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Investigation of Taste Neurobiology in *Drosophila*: From Peripheral Detection to Behavior

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ABSTRACT OF THE DISSERTATION

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by

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Drosophila melanogaster feed, mate and lay eggs on fermented compounds. Feeding on caloric-non-toxic foods is ideal, however flies primarily feed upon mixtures including attractive and aversive compounds. Investigating taste detection mechanisms is vital to understand feeding preferences. Utilizing *Drosophila* we have a unique opportunity to connect mechanisms of taste receptor function to feeding behaviors. We find that *Drosophila* exhibits a strong feeding behavior towards beer. Specifically, our data demonstrate that feeding preference towards fermentation products depends on gustatory receptor *Gr64e*, a receptor for glycerol. Glycerol is an attractive compound in yeast-fermented foods. Furthermore, *Drosophila* species that lack a functional copy of *Gr64e* have reduced responses to glycerol. Our data provide evidence that *Gr64e* may contribute to evolutionary shifts in food selection in *Drosophila* species.

We are beginning to understand gustatory receptor (Gr) response profiles, however mechanisms by which Grs function to encode specific compounds remain largely unknown. Peripheral detection of attractive compounds, such as sugars, is crucial for eliciting proper feeding behaviors. There are eight conserved Grs in the sweet clade expressed by sweet neurons. We characterize five Gr mutants using a panel of sweet compounds and find that each Gr mutant shows a loss in response profile to a sweet panel. In turn, these mutant data correlate with ectopic expression of corresponding single Grs in a novel *in vivo* system. Taken together our results indicate each Gr contributes to sugar detection and sugars can activate multiple Grs.

In addition to sugars, aversive compounds are also found in complex stimuli. Little is known about how flies taste acids despite their common occurrence in fruit sources. Here we explore the mechanism of acid detection in *Drosophila* and find that flies strongly avoid acids by a subset of bitter neurons. Furthermore, bitter neurons exhibit dose dependent activity increases in response to decreasing pH. Overall, our data sets a foundation for acid detection in *Drosophila* and facilitates further investigations for acid receptor identity. Our research demonstrates the power of *Drosophila* as a model system to characterize peripheral taste detection aiming to further understand how taste receptors function to encode taste compounds.

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Chapter 1:

Introduction

All animals rely on consuming food, an energy source, to fuel metabolic processes necessary for survival. How do animals know what is consumable and what is not? Animals rely on gustatory sensation, also referred to as the taste system, to evaluate food sources. The taste system evolved to detect various chemicals in the environment, allowing organisms to eat palatable foods while avoiding rotten or spoiled foods that may contain toxins.

Animal models of taste: Mouse and Drosophila melanogaster

Currently, our knowledge about the organization of the peripheral taste system comes from genetic model organism studies in mice and flies (*Drosophila melanogaster*). With the advantage of powerful molecular and genetic tools available in mice and flies, previous studies identify taste receptors for most of the classical modalities: sweet, savory (umami), salty, sour, and bitter. Additionally, careful physiological and behavioral studies show how both primary taste cells activate and send taste information to different regions in the brain[1,2]. We are beginning to understand the connections between peripheral detection and upstream neural circuits in the brain that integrate taste information and produce behavioral output. Mouse and human brains contain millions to billions of neurons and are extremely complex, which makes dissecting taste neural circuits difficult. However, the fly brain has ~100,000 neurons and about 1,000

peripheral taste neurons[3], making them a tractable organism to investigate in terms of the organization and function of the taste system.

Significance of studying insect taste biology

Drosophila detect many of the same classes of taste compounds as mammals and have similar feeding preferences to humans[4]. Examining mechanisms of taste behavior in *Drosophila* not only illuminates fundamental knowledge about general taste biology, but also provides potential solutions for controlling pest and vector insects. Insects present great costs to humans through crop and livestock damage as well as human disease transmission[5]. Pest and vector insects cause billions of dollars in damage for commercial food growers as well as for the millions of people affected by insect-born transmitted diseases[5,6]. Specifically, some examples include the Asian citrus psyllid and *Drosophila suzukii*, which are two of insects that significantly affect fruit crops. Among vector species, mosquitoes transmit deadly diseases like malaria, dengue, and chikungunya, all of which cause large numbers of deaths[6]. Many countries utilize insect control agents to kill and prevent the growth of pest and vector species. However, insects often gradually become resistant to insecticides, which are expensive and can be extremely toxic to humans and other animals. By studying general insect taste biology and attractive feeding behaviors toward food sources, entomologists may be able to exploit pest and

vector insect taste behaviors with more efficient chemical attractants or deterrents to lessen their destructive effects.

Feeding behaviors in *Drosophila*

Flies use their taste system to not only find food and avoid possible toxic chemicals, but also evaluate potential mating partners and oviposition sites. They accomplish these behaviors by detecting compounds in their environment with taste receptors found in peripheral neurons housed in taste organs. The taste signals initiated by these neurons transmit multimodal (e.g., olfactory information) and feeding state signals (e.g., hunger) to the brain where they stimulate appropriate feeding behaviors.

The primary taste organ in both mice and humans is the tongue, which is covered by numerous taste buds. The taste receptor cells (TRCs) inside the taste buds detect various taste stimuli. Taste receptors are also found in a variety of non-canonical taste organs, such as reproductive tissues in humans and flies[7,8]. Meyer et al. 2012 hypothesize that mice have taste receptors in their reproductive tissues so that sperm can navigate the oviduct and facilitate egg targeting[8]. By contrast, the fruit fly has an even greater variety of peripheral taste organs, including the proboscis (mouth), tarsi (legs), wings, and female ovipositor[9,10].

Previous work by Dethier 1976 has shown that flies' multiple taste organs inform the sequence in their feeding behaviors—a sequence that begins with them probing food sources with their legs. Once they evaluate food as being

acceptable for consumption, flies then extend their proboscis and initiate sucking and ingestion behaviors[11].

Flies generally feed on an array of fermented and rotten foods. These complex food sources contain various taste categories that must be sensed and integrated before feeding begins. To better understand how *Drosophila* both detect and process taste information into feeding behaviors, it is important to first explore the peripheral taste system's organization.

Peripheral taste organs in Drosophila

Flies' external taste organs are covered by hair-like structures called sensilla. The major taste organ at the tip of their proboscises is called the labellum, which contains approximately 31 sensilla per half. Sensilla are characterized into three types depending on their location and size: large (L), intermediate (I), and small (S)[12]. These sensilla are stereotypical from fly to fly and allow for precise characterization. L- and S-sensilla contain four gustatory receptor neurons (GRNs). Hiroi et al 2002, 2004 show that L-sensilla GRNs selectively sense compound profiles belonging to the sweet, salt (high and low), or water. They also show that S-sensilla GRNs selectively sense compound profiles belonging to the sweet, bitter, salt, or water. I-sensilla contain only two GRNs that selectively respond to sweet or bitter[13,14]. Additionally, every sensillum contains a mechanosensory neuron that senses touch and thus can detects and transmits multimodal signals containing taste and somatosensory information[12].

GRNs send dendrites up the length of the sensillum, where gustatory receptors (Grs)—expressed in GRNs—come in contact with compounds through a small pore at the tip of the sensillum.

It is intriguing that not all of the sensilla contain the same number of GRNs, possibly indicating an initial level of taste coding in the flies labeller architecture. There are also internal organs that are involved in detecting taste compounds. For example, when fly spreads its labial palps, there are peg neurons contained in pore-like structures on the internal side. Flies suck food into their esophagus, compounds are detected by three sets of internal pharyngeal neurons located in similar pore-like-structures along the length of the labellum, before the food is swallowed[10,15]. Thus fruit flies can sense taste compounds in multiple organs throughout the body, which suggests flies' ability to integrate taste information within and among multiple body parts.

Taste projections and organization in the brain

GRNs send their axons to the primary taste center within the subesophageal ganglion (SOG). The SOG contains a spatial organization, whereby neurons coming from the pharynx project dorsal-anteriorly, labellar neurons project medially and tarsal neurons project ventral posteriorly. One recent study in the mammalian gustatory cortex has shown non-overlapping taste modality dependent activity in the primary cortex, in which they propose a spatial map of taste perception encoded for sweet, bitter, umami and salt tastes[16].

Consistent with the mammalian system, the sweet and bitter neuronal projections are segregated in the SOG of flies. It will be interesting to compare higher taste processing centers between mammals and flies; however, the higher-order neurons that relay taste information from SOG to other brain regions have yet to be discovered.

Taste receptors in Drosophila

Clyne et al. 2000 first described a large diverse taste receptor clade of 68 gustatory receptors (Grs)[17] in *Drosophila melanogaster*. Grs were discovered around the same time olfactory receptors (Ors) were discovered. Over the past fourteen years since we have discovered Grs in *Drosophila*, many olfactory receptors (Ors) have been decoded with the empty neuron system[18] as well as how they function with a co-receptor (Orco)[19]. In contrast, very few Grs have known receptor function and we have yet to uncover the functional composition of Grs in flies. The first confirmation of Gr function in flies was found in the gene *Gr5a*. Mutants for *Gr5a* have lost trehalose sensitivity[20].

Additionally, there are more than just Grs that respond to chemical stimuli in the peripheral taste neurons affecting behavior. Three major families of receptor/channels are involved and thought to be associated with peripheral detection of attractive and aversive stimuli. A large family that was recently discovered is ionotropic receptors (Ir)s[21]. Irs were first described as olfactory receptors, however they are not related to Ors, but have evolved from ionotropic

glutamate receptors. Primarily these receptors detect various categories of odors in the olfactory system[21,22], however more recently, they are expressed[23] and functional in the GRNs. Ir20a clade may be involved in pheromone detection[23] and Ir76b is a receptor for low salt detection[24]. Much less is known about the roles of Irs in GRN detection and feeding behavior, which allows speculation of Irs to have novel taste functions, yet to be discovered. Another family that has importance to feeding behavior is the pickpocket (ppk) channels. The function of Degenerin/epithelial sodium channels or ppks in flies is largely unknown, however ppk channels have been observed to be important for water detection, pheromone detection and mechanosensory nociceptive stimuli[25]. Finally, the Transient receptor potential (TRP) channel family is important for temperature sensing in both humans and flies, however TRPs have also been shown to mediate noxious chemicals in fly bitter GRNs[26,27].

Taken together there are many receptor families mediate taste detection. Of the known receptor/channel families, we still do not know what each receptor responds to and further whether receptors found in overlapping expression patterns functionally interact. We are beginning to understand and characterize genes that have roles in peripheral gustatory receptor neuron detection. Each of these families (Grs, ppks, TRPs and Irs) appear to be largely functionally distinct, however can share stimulus detection[28,29]. Lastly, there are still genes that have not fully characterized in the *Drosophila* genome. Among these transmembrane proteins, “orphan” and not-yet discovered receptor/channels, future studies will

be needed to investigate whether these unknown and undiscovered genes have a role in gustatory detection.

Defining taste and taste coding theories

We, as humans are able to taste various categories of compounds, however until recent advances in our understanding of taste receptor function, there remained some qualifying definition of what taste is and furthermore the idea of "basic tastes". What is "taste" or "basic tastes?" Definitions have been put forward to describe taste sensation and two schools of thought have emerged to test these definitions of taste.

One view "across-fiber pattern," states that taste is a continuum of sensation e.g. we do not taste sugar-salt mixtures as sugar and salt alone, but a perception of both. Across-fiber pattern hypothesizes that the quality of taste information is encoded in a pattern of activity across populations of neurons with varying amounts of activity that are integrated in the brain[30]. Evidence of this theory was obtained as taste cells in both mice and frogs were shown to respond to most of the four basic taste categories, however each cell might have a "best" taster response e.g. sugar[31,32]. Across-fiber theory is generally agreed upon to explain how the olfactory system codes smell and may also describe taste coding. The second theory is "labeled-line" theory, which proposes that taste is not a continuum, but segregated into taste modalities where neurons code taste information intrinsically. Labeled-line predicts that sugars and salts are sensed by different dedicated neurons, which individually code for each taste, sending

information that is integrated upstream in the brain. Strong evidence for labeled-line coding is demonstrated for sweet and bitter detection in both flies and mammals[33,34].

