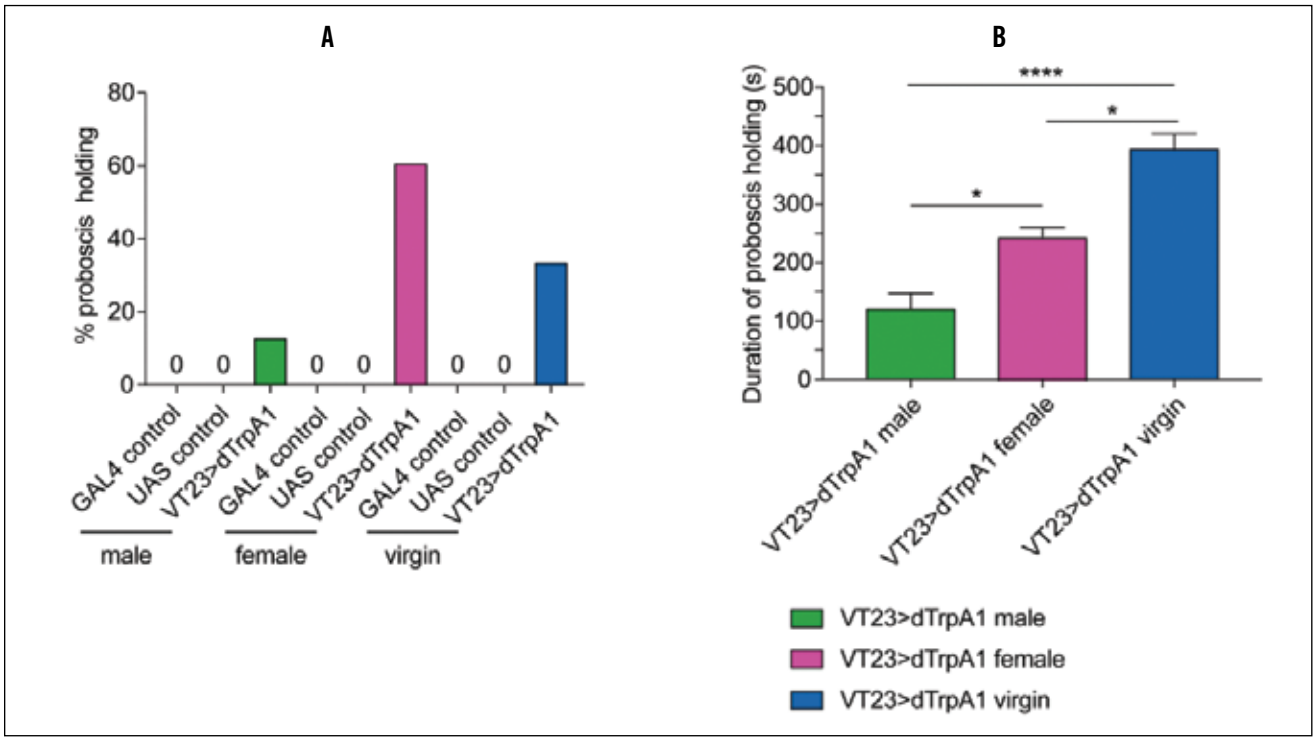

Figure 2. Activation of VT23-labeled neurons induces sexual dimorphic proboscis holding.
A. *Flies were tested via the heat-activated proboscis extension reflex (PER) assay and their responses were recorded. An extension greater than one minute was counted as proboscis holding. VT23>dTrpA1 flies showed varying degrees of participation among males (green), mated females (magenta), and virgin females (blue). The GAL4 and UAS transgene controls showed no proboscis holding. N = 45-172.*
B. *Flies were tested via the heat-activated PER assay and the duration of their proboscis holding was timed. N = 10- 104. Differences between means of different groups were evaluated for statistical significance with the Kruskal-Wallis Test followed by the post hoc Dunn's multiple comparison test. * $p<0.05$, **** $p< 0.0001$*

DISCUSSION

In this study, we characterized the role of a subset of brain neurons labeled by *VT041723-GAL4 (VT23-GAL4)* in feeding behavior in adult *Drosophila.* Thermogenetic activation of these brain neurons through expression of a heat-activated cation channel, dTrpA1, leads to the proboscis holding phenotype. Interestingly, the proboscis holding phenotype is sexually dimorphic. We observed that female *VT23>dTrpA1* flies exhibit proboscis holding more than males. In addition, the duration of proboscis holding is longer in both mated and virgin females than males.

The proboscis holding phenotype has not been described in literature. As reported by other research groups, when sugar-sensing neurons are thermogenetically activated, flies extend their proboscis many times as an indication of food acceptance (Du et al., 2016; Inagaki et al., 2014). In our study, we observed proboscis extension only one time without retraction when activating *VT23-GAL4* labeled brain neurons. Notably, many flies exhibited proboscis holding throughout the whole assay time (7 minutes). It is unclear whether proboscis holding indicates an attraction or aversion response. Given that there are multiple neurons labeled by this *VT23-GAL4* line, one way to interpret proboscis holding as an attractive response is that activation of some *VT23-GAL4* labeled brain neurons activate the appetitive taste circuit, leading to extension of the proboscis, while activation of other *VT23-GAL4* labeled brain neurons inhibit the motor program, preventing flies from retracting the proboscis. Alternatively, one could interpret proboscis holding as an aversive response, such as vomiting. Future research will need to be conducted to dissect these possibilities. For example, pre-feeding the flies before the heat-activated PER assay could potentially examine if food is vomited while proboscis holding.

Even though we do not have a conclusive answer for whether proboscis holding is an attraction or aversion response, we did reveal its sexually dimorphic nature. Female flies show proboscis holding significantly more than male flies. Why the sexual dimorphism exists will be another future research focus. Other research has found instances in which the neural circuitry of the fly brain differs between sexes (Kimura et al., 2005). Further experiments can compare the anatomy of *VT23-GAL4* labeled brain neurons in male and female flies. If the number or pattern of *VT23-GAL4* labeled neurons is different between sexes, this could potentially account for the sexually dimorphic proboscis holding and provide an entry point for further investigation of which neurons are responsible for the behavior. In addition to mated females and males, we also tested whether the mating status would affect the probability of flies showing proboscis holding. Since there were much fewer virgin female trials, more trials for virgin females need to be done in the future to confirm if the results we obtained were significant. However, our initial results still suggest the sexually dimorphic nature of proboscis holding, since both mated females and virgin females showed more and longer duration of the behavior than males.

In this study, we establish the foundation for future analysis of simple sensory behavior through the genetic model organism *Drosophila melanogaster,* specifically in the taste circuit. We focused on one candidate transgenic line *(VT23-GAL4)* that might be involved in feeding behaviors. By utilizing the *GAL4/UAS* binary expression system and performing the heat-activated proboscis extension reflex (PER) assay, we uncovered a unique proboscis holding phenotype when activating these *VT23-GAL4* labeled brain neurons. We also demonstrated that proboscis holding is a sexually dimorphic behavior. The ample genetic resources and reagents in Drosophila will allow us to further narrow down *GAL4* expression to single neuron resolution, such as transcription repressor GAL80 or split-GAL4 (Pfeiffer et al., 2010; Suster et al., 2004). This will allow us to pinpoint which individual neurons are involved in proboscis holding. Our results demonstrate a simple screening strategy that can be applied to other *VT-GAL4* lines to uncover different brain neurons processing taste information and mediate feeding behaviors.

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