Labeled-line coding in the taste system

Currently, labeled-line is the prevailing theory, as mounting evidence for labeled-line coding is demonstrated in both mice and *Drosophila*. Both mammals and flies cells involved in taste detection express non-overlapping repertoire of receptors. Mice and *Drosophila* receptors that are activated by sugars or bitters are expressed in separate taste cells and projected to distinct brain regions. This suggests cells are activated by a subset of taste compounds such as sweet or bitter, but not to both[34-36]. This idea of cell specific taste has also been shown for all five "basic tastes," sweet[33,36-39], bitter[33-35,38], umami (savory)[39,40], salty[41,42], and acids (sour)[43] tastes. Finally, labeled-line theory suggests that the neuron, irrespective of receptors within, contributes to stereotypical behaviors. Sweet and bitter tastes lead to attractive and aversive behaviors, respectively. In mice expressing an artificial GPCR receptor (receptor activated solely by a synthetic ligand; RASSL) in bitter or sugar cells, behavioral aversion or attraction is observed respectively to a previously tasteless compound, spiradoline[34]. Furthermore, in flies capsaicin is not found to be behaviorally relevant; however, when a capsaicin receptor (vanilloid receptor, VR1) is expressed in fly sugar neurons, capsaicin elicits attractive feeding behaviors, but when VR1 is

expressed in bitter neurons, capsaicin elicits aversive feeding behaviors [33]. All of these studies provide strong evidence for labeled-line coding.

Taste Modalities

Labeled-line coding hypothesizes that tasting different modalities is based on cell-type specific activation to that particular stimulus. Overall there is strong evidence for five taste modalities: sweet, umami (savory), salt, bitter and sour/acid detection in mammals[1]. Sweet and umami are attractive tastes that promote consumption of nutritious and caloric foods. In contrast, bitter and sour are aversive tastes that prevent feeding of possible spoiled or toxic foods. Studies demonstrate that salt can be either attractive or aversive depending on concentration, where low salt concentrations appear to be attractive and high salt concentrations lead to aversive feeding behaviors[24,44], which may suggest salt detection may be described by across-fiber theory as a salt activity and behavior changes depending on a concentration continuum.

In addition to the five classical modalities of taste there is also evidence in flies that show they detect water[45], carbon dioxide[46,47], and fatty acids[48]. Discoveries of new modalities of taste are important in characterizing detection, which can influence peripheral activity and the overall organism's taste behaviors.

Gustatory receptor signaling

Though both flies and mammals detect similar compounds, they do not have similar mechanisms of detection. Mice detect sweet, bitter, and umami by G protein-coupled receptors (GPCRs) through canonical GPCR signal transduction pathways, while flies detect sweet and bitter compounds via gustatory receptors (Grs) that share little homology to typical GPCRs. Indeed, both Grs and Ors have been shown to have an inverted topology to GPCRs and have ligand-gated ion channel function. Moreover, one recent study has shown that stimulating the fructose receptors (Gr43a) produced nonselective cation currents, which suggests ionotropic functionality of insect Grs[49]. However, there is also involvement of G proteins in Gr signaling (e.g. G-a)[50,51]. The addition of a gustatory "empty neuron" system is needed to illustrate what forms a functional receptor and how insect Grs signal *in vivo*.

Sweet detection:

Sweet detection neurons are found broadly in both mammalian taste buds on the tongue and in multiple taste organs of *Drosophila* including the proboscis (both internally, pegs and pharynx and externally, labellum), and legs. Mammals contain a single heteromeric sweet receptor, which is broadly tuned to the majority of sweet compounds[39]. However, in flies there are 8 gustatory receptors (Grs) in the "sweet clade, including *Gr5a, Gr61a, Gr64a-f*. *Gr5a* and *Gr64a* are necessary for all tested sugars in flies[37], however we know far less about the

remaining sugar Grs. *Gr64f* is shown to be involved in the detection of sugars with both *Gr5a* and *Gr64a*, suggesting heteromeric detection of trehaolse and possibly all sugars in general [52]. Furthermore, the combination of Gr64f with either Gr64a or Gr5a expression in bitter GRNs is not sufficient to confer sugar responses, suggesting that additional machinery is required[52].

Bitter detection:

Recently, bitter characterization of taste sensilla in the labellum was performed. Through the screening of a panel of molecularly different bitter compounds, Weiss et al. discovered L-, I- and S-type sensilla are categorized into five groups of bitter neuron responses. Of the three classes of sensilla in the labellum, the S- and I-type sensilla both contain bitter-activated neurons whereas L-type do not contain a canonical bitter neuron[53]. Additionally, S- and I-type show functional heterogeneity in bitter detection and are further grouped into sub-classes by the qualitative and quantitative differences in their responses to the bitter compound panel. S-class sensilla are separated into S-a and S-b classes and have the broadest tuning to bitter compounds in comparison to I-type sensilla, which have a restricted range of activation to the bitter panel tested. I-type sensilla are also separated into I-a and I-b classes, in which each respond to non-overlapping panels of bitter compounds, e.g. I-a responds to denatonium but not caffeine where as I-b responds to caffeine but not denatonium. Although

S-a and S-b bitter neurons showed responses to all tested bitter compounds, the major difference is that responses of S-b neurons are significantly stronger[53].

The location of bitter Grs was described based on GAL4 reporter analysis, where thirty-three bitter associated Grs have been found to fall into the four (S-a S-b, I-a, and I-b) functional sets of bitter neurons. Of the thirty-three putative bitter Grs that were found, only a few bitter receptors have been identified in bitter taste detection.

The analysis of bitter Gr mutations has yielded *Gr33a*, *Gr66a* and *Gr93a* as receptors necessary for response to and behavioral avoidance of caffeine[54,55]. Furthermore, DEET, a robust insect deterrent, is detected by bitter taste neurons in *Drosophila* mediated by *Gr33a*, *Gr66a* and *Gr32a*[56]. These bitter-sensing Grs when expressed in sugar neurons have not yielded functional response to caffeine nor DEET[56], again suggesting that either other Grs are necessary for functional receptor to these compounds or the signaling molecules are absent. The fact that neither sugar Gr expression in bitter neurons nor bitter Gr expression in sugar neurons confers appropriate detection suggests that regulatory mechanisms at the level of expression, translation or trafficking to the membrane may prevent functional, ectopically expressed Grs.

There are five broadly expressed Grs including Gr32a, Gr33a, Gr39a.a, Gr66a, Gr89a, which are in all bitter neurons in the labellum[53] suggesting “core” receptors for bitter detection. While these Grs may contain broad tuning to bitter compounds, there are also receptors that only respond to a single stimulus e.g.

Gr93a or Gr8a, which responds to caffeine or L-canavanine respectivly, but are not necessary for detection of other tested bitter compounds[55,57]. These data suggests flies may use core set of Gr heteromers for bitter compound detection.

Umami detection:

Amino acids are attractive for both mammals and flies. In mammals, T1R1 and T1R3 together form a broadly tuned amino acid receptor[40]. Amino acids are more attractive when female flies have been mated through sex peptide receptor activation in *pickpocket* positive neurons and associated with (target of rapamycin) TOR/S6K (RPS6-p70-protein kinase) signaling[58]. However in flies, a receptor in the taste system responsible for amino acid detection has yet to be discovered. Although no detection of amino acids in labellum is observed[37], flies show feeding behaviors towards amino acids when they are deprived of them[59]. It is possible that either the legs or internal pegs might detect amino acids; however, no convincing evidence is reported.

Acid detection:

Currently there is no definitive molecular mechanism for acid detection in either mammals or flies. In the mammalian system polycystic kidney disease-1-like 3 (PKD1L3) and PKD2L1 expression have marked acid sensitive taste cells in mice [43]. When PKD1L3 cells are ablated no acid detection is observed[60]. Interestingly PKD1K3 and PKD2L1 can form a heteromer that is acid sensitive[61].

The molecular roles that PKD1K3 and PKD2L1 play in activation of cells to acids is in an “off-response” mechanism. During acidic conditions in the mouth PKD1K3 and PKD2L1 acid channels are inactive, however with the release or increase in pH following acid presentation PKD1K3+PKD2L1 channels open activating acid cells[62]. However, acid detection is still observed in mutations of PKD1K3 and PKD2L1[60] suggesting an additional acid “on-response” receptor[63]. Despite, the discovery of a bonafied acid receptor there is a specific reduction in acid cell mediated activity in mutations of PKD1K3 and PKD2L1[60], suggesting they still play an overall role in taste detection of acids.

There is evidence suggesting that there are intracellular and extracellular mechanisms involved in acid sensing. One mechanism proposes is that acids are detected by extracellular receptors or channels peripherally and another mechanism suggests an intracellular acidification that may act on intracellular channels to depolarize acid activated cells[47,64,65]. It entirely possible that there are multiple mechanisms and receptors that mediate acid detection. This hypothesis would suggest that by the inhibition of a single mechanism or deletion of a single receptor involved in acid detection may affect but not abolish acid signaling.

Acid detection observed in insects suggests two possible mechanisms, activation of a neuron and inhibition of sugar detection. Blowflies contain neurons that respond to acids and generally increase neuronal activities to decreasing pH demonstrated by electrophysiology recordings in taste hairs[66]. Additionally, in

other insects including blowfly, flesh fly, and moth (*Manduca sexta*) acids and pH have been shown to inhibit sugar neuron firing[67,68], and modify salt detection[69]. These data suggest two mechanisms of acid detection in insects: possible activation of a deterrent neuron and possible inhibition of attractive neurons.

Salt detection:

Salt detection is unlike the other classical modalities as it can change behavioral activity depending on concentration. Similar to mammals, flies detect low salt as attractive and high salt as aversive in two distinct cell populations. *Drosophila* contains two types of neuron that respond to either, low or high salt concentrations [13]. Interestingly, high salt neuron activation is specific to salt and not activated by any canonical bitter compounds suggesting a separate mechanism for detecting aversive compounds[53]. Recent work in flies and *C. elegans* has revealed mechanisms of low and high salt sensing. Ionotropic receptor 76b (Ir76b) is necessary and sufficient for activation to low concentrations of sodium in flies[24] and trans-membrane channel like (TMC-1) in *C. elegans* controls high sodium avoidance[70]. It is an intriguing possibility that high salt aversion in flies might also depend on related TMC-1 channels.

Dissertation overview

Using *Drosophila melanogaster*, we investigate peripheral taste physiology and behavioral responses to attractive and aversive compounds found in yeast fermentation. In Chapter 2, we find that flies are attracted to beer,

a fermented complex stimulus and show one gustatory receptor, *Gr64e*, mediates this behavior. We discover that a previously unknown sugar receptor *Gr64e* and *Gr64a* detect an attractive compound, glycerol, highly associated with yeast and yeast fermentation products. Additionally, loss of *Gr64e* found in two related species correlates with a loss of glycerol sensitivity. Our results suggest that *Gr64e* may contribute to *Drosophila* species-specific variation in glycerol selectivity, a cue that is associated with natural food sources, yeast.

Furthermore in Chapter 3, we characterized sweet clade Grs and demonstrate that all sweet Grs are used to form a breadth of tuning to sweet compound detection by the sweet neurons. We also propose a modulatory mechanism for peripheral sugar detection, which may add another layer to the complexity of homeostatic regulation during starvation or deprivation.

Finally in Chapter 4, we investigate the cellular and molecular detection of acids, a common stimulus associated with fermenting and rotten foods. We find that acids are detected by a subset of bitter neurons in the labellum in a pH dependent manner. We find two separate mutant candidates that have a loss in pH detection for further investigation of genes involved in acidic pH detection. Taken together, these results lay a foundation for future experiments from characterizing peripheral taste neuron detection to uncovering functional Grs, homeostatic modulatory mechanisms, and the acidic pH receptor(s).

Chapter 2:

Evolutionary differences in food preference rely on Gr64e, a receptor for glycerol.

Abstract:

Very little is known about how complex stimuli such as beer, which are typically not rich in sugars, trigger attractive gustatory responses in *Drosophila*. Here we identify a member of the gustatory receptor family, *Gr64e*, as a receptor required for feeding preference for beer and other food sources that contain fermenting yeast. We find that *Gr64e* is required for both neuronal and behavioral responses to glycerol, an abundant component of growing yeast and fermentation products. We demonstrate that ectopic expression of *Gr64e* in an olfactory neuron confers responsiveness to glycerol. We also show that *Drosophila* species predicted to carry pseudogenes of *Gr64e* have reduced glycerol sensitivity. Our results provide insight into the molecular mechanisms of feeding acceptance of yeast products and raise the possibility that *Gr64e* contributes to specific evolutionary variations in food choice selectivity across *Drosophila* species to yeasts.

Introduction:

The gustatory system is essential to animal survival as it facilitates the discrimination between high caloric food sources and toxins found in the environment. Detection of attractive cues is essential to elicit ingestive feeding behavior. Sugars that are found at high concentrations in fruits, are one of the

best-characterized attractive food sources of the fly *Drosophila melanogaster*[4,71]. Studies have shown, however that there is a strong attraction to beer, yeast and fermented fruits, which have low sugar content[72]. Not much is known about the molecular and cellular mechanisms underlying detection of attractive non-sugar compounds that may be important for signaling ingestion of yeast-associated foods.

There are two classes of sensory neurons in the proboscis that have been linked to triggering taste acceptance behavior in *Drosophila*. One population of neurons is found in the external taste hairs that are labeled by the sugar receptor Gr5a and are responsive to a number of different sugars[33,37,38,73]. The second is an internal group of taste peg neurons labeled in *E409-GAL4* enhancer trap line, which has response to carbonation but not sugars[46]. Although carbonation by itself has not been shown to stimulate food intake, artificial activation of each *Gr5a* and *E409* neurons results in ingestion[33,46].

Members of a conserved clade of Gr5a-related receptors of the Gustatory receptor (Gr) family[74,75] mediate responses to sugars and sugar alcohols in taste neurons [20,37,52,76,77]. Gr5a and Gr64a are together required for responses to all tested sugars, including several mono-, di-, and oligosaccharides[37]. Recently, another member of this group, Gr64f, was implicated as a heterodimeric partner for both Gr5a and Gr64a[52]. Five other receptors are closely related to Gr5a[75] and their expression is associated with Gr5a-labeled neurons [37,53,77], but whether they

function in concert with Gr5a or Gr64a, or mediate recognition of other classes of attractive taste cues is not yet known.

In this study, we investigated taste acceptance of low-sugar stimuli, including beer, fermented fruit and yeast extract, and identified a *Gr* gene, *Gr64e*, that is necessary for feeding preference for these stimuli. Using a *Gr64e-GAL4* reporter fly we found that its expression is associated with two classes of taste acceptance neurons reported to have distinct response selectivities. We found that *Gr64e* is necessary for cellular and behavioral responses to glycerol, a byproduct of yeast fermentation that is present in beer, wine, and fermenting fruit. Importantly, ectopic expression of *Gr64e* in a heterologous chemosensory neuron conferred sensitivity to glycerol demonstrating a direct role for Gr64e in glycerol recognition. Psuedogenization of *Gr64e* in two *Drosophila* species of the obscura group, *D. pseudoobscura* and *D. persimilis*, correlated with a loss of glycerol sensitivity in labellar sugar-sensing neurons. We postulate that *Gr64e* function may contribute to *Drosophila* species-specific differences in taste selectivity to a cue that is associated with yeast, an important natural food source.

Materials and Methods:

Fly stocks

Flies were reared on standard dextrose-cornmeal-agar diet at 22–25°C. Δ*Orco* mutants were and *Minos*^{MB03533} flies were obtained from the Bloomington Stock Center (#23129 and 23628 respectively), *Drosophila* species were

obtained from the UCSD *Drosophila* Species Stock Center, and the *E409-GAL4* stock was a generous gift from Kristin Scott (UC, Berkeley). Unless otherwise noted, wild type flies were w^{1118}.

Chemosensory recordings

Extracellular tip recordings from single taste sensilla were obtained from adult males aged 5–15 days using TasteProbe (Syntech), as described previously[37]. All compounds were obtained from Sigma/Aldrich and were dissolved in either 1 mM KCl or 30 mM tricholine citrate (TCC). Neural response was measured and quantified by counting the number of spikes in first 500 ms upon contact with the recording electrode and multiplied by two to obtain firing rate in spikes per second.

Single-sensillum recordings from the ab1 olfactory sensilla were as described[78] with the following modifications: Two recording electrodes, one in which the glass micropipette was filled with electrolyte alone (sensillum lymph Ringer – SLR) and a second in which the micropipette was filled with stimulus solution in SLR (stimulus) were held on the same manipulator. Recordings with SLR were first obtained from three ab1 sensilla for ~6-s in order to measure baseline activity of the ab1 neurons. Subsequently, the recording electrode was switched to the one containing the stimulus and ~6-s recordings were obtained again from the same three sensilla. Up to three different stimuli were sequentially tested on a single fly; each stimulus was tested on an independent group of three

sensilla (i.e. a total of up to 18 recordings – 9 SLR and 9 stimulus – per fly). Action potentials of the ab1C neuron were counted in the 2-s period after establishing electrical contact with the sensillum and divided by 2 to obtain a firing rate in spikes per second. In each case, baseline SLR activity of the ab1C neuron was subtracted from the stimulus-evoked response recorded from the same sensillum.

Behavioral assays

For all behavioral tests, flies aged 3–6 days old were transferred to fresh culture vials for at least 24 hours and subsequently starved on water-saturated tissues prior to testing. Behavior experiments were performed between 2–6 PM to control for circadian variations in feeding behavior. Flies that had antennae removed were allowed to recover on fresh food for 24 hours before testing. Starvation times varied for the different species and were determined from their starvation survival curves: *D. melanogaster* (24–26 hours), *D. simulans* (32–34 hours), *D. yakuba* (14–16 hours), *D. pseudoobscura* (25–27 hours), *D. persimilis* (25–27 hours), and *D. virilis* (96–98 hours). Proboscis extension responses of male flies were tested in a controlled environment room (22–25^{o}C, 25–45% humidity) as described previously[37]. Two-choice feeding preference tests were performed using tight-fit Petri dishes (Falcon 35-1006). Solutions of 0.75% agarose containing the stimuli and either 0.25 mg/ml Indigo Carmine (Sigma I8130) or 0.5 mg/ml Sulforhodamine B (Sigma 230162) were prepared fresh and

spotted on the Petri dishes. Starved flies were placed in the Petri dishes in a humidified box at 25°C for 2 hours, after which they were frozen and scored for abdomen coloration within 24 hours. For *D. pseudoobscura*, abdomens were dissected for scoring because of the difficulty to see color of food choice under natural red abdomens. Only trials in which at least 50% of the flies participated were included for data analysis. Preference index (PI) values for the variable stimulus were calculated using the formula (Nr-Nb)/(Nr + Nb + Np) or (Nb-Nr)/(Nr + Nb + Np) as appropriate, where Nr, Nb and Np are the number of flies with red, blue and purple abdomens, respectively.

RT-PCR analysis

For RT-PCR experiments, total RNA was isolated from ~50 heads or ~100 proboscises using TRIzol reagent (Invitrogen) and used for cDNA synthesis. PCR products were obtained after 35–38 thermocycles, cloned into pJET (Fermentas) and sequenced to determine intron-exon junctions and predicted protein sequences. Clones for sequencing were obtained from at least two independent PCR amplification reactions for each sample.

Immunohistochemistry

Whole brains were dissected, fixed and stained as described [37]. Antibodies were used at the following concentrations: mouse α-nc82 (1:20), rat α-CD8a (1:100), Alexa-488 α-rat (1:150), and Alexa-568 α-mouse (1:150).

Confocal *z*-stacks were acquired using a Zeiss LSM510 and analyzed using ImageJ.

Statistical analyses

Unless otherwise indicated, one-way ANOVA and Tukey's *post hoc* tests were used for statistical analyses. For all graphs, error bars indicate standard errors of the mean.

Results:

Beer drives a strong ingestion response

To uncover mechanisms of taste acceptance of food sources that are not rich in sugar content we chose to test beer, a complex stimulus that is strongly attractive to *Drosophila* flies for feeding and breeding [72]. We selected a pale ale (Bass) as pale ales have very low sugar content[79], and characterized how flies responded to it in a binary feeding preference assay. Given a choice between sucrose and beer, we observed that flies preferentially ingested beer across a range of concentrations (**Figure. 2.1a**), in spite of its insignificant sugar content, as well as the presence of hop-derived acids that taste bitter to humans[80].

As a stimulus, beer also has a strong olfactory component. We wished to evaluate whether flies without functional olfactory sensory neurons (OSNs) retained feeding preference for beer. For these experiments we tested Δ*Orco* mutants[19,81], which lack olfactory input from Odor receptor (Or)-expressing neurons. We also tested Δ*Orco* flies in which the antennae had been surgically

removed, since the antennae house a number of OSNs that express members of the ionotropic receptor (Ir) family and function independently of Orco[21,82]. There was no difference in beer preference of Δ*Orco* mutants with intact antenna and that of wild type flies; moreover, flies lacking both Or and Ir classes of OSNs still displayed a preference for beer, although at a reduced level (**Figure 2.2a**). Our results support the view that gustatory input has a significant contribution towards the preferential ingestion of beer.

The *Drosophila* sibling species provide an excellent model to examine the molecular basis of variations in chemosensation. To begin to investigate the mechanisms underlying beer preference, we used the two-choice assay to compare beer preference in *D. melanogaster* with that in *D. pseudoobscura*, a species belonging to the obscura group. Interestingly, *D. pseudoobscura* showed a remarkable decrease in beer preference (**Figure 2.1b**). Feeding selectivity is often correlated with taste sensitivity (Li Plos Gen 2005), raising the possibility that variations in appetitive behavior towards beer arise from differences in chemoreceptor gene function between *D. melanogaster* and *D. pseudoobscura*.

Gr64e mutants show reduced preference for yeasty foods

The genome of *D. pseudoobscura* has two notable differences with respect to the clade of eight *Gr5a*-related *Gr* genes expressed in taste acceptance neurons; *Gr5a* is absent, and *Gr64e* is predicted to be pseudogenized by a nucleotide polymorphism in the donor splice site of the

penultimate intron[74,83]. To begin to investigate the underlying gustatory mechanism of the species difference, we asked whether mutations in either *Gr5a* or *Gr64e* reduced the feeding preference for beer. Flies lacking *Gr5a*, which we have previously shown to be necessary for taste responses to a subset of sugars[20,37], preferred 30% beer, a concentration that wild type flies displayed a marked preference for (**Figure 2.1c**). By contrast, flies carrying a *Minos*^{MB03533} insertion in the fourth exon of the *Gr64e* gene (hereafter referred to as *Gr64e*^{MB03533} or *Gr64e* mutant) showed a dramatic loss in their preference for 30% beer (**Figure 2.1c**).

Interestingly, *Gr64e*^{MB03533}, but not Δ*Gr5a*, flies also had significantly reduced preference for yeast extract and fermented grape; in both mutants the preference for ripe banana was indistinguishable from that of wild type flies (**Figures 2.1c, 2.2b**). Overall, these results suggest that *Gr64e* mutants do not suffer from a general defect in food selection or intake, but rather that they fail to recognize some by-product of yeast metabolism that is common to beer, fermenting fruit, and the water soluble portion of autolyzed yeast.

Gr64e-GAL4 is expressed in taste acceptance neurons

We examined the expression of *Gr64e* in taste neurons using the bipartite *GAL4/UAS* system, which we used to analyze the expression of virtually all members of the Gr family[53]. Using a GFP reporter we found that *Gr64e-GAL4* showed widespread expression in neurons of both internal and external taste

organs including the pharyngeal organs, the labellum, and the legs (**Figure 2.3**). The distribution of GFP-labeled axon termini in the sub-esophageal ganglion (SOG) in the brain showed projections via the labial and pharyngeal nerves and the central connective, in correspondence with peripheral expression patterns and previously published studies [9,38,46,73] (**Figure 2.3**).

In the labellum, we previously determined that *Gr64e-GAL4* is expressed in most if not all sugar-sensing neurons based on the overlap between *Gr64e-GAL4* and *Gr5a-GAL4* expression in flies carrying both *GAL4* transgenes[53]. This finding is confirmed by our current observation that axon termini of *Gr64e-GAL4* neurons are found in an area of the SOG that represents a sugar recognition center[33,38,73](**Figure 2.3**). In addition, we observed that *Gr64e-GAL4* labeled neurons in a large population of neurons that innervate another type of sensilla, the taste pegs that are found in the folds between the pseudotrachea on the oral surface of the labellum[10] (**Figure 2.3**). This expression pattern is reminiscent of that reported for *E409-GAL4*, which labels a class of taste acceptance neurons that respond to carbonation [46]. We therefore tested whether *Gr64e-GAL4* and *E409-GAL4* are co-expressed by examining the number of labeled cells in flies that carry both *GAL4* drivers. We found no significant increase in the number of taste peg neurons in such flies (23.4±6.1 in males, n=5) as compared to those that were labeled by *E409-GAL4* alone (20.8±3.9 in males, n=6) suggesting that the *Gr64e* and *E409* drivers mark largely overlapping populations of neurons innervating peg sensilla. Expression of *Gr64e-GAL4* in two different classes of

taste neurons whose activation triggers ingestion is consistent with a role for this receptor in mediating the recognition of “sweet” compounds.

Gr64e mediates glycerol response in sweet taste neurons

Previous studies show that *Gr5a* and *E409* neurons have non-overlapping response properties: *Gr5a* neurons respond to sugars and sugar alcohols[37,46], whereas *E409* neurons selectively respond to carbonated water[46]. The putative association of *Gr64e* with two attractive neuronal populations raises interesting questions about the identity of its ligands. Our observation that *Gr64e*^{MB03533} flies have reduced feeding preferences to yeast extract and yeast fermentation products led us to investigate chemicals that are associated with yeast.

An abundant product of yeast fermentation is glycerol, found in addition to ethanol and carbon dioxide. Yeasts grown on glucose media can have intracellular levels of glycerol that are as high as ~8%[84]. Insects were previously thought to be indifferent to glycerol[85], but recent studies show that *Drosophila* has robust physiological and behavioral responses to glycerol [4,86,87]. We measured free glycerol content in the yeast fermentation products that we tested, and detected higher concentrations of glycerol in beer, fermented grape and yeast extract, as compared to banana (**Figure 2.2c**). Thus *Gr64e* appears to be required for preference to stimuli with higher glycerol content. We therefore centered on the hypothesis that *Gr64e* encodes a receptor for glycerol. Moreover, in feeding choice experiments with 5 mM sucrose as a standard,

neither ethanol (tested at 1.65%, which is the concentration in 30% beer) nor carbonated water, induced strong ingestive responses in wild type flies (PI for ethanol=–1, n=10; PI for carbonated water=–0.75±0.09, s.e.m., n=6).

In order to test whether Gr64e mediates responses to glycerol, we performed extracellular recordings from I-type sensilla (**Figure 2.4a**) that house two taste neurons, one of which senses sugars, salts and glycerol[13,14,86]. We found that response to 10% glycerol is virtually absent in *Gr64e* mutants, whereas control flies showed a strong response (**Figure 2.4b**). *Gr64e* mutants do have a responsive sugar neuron because a stimulus of 100 mM sucrose elicited a firing rate that is comparable to that observed in control flies (**Figure 2.4b**).

The *Gr64e* gene lies within a tightly linked cluster of six genes that encode the Gr64a-Gr64f receptors[75] (**Figure 2.5a**). Expression of these genes has been examined by RT-PCR, 5' and 3' RACE, mRNA tagging and Northern analysis, some of which suggest the presence of polycistronic mRNAs that encode two or more receptors of the Gr64a-Gr64f group [37,76,77]. RT-PCR analysis of transcripts derived from proboscis tissue of *Gr64e*^{MB03533} flies confirmed that the insertion disrupts the protein-coding region of *Gr64e* (**Figure 2.5b**). Notably, cDNA products of the predicted sizes can be amplified for *Gr64a*, *Gr64b*, *Gr64c*, *Gr64d*, and *Gr64f* (**Figure 2.5c**) showing that expression of other *Gr* genes in the cluster is not affected.

To ascertain whether the reduction in glycerol response is a consequence of the loss of *Gr64e* function we drove *Gr5a-GAL4*-dependent expression of

Gr64e cDNA in sugar taste neurons of *Gr64e* mutants and tested their electrophysiological responses. Tip recordings with glycerol showed that *Gr64e* expression restored glycerol sensitivity in these "rescue" flies (**Figure 2.4b**). Rescue was achieved solely in the presence of both *GAL4* and *UAS* transgenes; either transgene alone was not capable of restoring glycerol response (**Figure 2.4b**).

In order to determine the specificity of the electrophysiological defects in *Gr64e* mutants, we examined responses to three sugars – sucrose, glucose and fructose – in wild type, *GAL4* and *UAS* controls, *Gr64e* mutants, and *GAL4/UAS* rescue flies. For these experiments we performed tip recordings from L-type sensilla (**Figure 2.4a**), which we selected for two reasons. First, successful recordings from L-type sensilla are obtained in a higher proportion as compared to I-type sensilla[13]. Second, the electrophysiological responses of sugar neurons in L-type sensilla have been characterized in more detail[13,37,88]. L-type sensilla in $Gr64e^{MB03533}$ mutants lacked a response to 10% glycerol, which was rescued by *Gr5a-GAL4*-dependent expression of *Gr64e* (**Figure 2.4c**), supporting the idea that the Gr64e receptor is broadly required in different morphological types of sensilla. By contrast, mean responses to sucrose, glucose, and fructose were not significantly different between wild type, *GAL4* control, mutant, and rescue flies, although responses to sucrose were somewhat reduced in *UAS* control flies (**Figure 2.4d**). Together, these experiments demonstrate that *Gr64e* is necessary for glycerol detection in sweet-sensing taste neurons.

We also generated flies in which *Gr64e* was overexpressed via *Gr5a-GAL4* in an otherwise wild type background. Electrophysiological and behavioral analyses of these flies showed that they do not have exaggerated responses to glycerol (**Figures 2.6 a,b**), suggesting that the levels of *Gr64e* are not a limiting factor for glycerol sensitivity of taste neurons in wild type flies.

Gr64e is necessary for behavioral responses to glycerol

To determine the extent to which *Gr64e* contributes to behavioral responses to glycerol, we characterized the responses of wild type, *GAL4* and *UAS* controls, *Gr64e* mutants, and *GAL4/UAS* rescue flies to a range of glycerol concentrations using two well established, independent assays: the proboscis extension response (PER), and a binary feeding preference test [71]. Wild type flies exhibited a robust, dose-dependent extension of the proboscis upon stimulation of labellar taste hairs with glycerol, consistent with previous studies [86]. In contrast, *Gr64e* mutants showed greatly reduced proboscis extension responses to glycerol at all concentrations tested, which were rescued by expression of *Gr64e* in sugar neurons (**Figure 2.7a**). Rescue only occurred with *GAL4*-dependent expression of *Gr64e* and not *GAL4* or *UAS* alone. In feeding preference assays in which populations of flies were presented 5 mM sucrose against varying concentrations of glycerol, wild type flies showed strong preferences for 1% and 10% glycerol, which were virtually abolished in the *Gr64e* mutants (**Figure 2.7b**). Behavioral responses to sucrose were similar in wild

type, *Gr64e* mutants and rescue flies in both assays (**Figures 2.7c**,**d**). Additionally, mean participation rates of the *Gr64e* mutant flies (83% across all trials) were comparable to those of wild type (82% across all trials) and control flies (91% for *GAL4* and 93% for *UAS* across all trials) demonstrating that the mutants do not suffer from non-specific defects in consumption. Feeding preference to glycerol was rescued by driving *Gr64e* expression in *Gr5a* neurons, however only partial rescue was obtained at the intermediate concentration tested (1% glycerol: control PI=0.9±0.03, n=13; mutant PI=–0.76±0.13, n=14; rescue PI=–0.02±0.08, n=14; **Figure 2.7b**). It is possible that *Gr64e* expression in neurons that do not express *Gr5a*, such as those in the legs, labellar taste pegs and pharynx may be important to achieve complete rescue. However, we chose not to employ *Gr64e-GAL4* for these experiments in order to avoid confounding effects caused by overexpression of other *Gr* genes, whose coding sequences are included in the *Gr64e-GAL4* construct [53]. Our results establish that Gr64e-dependent recognition of glycerol is necessary for behavioral sensitivity towards this compound.

Gr64e underlies species variations in glycerol response

In order to determine whether molecular changes in *Gr64e* correlate with glycerol sensitivity in *Drosophila* species that are separated by >40 million years of evolution, we used electrophysiological analysis to compare glycerol responses across ten species comprising members of the melanogaster,

obscura, willistoni, repleta, and virilis groups (**Figure 2.8**). We were particularly interested in examining the two sibling species of the obscura group, *D. pseudoobscura* and *D. persimilis*, both of which are predicted to carry a pseudogenized copy of *Gr64e*[74,83].

We sequenced the *Gr64e* gene region in both *D. pseudoobscura* and *D. persimilis* and confirmed that there is no evidence for mutations that disrupt the coding region, but that the sequence of the predicted splice donor site of the penultimate intron (#8) is GAAAGC, which differs from canonical sites that start with GT. To determine whether this sequence polymorphism affects splicing, we performed RT-PCR analyses using RNA extracted from proboscis tissue of *D. pseudoobscura* flies. A PCR reaction performed with a downstream primer that hybridized to sequences in intron #8 yielded a product of the predicted size, confirming that this intron is retained, at least in some *Gr64e* transcripts (**Figure 2.9a**). Amplification with primers that hybridize to DNA sequences flanking intron #8 yielded fragments of multiple sizes, revealing the presence of alternatively spliced *Gr64e* mRNAs. To further examine the intron-exon structures of the transcripts, we amplified the protein-coding region of *D. pseudoobscura Gr64e* cDNA, cloned the PCR product and sequenced ten independent clones. The predominant product matched the predicted size for a transcript in which intron #8 is not spliced out (**Figure 2.9b**). Sequence analysis confirmed the presence of two alternatively spliced transcripts in addition to a correctly spliced version: one in which the penultimate intron is retained, and a second in which the preceding

exon is skipped (**Figures 2.9b**,**c**). The former is predicted to encode a Gr64e protein that differs from its wild type counterpart across ~80 amino acids at the carboxy terminal end, whereas the latter is predicted to encode a truncated receptor (**Figure 2.9d**). Taken together, our results support the hypothesis that *Gr64e* is functionally compromised in *D. pseudoobscura*, and by extension, in *D. persimilis*.

We next tested glycerol responses in the ten species including *D. pseudoobscura* and *D. persimilis* by performing tip-recordings from L-type sensilla, which are easily identified despite variations in the total number of sensilla in the different species. The recordings showed that as in *D. melanogaster*, 10% glycerol evoked mean responses of >20 spikes per second in all *Drosophila* species predicted to carry functional copies of *Gr64e*. However, mean responses to 10% glycerol were greatly reduced in *D. pseudoobscura* (8.73±1.02, s.e.m., n=11) and *D. persimilis* (7.82±1.61, s.e.m., n=11) (**Figures 2.8b**,**c**). We also recorded with 100 mM sucrose (**Figures 2.8b**,**c**), maltose and maltotriose (**Figure 2.10**), and observed robust responses in every species, although they varied widely across the ten species (for 100 mM sucrose: 40.16±3.29≤mean response (spikes per second)≤93±13.87, s.e.m., 8≤n≤14). As predicted, recordings with 100 mM trehalose evoked a mean responses in the range of ~10–25 spikes per second in every species except *D. psuedoobscura* and *D. persimilis*, which lack the *Gr5a* trehalose receptor gene[74,83] (**Figure 2.10**).

To investigate whether *Gr64e* function underlies species-specific variations in behavioral sensitivity to glycerol, we examined proboscis extension responses of *D. pseudoobscura* and *D. persimilis* to a range of glycerol concentrations, and compared them with the responses of *D. melanogaster* and three other species that showed electrophysiological sensitivity to glycerol. Responses to 100 mM sucrose and water were first used as positive and negative controls respectively, after which water, and concentration series' of glycerol (1%, 5%, 10%) and sucrose (10 mM, 100 mM) were tested blind. We observed that mean behavioral responses to glycerol, but not sucrose, were consistently reduced in *D. pseudoobscura* and *D. persimilis* species (**Figures 2.11 a**,**b**), in agreement with the reduction in electrophysiological responses to glycerol in labellar taste neurons. Moreover, when given a choice between 5 mM sucrose and 1% glycerol in feeding choice assays, *D. pseudoobscura* and *D. persimilis* displayed significantly reduced preferences for glycerol (**Figure 2.11c**). By contrast, glycerol feeding preferences in *D. simulans*, *D. yakuba*, and *D. virilis*, which are among the species predicted to encode functional copies of *Gr64e*, were indistinguishable from that observed for *D. melanogaster*. Taken together, our results suggest that the loss of *Gr64e* function in *Drosophila* species of the obscura group reduces their sensitivity to glycerol and may account for the relative indifference of *D. pseudoobscura* to beer.

Gr64a mediates glycerol response in sweet taste neurons

We found that glycerol detection was lost in *ΔGr64e* mutants and this result lead us to investigate whether other Grs were necessary for glycerol detection. Grs have been implicated to function in heteromeric complexes. Studies have looked into how sweet Grs interact with each other and have found that Gr64f, along with Gr5a, is required for cellular and behavioral response to trehalose[52]. Here we discovered using the Proboscis Extension Response (PER) that *Gr64a* mutants had a severe deficiency in detecting glycerol (**Figure 2.12a**), but not m-a-glucoside (**Figure 2.12b**), a known Gr5a sugar. Our behavioral results were confirmed as we find that *Gr64a* mediates the detection of glycerol by performing extracellular recordings from L-type sensilla (**Figure 2.12c**). We found that response to 10% glycerol is virtually absent in *Gr64a* mutants, whereas wild type flies demonstrate a strong response (**Figure 2.12c**). *Gr64a* mutants have a responsive sugar neuron as a Gr5a sugar m-a-glucoside tested at 100 mM had no significant difference ($p > 0.05$) in firing rates to that observed in wild type flies[37]. To determine whether the reduction in glycerol response is an effect of the loss of *Gr64a* function we drove *Gr5a-GAL4*-dependent expression of *Gr64a* cDNA in sugar taste neurons of *Gr64a* mutants and tested both PER behavior and electrophysiological responses. Tip recordings with glycerol showed that *Gr64a* expression in sweet neurons restored glycerol sensitivity, shown in rescue (**Figure 2.12**). Our data confirms that Gr64a, along with Gr64e, is necessary for glycerol detection.

Discussion:

We found the molecular basis for glycerol detection. Glycerol is found in preferred foods, beer and yeast. We used electrophysiology and behavior assays to show that Gr64e and Gr64a function are necessary for the cellular and behavioral response to glycerol.

Despite finding the Gr family fourteen years ago we still have no direct evidence of what a sufficient Gr is. Gr neurons express a suite of receptors and there are examples of bitter responses that are dependent on multiple bitter Grs[55,56]. This makes the endogenous neuron system difficult to use to isolate specific Gr function from other Grs that are also expressed in that neuron. Mutant studies have been able to show necessity of receptor for ligand detection, however there are few successful heterologous systems to test the basic components of what makes up a functional Gr.

The first taste receptor in flies to be expressed in a heterologous system was Gr5a by the Carlson Lab[89]. However, very few publications of other Grs expressed in cells have been shown to confer response to stimuli. Over the last ten years finding a suitable system to functionally express and decode Grs in either cell systems or ectopically has met with little success. Besides Gr5a only a handful of receptors have been functionally expressed. Gr43a, a sugar receptor responds to fructose[49]. Others have miss expressed Grs and seen altered response in the taste system. Linnea et al. 2011 found 4 distinct classes of bitter sensilla when testing a panel of bitter compounds. Gr59c, a bitter receptor is

expressed in I-a class of bitter neurons, which respond to a limited panel of compounds, the strongest responses being from berberine, lobeline and denatonium. When Gr59c was miss expressed in all bitter sensilla types they found that Gr59c confers physiological responses of I-a compounds in all bitter sensilla classes[53]. The main caveat being that none of these neurons were devoid of other bitter Grs, which still does not show that Gr59c is a functional receptor on its own.

Our lab however is the first to show that that ectopic expression of Grs that are necessary for taste detection can confer novel ligand recognition in a heterologous neuron[90,91]. Expression of *Gr64e* or *Gr64a* in the CO_2-sensing neuron in the olfactory system, antennal basiconic sensillum 1C (ab1C) confers a specific and dose dependent response to glycerol. This validation of Gr64e and Gr64a shows that it is a receptor for glycerol, however Gr21a and Gr63a, receptors that confer CO_2 sensitivity are also present in this neuron. We do not know what specific architecture or features allow for functional role of Gr64e or Gr64a in this neuron. Our results suggest that Gr64e and Gr64a partner to function *in vivo* and it is possible that Gr21a could facilitate this role of heteromeric partnership. Without a double mutant *ΔGr63a* and *ΔGr21a* we cannot definitively say that Gr64e or Gr64a does not require other Grs to confer sensitivity to glycerol. Additionally, when *Gr63a* and *Gr21a* were expressed ectopically in the ab3A neuron with $G\alpha_q$, only with all three both Grs and $G\alpha_q$ could CO_2 sensitivity be restored to wild type levels[92]. This finding raises the

possibility that $G\alpha_q$ may be supporting functional Gr64e or Gr64a activity in the ab1C neuron, however this has yet to be directly tested.

To further confirm the species comparison of Gr64e function a future experiment that was not tested would be to express *D. melanogaster Gr64e* in *D. pseudoobscura* to see if the physiology and behavior to glycerol is rescued. This experiment could also be done in reverse and the hypothesis would be that *D. pseudoobscura Gr64e* would not rescue *D. melanogaster* physiology or behavior to glycerol. However, more precisely we could express *D. pseudoobscura Gr64e* in the ab1C ectopic system and the hypothesis would be that glycerol responses would be greatly reduced.

It is interesting to note that *Drosophila* have such a robust response to glycerol. *Drosophila* species are polyphagous, and most species feed on a variety of fruit and various fermenting substances. Additionally, different species of yeasts are favored to different extents by various *Drosophila*[93-95]. Given that specific chemosensory cues arising from yeast growth and fermentation are likely to fluctuate according to yeast species as well as fermentation substrates, variations in sensitivity to such cues may have extensive influences on feeding selectivity and niche diversification.

Glycerol is abundant in and around yeast[84], which is an important natural food source of *Drosophila*. Glycerol production by yeast during wine-making has been studied extensively and aside from CO_2 and ethanol, it is the most abundant product in fermenting grape juice with levels ranging from ~0.1–1.5%;

its content in beer and wine can be just as high [96-98]. Interestingly, natural yeasts exhibit variations in their production of glycerol (Brandolini World J Microbiol Biotech 2002). One of the most commonly found yeasts on grape surface is *Kloeckera apiculatas*[99], which produces less glycerol during fermentation than *Saccharomyces cerevisiae*[98,100]. Analysis of yeast flora from digestive tracts of wild-caught *Drosophila* suggested that *K. apiculata* is found in all species, but that its proportion can vary between species[93]. Although it is likely that multiple sensory cues are involved in this choice, an intriguing possibility is that *Gr64e* function is a component of evolutionary shifts in yeast preference in *Drosophila* species.

Our data suggest that there are other compounds besides glycerol that are found to be attractive in beer and yeast. What are those compounds? Recently a study found there was an attraction to amino acids when depriving the flies of yeast [59]. Amino acids, found in both beer and yeast, are important for the building blocks of proteins that all organisms need. Interestingly, no one yet has found a molecular mechanism of detection of amino acids in an age of where we have the tools to measure activity of neurons to amino acids. Which raises a possible explanation that either amino acids are not detected at the periphery, but only internally. However, another possibility is that amino acids alone are not enough to elicit firing of attractive neurons and only in combination with glycerol or other sugars have an additive effect that surpassed the threshold of activity.

Key questions remain to be answered. What Grs make up the sufficient receptor complex for glycerol detection or furthermore all other sugars? Are other sweet clade Grs involved in detection of attractive compounds and if so what compounds do they detect?

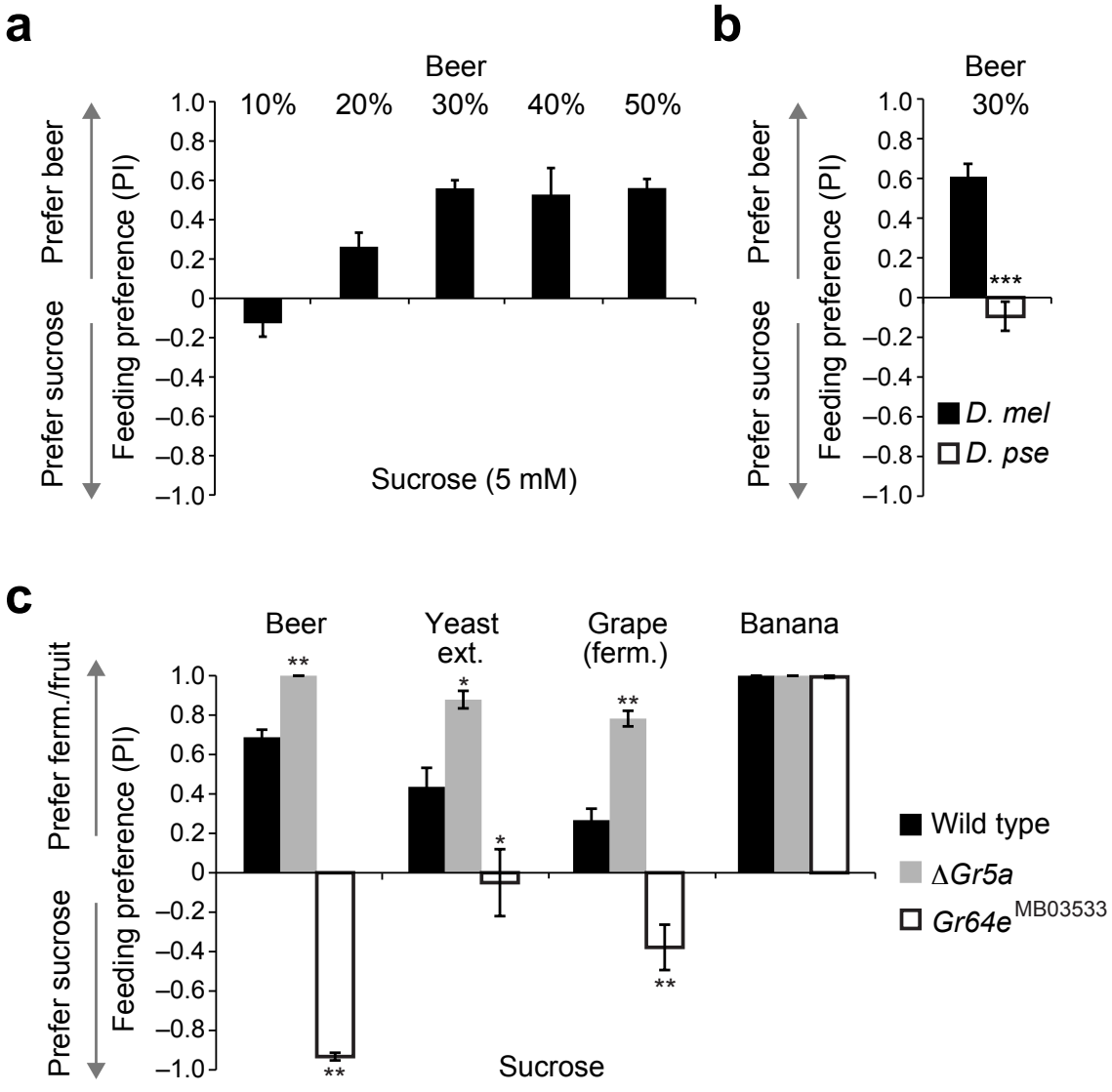
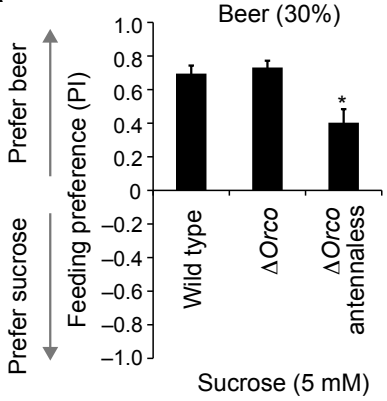
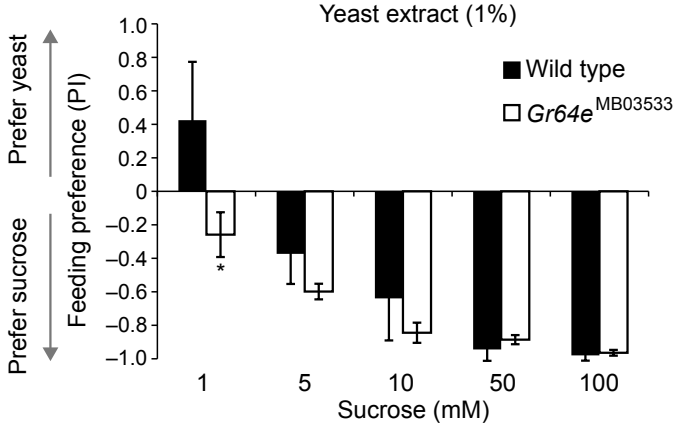

a
Beer
10%
20%
30%
40%
50%
Prefer beer
Prefer sucrose
Feeding preference (PI)
1.0
0.8
0.6
0.4
0.2
0
–0.2
–0.4
–0.6
–0.8
–1.0
Sucrose (5 mM)
b
Beer
30%
***
D. mel
D. pse
c
Beer
Yeast ext.
Grape (ferm.)
Banana
Prefer ferm./fruit
Wild type
ΔGr5a
Gr64e MB03533
Sucrose

Figure 2.1 Feeding preference to yeast fermentation products is reduced in *Gr64e* mutants. (**a**) Feeding preference of wild type flies (w^{1118}) for beer (Bass Co. Pale Ale) in a binary choice assay. For each concentration, n=6. (**b**) Feeding preference for beer, tested against 5 mM sucrose, in *D. melanogaster* Canton-S (*D. mel*) and *D. pseudoobscura* (*D. pse*). n=8 trials. *t*-test, ***p<0.0001. (**c**) Feeding preferences of wild type (w^{1118}), Δ*Gr5a* (Δ*EP-5*), and $Gr64e^{MB03533}$ to yeast products and fruit stimuli. Beer, fermented grape and ripened banana were each tested at a final concentration of 30% against 5 mM sucrose. Yeast extract (1%, Sigma Y1625) was tested against 1 mM sucrose; the sucrose concentration was selected on the basis of a dose response analysis (**Figure 2.2b**). Grapes were crushed and fermented with *S. cerevisiae* for 5–7 days at room temperature. n=7–21. Asterisks indicate responses that are significantly different from wild type: *p<0.05, **p<0.001. Error bars indicate s.e.m.

a



b



c

Glycerol content

Source	Beer	Yeast ext. (10%)	Grape (ferm)	Banana
Glycerol (%)	0.256%	0.032%	1.198%	0.015%

Figure 2.2 Behavioral activity and glycerol content analysis of yeast fermentation products. (**a**) Feeding preference to beer is reduced but not abolished with the lack of olfactory input. Beer feeding preference of wild type flies (w^{1118}), *Orco* mutants (Δ*Orco*), and *Orco* mutants lacking antennae (Δ*Orco* antennaless). Antennae were removed surgically and flies were allowed to recover for 48 hours prior to starvation for experiments. (**b**) Feeding preferences of wild type (w^{1118}) and $Gr64e^{MB03533}$ flies to 1% yeast extract when presented as a choice against indicated concentrations of sucrose. In both (a) and (b), the asterisk indicates responses that are significantly different from those observed for wild type, $*p<0.01$. Error bars indicate s.e.m. (**c**) Empirical determination of glycerol content in fermented and fruit stimuli used in (**Figure. 2.1**). Glycerol content was measured using the Free Glycerol Reagent from Sigma (F6428), and is within the range observed in previous studies[97,98].

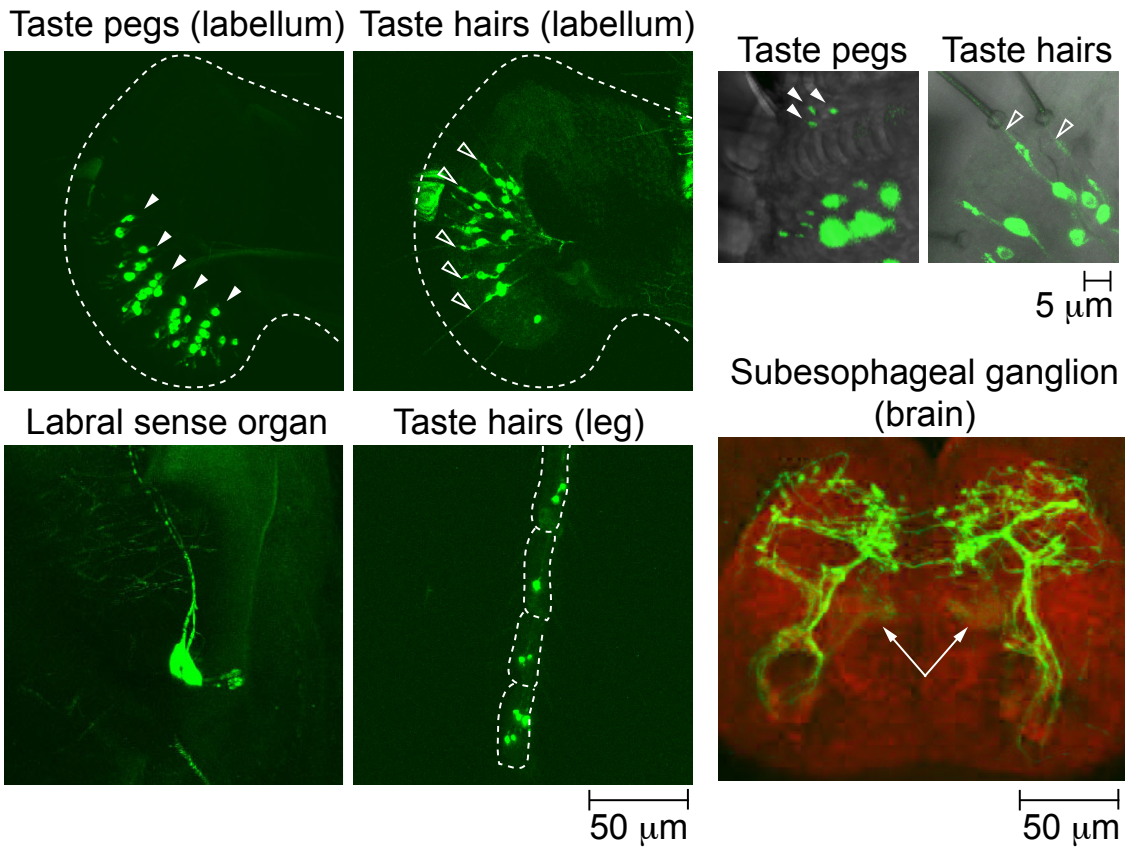

Taste pegs (labellum)
Taste hairs (labellum)
Taste pegs
Taste hairs
5 μm
Subesophageal ganglion (brain)
Labral sense organ
Taste hairs (leg)
50 μm
50 μm

Figure 2.3 *Gr64e-GAL4* is broadly expressed in taste acceptance neurons. *Gr64e-GAL4*-driven expression of GFP in peripheral taste organs as indicated, shown also in enlarged views overlaid on brightfield images (right). Arrowheads indicate neurons innervating taste peg sensilla (closed) and dendrites of some of the neurons that innervate taste hairs (open). The composite pattern of labeled axon termini (green, α-CD8) is seen in a *z*-projection of optical sections of the sub-esophageal ganglion in the brain (right, bottom); arrows indicate projections of labellar sugar neurons. Neuropil is stained with α-nc82 (red). Genotype is *Gr64e-GAL4/Gr64e-GAL4; UAS-mCD8:GFP/UAS-mCD8:GFP*.

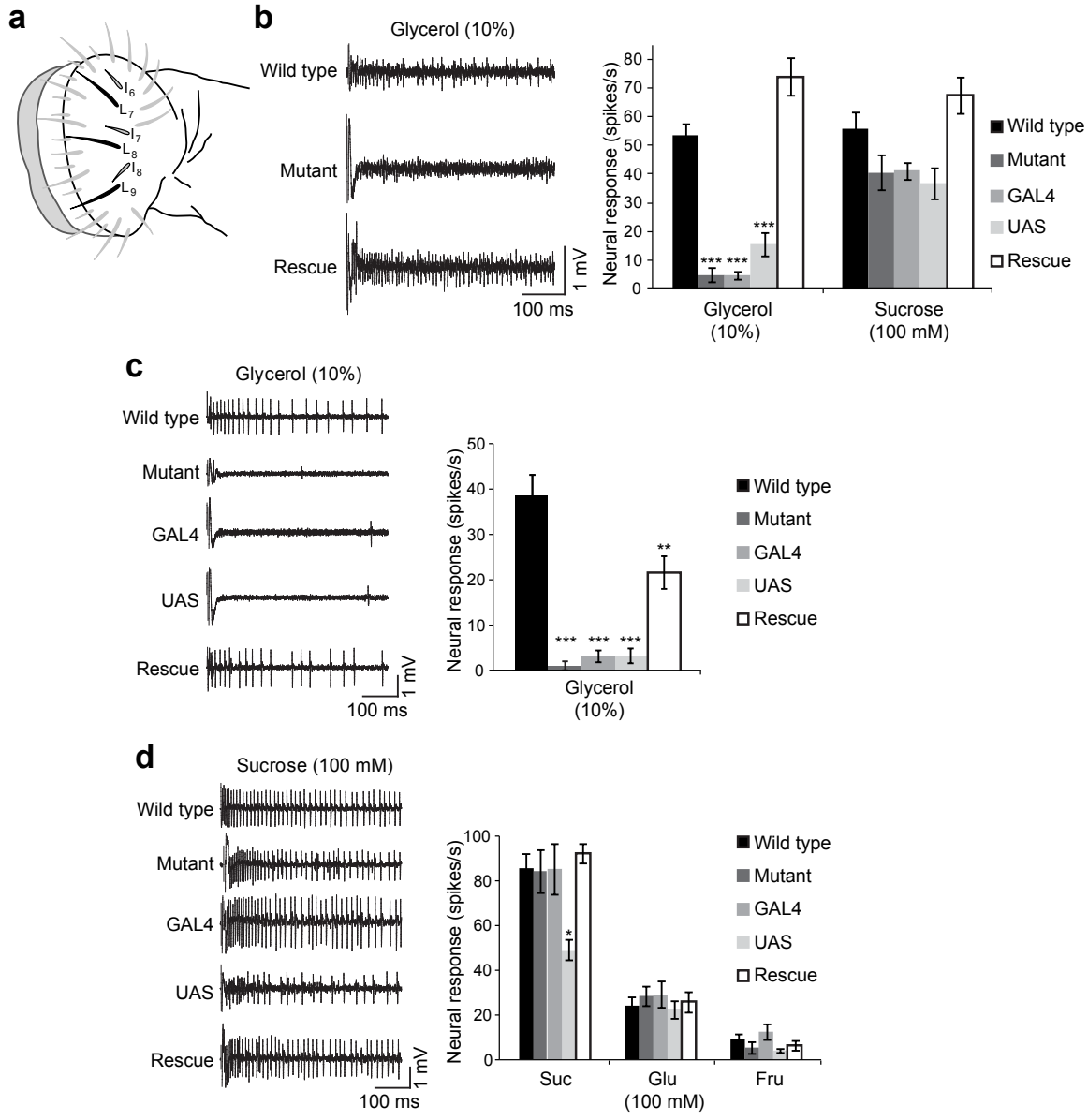
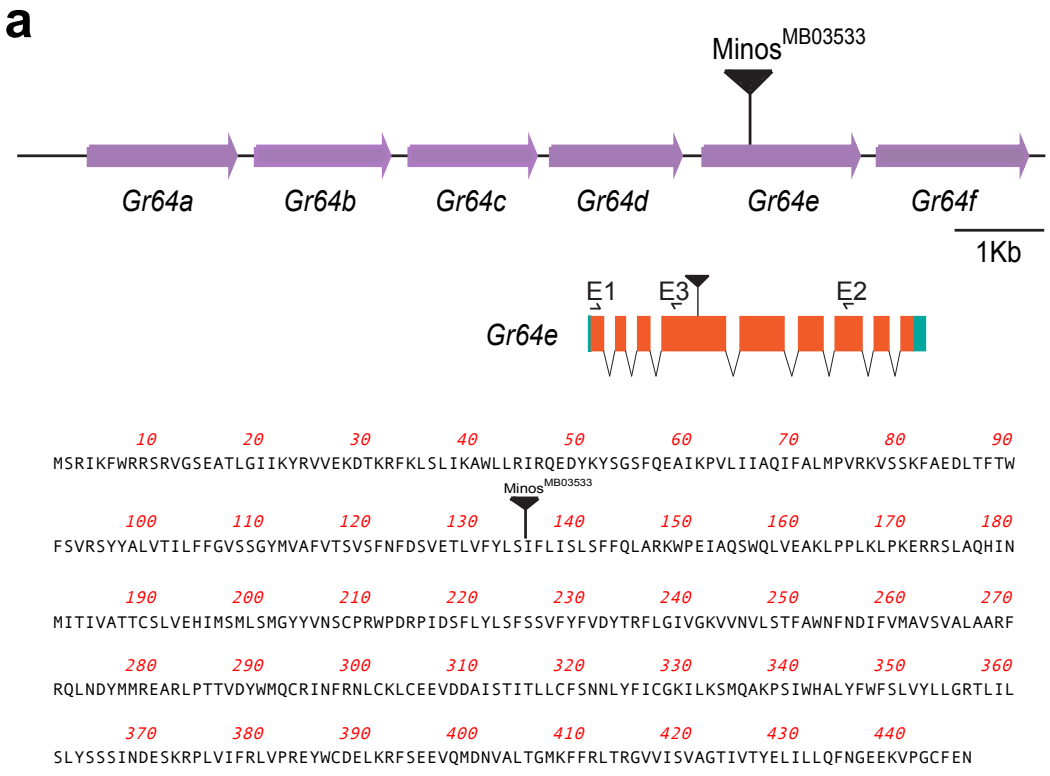

a
b
Glycerol (10%)
Wild type
Mutant
Rescue
1 mV
100 ms
Neural response (spikes/s)
Wild type
Mutant
GAL4
UAS
Rescue
Glycerol (10%)
Sucrose (100 mM)
c
Glycerol (10%)
Wild type
Mutant
GAL4
UAS
Rescue
1 mV
100 ms
Neural response (spikes/s)
Glycerol (10%)
d
Sucrose (100 mM)
Wild type
Mutant
GAL4
UAS
Rescue
1 mV
100 ms
Neural response (spikes/s)
Suc
Glu
Fru
(100 mM)

Figure *Gr64e* is necessary for glycerol recognition in sugar-sensing taste neurons. (**a**) Illustration showing L- and I-type labellar sensilla that were selected for recordings. (**b**) Sample traces and mean responses obtained from recordings of I-type sensilla in wild type (w^{1118}), mutant ($Gr64e^{MB03533}$), GAL4 (*Gr5a-GAL4*; $Gr64e^{MB03533}$), UAS (*UAS-Gr64e*; $Gr64e^{MB03533}$), and rescue (*Gr5a-GAL4/UAS-Gr64e*; $Gr64e^{MB03533}$) flies. Compounds were tested in a 1 mM KCl electrolyte solution. Rescue recordings were obtained from two independent *UAS-Gr64e* insertion lines. $10 \leq n \leq 19$. (**c**) Sample traces and mean responses obtained from recordings of L-type sensilla in genotypes as in (b). Glycerol was tested in 30 mM tricholine citrate (TCC), which was used to suppress the response of the water-sensing neuron in L-type sensilla[32]. $10 \leq n \leq 24$. (**d**) Sample traces and mean sugar responses in L-type sensilla of genotypes as in (b). Sugars were tested in 30 mM TCC. $9 \leq n \leq 24$. In all graphs, asterisks indicate responses that are significantly different from wild type: $*p<0.05$, $**p<0.005$, $***p<0.001$. Error bars indicate s.e.m.


a
Minos MB03533
Gr64a
Gr64b
Gr64c
Gr64d
Gr64e
Gr64f
1Kb
E1
E3
E2
Gr64e
10 20 30 40 50 60 70 80 90
MSRIKFWRRSRVGSEATLGIIKYRVVEKDTKRFKLSLIKAWLLRIRQEDYKYSGSFQEAIKPVLIIAQIFALMPVRKVSSKFAEDLTFTW
Minos MB03533
100 110 120 130 140 150 160 170 180
FSVRSYYALVTILFFGVSSGYMVAFVTSVSFNFDSVETLVFYLSIFLISLSFFQLARKWPEIAQSWQLVEAKLPPLKLPKERRSLAQHIN
190 200 210 220 230 240 250 260 270
MITIVATTCSLVEHIMSMLSMGYYVNSCPRWPDRPIDSFLYLSFSSVFYFVDYTRFLGIVGKVVNVLSTFAWNFNDIFVMAVSVALAARF
280 290 300 310 320 330 340 350 360
RQLNDYMMREARLPTTVDYWMQCRINFRNLCKLCEEVDDAISTITLLCFSNNLYFICGKILKSMQAKPSIWHALYFWFSLVYLLGRTLIL
370 380 390 400 410 420 430 440
SLYSSSINDESKRPLVIFRLVPREYWCDELKRFSEEVQMDNVALTGMKFFRLTRGVVISVAGTIVTYELILLQFNGEEKVPGCFEN

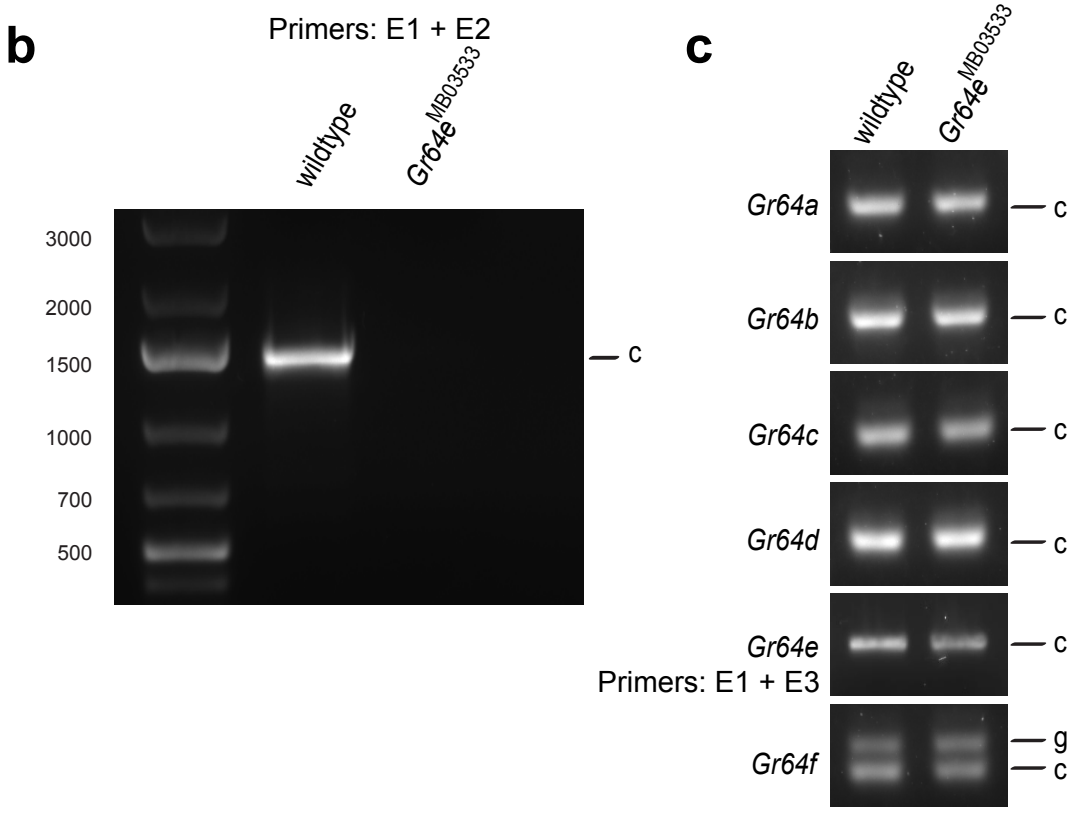

b
Primers: E1 + E2
wildtype
Gr64e MB03533
3000
2000
1500
1000
700
500
c
c
wildtype
Gr64e MB03533
Gr64a
Gr64b
Gr64c
Gr64d
Gr64e
Primers: E1 + E3
Gr64f
c
c
c
c
c
g
c

Figure 2.5 Expression analysis of *Gr* genes in *Gr64e*^{MB03533} flies. (**a**) Schematic showing the *Gr64a-Gr64f* gene region. The inverted triangle indicates the location of the MB03533 *Minos* insertion. Shown below is the transcript structure for *Gr64e*, indicating protein coding regions (orange) and untranslated sequences (blue), and wild type protein sequence of *Gr64e*, indicating the location of the *Minos* insertion site. (**b**) RT-PCR analysis of *Gr64e* in wild type (w^{1118}) and *Gr64e*^{MB03533} flies as indicated using primers that hybridize to sequences flanking the *Minos* insertion site (locations indicated in (**a**)). cDNA template was synthesized from total RNA that was extracted from ~100 proboscises of named genotypes. Primer sequences were: E1 (5'-TCAAGTTCTGGCGAAGATCGC) and E2 (5'-ATGAGGGTGCGACCAAGTAG). Predicted size for cDNA product is indicated (right). (**c**) RT-PCR analysis of *Gr64a-Gr64f* in wild type (w^{1118}) and *Gr64e*^{MB03533} flies as indicated. Primers sequences were as follows: *Gr64a* (5'-GGCGTTAAGCAGGTGGAGAG and 5'-CCAGATTCGAACAACTGCTGG), *Gr64b* (5'-TTAGCAATGTCCGTGCTCTGG and 5'CACATGATGAAGCAGTCGGTG), *Gr64c* (5'-ACCAGAAACACGCTTCAGCA and 5'-CGTTCGACGGATGATGTATGG), *Gr64d* (5'-CGGTCAGTGCAGGAGAATACC and 5'-CTTCCTGGTTCGCATAGCAGG), *Gr64e* E1 (5'-TCAAGTTCTGGCGAAGATCGC) and *Gr64e* E3 (5'-CTGAACCAGGTGAAGGTTAGG), and *Gr64f* (5'-CTGCTGGGAATGATGCTGTCC and 5'-CTCTGACCAGTAAGATGGAGC). Positions of amplicons from cDNA (c) and genomic DNA (g) are indicated (right).

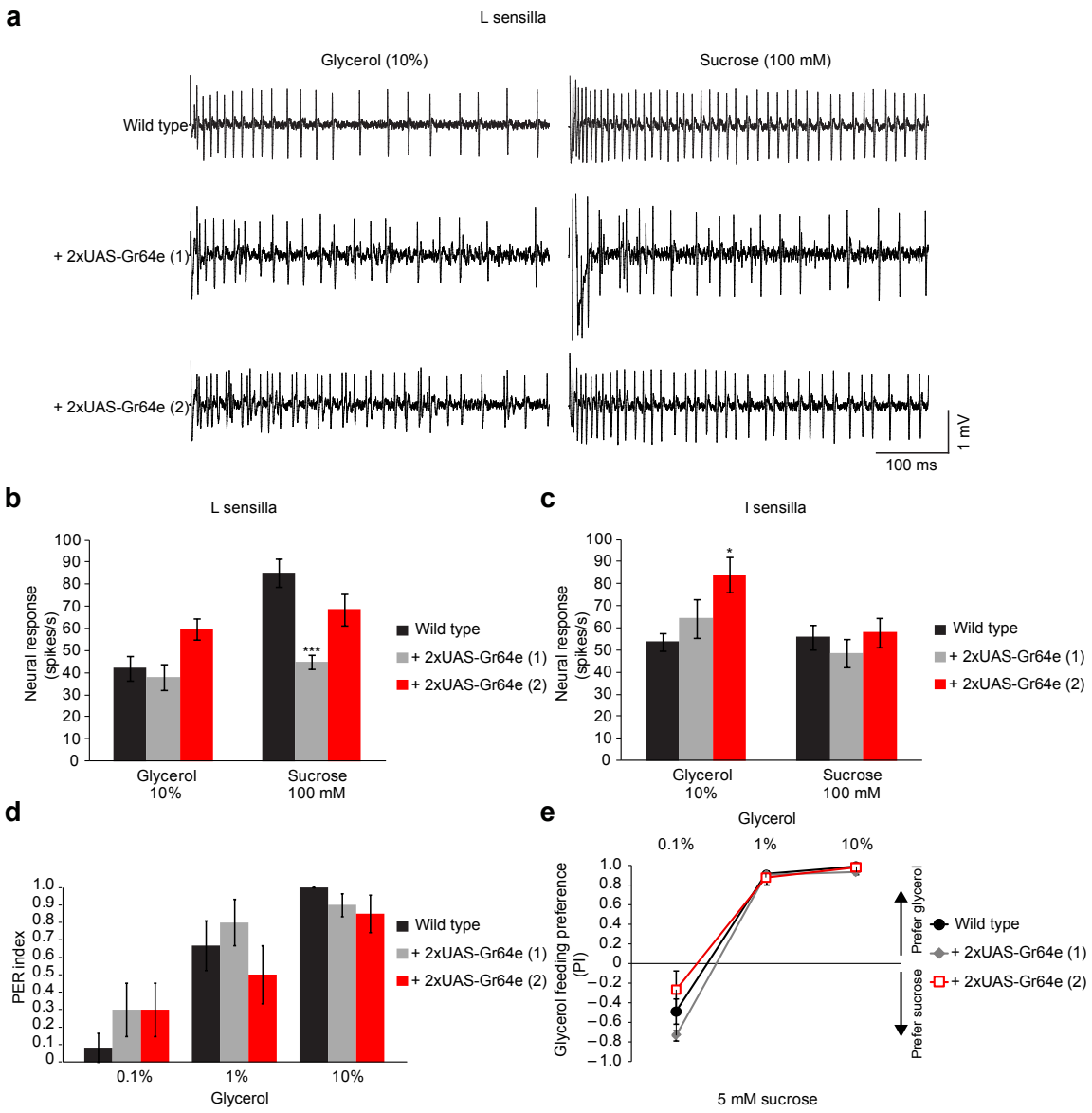

a
L sensilla
Glycerol (10%)
Sucrose (100 mM)
Wild type
+ 2xUAS-Gr64e (1)
+ 2xUAS-Gr64e (2)
1 mV
100 ms
b
L sensilla
Neural response (spikes/s)
Glycerol 10%
Sucrose 100 mM
***
Wild type
+ 2xUAS-Gr64e (1)
+ 2xUAS-Gr64e (2)
c
I sensilla
Neural response (spikes/s)
Glycerol 10%
Sucrose 100 mM
*
Wild type
+ 2xUAS-Gr64e (1)
+ 2xUAS-Gr64e (2)
d
PER index
0.1%
1%
10%
Glycerol
Wild type
+ 2xUAS-Gr64e (1)
+ 2xUAS-Gr64e (2)
e
Glycerol
0.1%
1%
10%
Glycerol feeding preference (PI)
Prefer glycerol
Prefer sucrose
5 mM sucrose
Wild type
+ 2xUAS-Gr64e (1)
+ 2xUAS-Gr64e (2)

Figure 2.6 Overexpression of *Gr64e* via *Gr5a-GAL4* in otherwise wild type flies does not alter responses to glycerol. Sample traces (**a**) and mean responses (**b,c**) obtained from recordings of L-type and I-type sensilla in w^{1118} (wild type), *UAS-Gr64e/UAS-Gr64e; Gr5a-GAL4/TM3* (+2xUAS-Gr64e (1)), and *Gr5a-GAL4/Gr5a-GAL4; UAS-Gr64e/UAS-Gr64e* (+2xUAS-Gr64e (2)). Compounds were tested in 30 mM TCC (L sensilla) or 1 mM KCl (I sensilla. $10 \leq n \leq 14$). Proboscis extension responses ($10 \leq n \leq 12$) (**d**) and feeding preference tests ($6 \leq n \leq 19$) (**e**) performed as in (**Figure 2.7**). Asterisk indicates responses that are significantly different from those observed for wild type, one-way ANOVA with Tukey's *post hoc* analysis, ***$p<0.001$. Error bars indicate s.e.m. Mean differences in electrophysiological and behavioral responses to glycerol between the three genotypes are not significant at the 0.05 level.

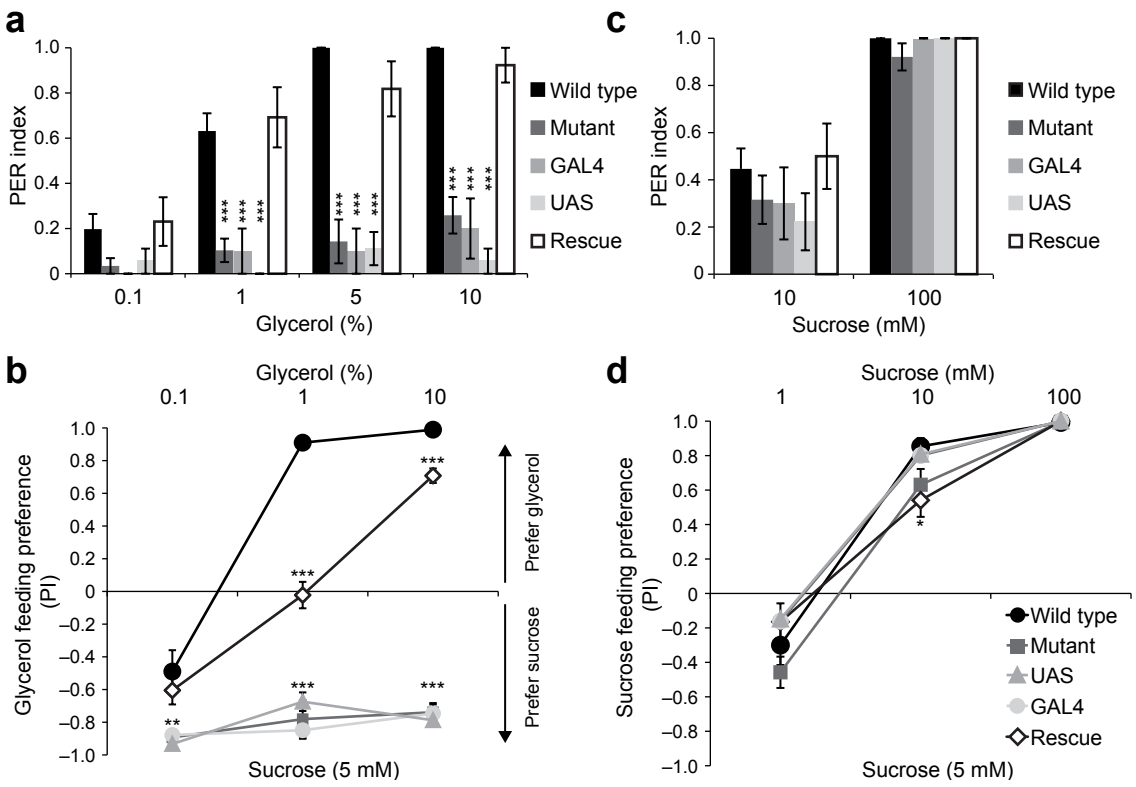

a
PER index
1.0
0.8
0.6
0.4
0.2
0
0.1
1
5
10
Glycerol (%)
***
Wild type
Mutant
GAL4
UAS
Rescue
c
PER index
10
100
Sucrose (mM)
b
Glycerol (%)
0.1
1
10
Glycerol feeding preference (PI)
Prefer glycerol
Prefer sucrose
**
Sucrose (5 mM)
d
Sucrose (mM)
1
10
100
Sucrose feeding preference (PI)
*
Sucrose (5 mM)

Figure 2.7 *Gr64e* is necessary for behavioral responses to glycerol. (**a**) Proboscis extension responses of wild type (w^{1118}), mutant ($Gr64e^{MB03533}$), UAS (*UAS-Gr64e*; $Gr64e^{MB03533}$), GAL4 (*Gr5a-GAL4*; $Gr64e^{MB03533}$), and rescue (*Gr5a-GAL4/UAS-Gr64e*; $Gr64e^{MB03533}$) flies to indicated concentrations of glycerol. Responses were graded for full extension (1), partial extension (.5), and no movement upon stimulus application (0). $10 \leq n \leq 38$. (**b**) Feeding preference to glycerol. Stimuli were tested at indicated concentrations against a standard of 5 mM sucrose. $10 \leq n \leq 16$. (**c**) Proboscis extension ($13 \leq n \leq 19$) and (**d**) feeding preference ($9 \leq n \leq 19$) to sucrose. For all graphs, asterisks indicate responses that are significantly different from wild type: $*p<0.05$, $**p<0.01$, $***p<0.001$. Error bars indicate s.e.m. Glycerol preference of *Gr64e* mutants is not significantly different from that of *GAL4* and *UAS* control flies at any concentration; in (d), there is a significant difference in the preference to 10 mM sucrose between wild type and rescue flies, $p=0.027$.

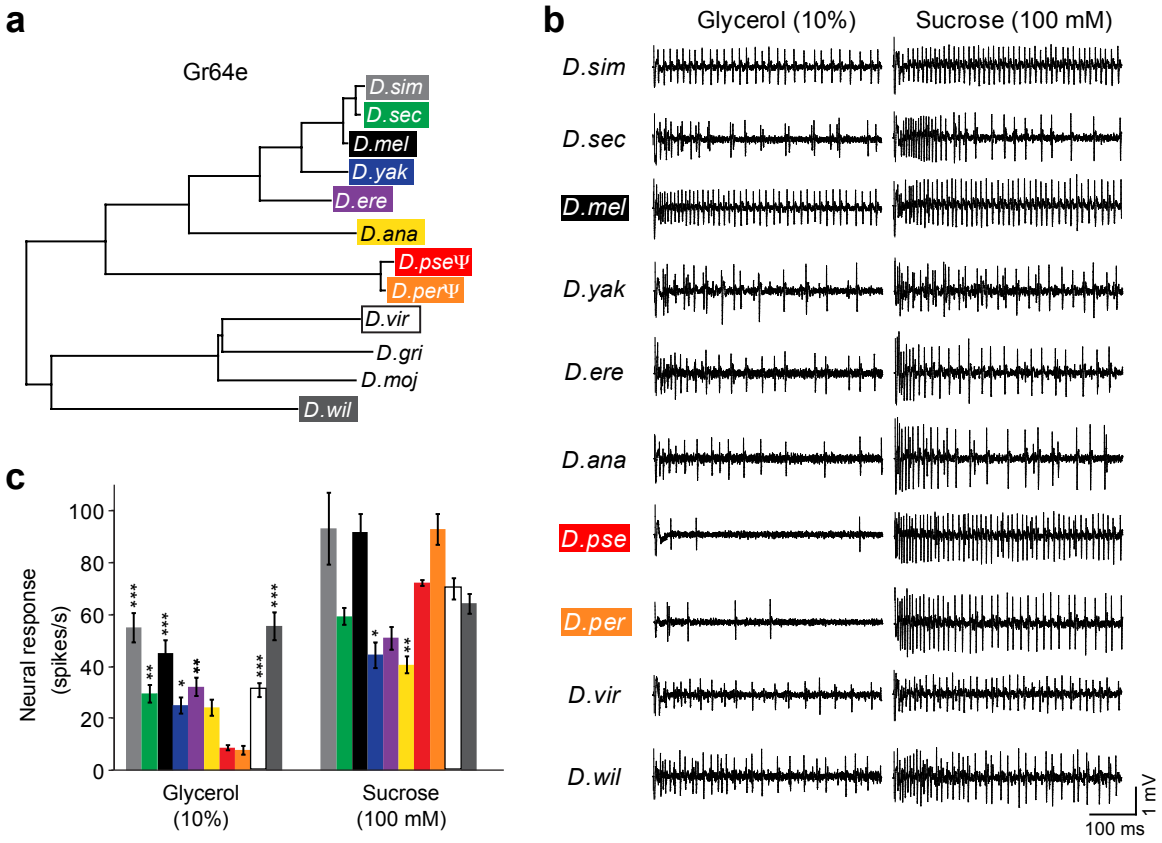

a
Gr64e
D.sim
D.sec
D.mel
D.yak
D.ere
D.ana
D.pseΨ
D.perΨ
D.vir
D.gri
D.moj
D.wil
b
Glycerol (10%)
Sucrose (100 mM)
D.sim
D.sec
D.mel
D.yak
D.ere
D.ana
D.pse
D.per
D.vir
D.wil
1 mV
100 ms
c
Neural response (spikes/s)
100
80
60
40
20
0
Glycerol (10%)
Sucrose (100 mM)

Figure 2.8: *Gr64e* pseudogenization correlates with absence of cellular response to glycerol. (**a**) Phylogenetic relationship of *Gr64e* orthologs from twelve *Drosophila* species (adapted from ref. 10). "Ψ" indicates predicted pseudogenes. Sample traces (**b**) and mean responses (**c**) of extracellular tip recordings from L-type sensilla of *D. simulans* (*D.sim*), *D. sechellia* (*D.sec*), *D. melanogaster* (*D.mel*), *D. yakuba* (*D.yak*), *D. erecta* (*D.ere*), *D. ananassae* (*D.ana*), *D. pseudoobscura* (*D.pse*), *D. persimilis* (*D.per*), *D. virilis* (*D.vir*), and *D. willistoni* (*D.wil*) with glycerol and sucrose, both tested in 30 mM TCC. $9 \leq n \leq 17$. Colors for the bar graph correspond to those indicated for each species in (a). Asterisks indicate responses that are significantly different from that observed in *D.pse*, $*p<0.05$, $**p<0.01$, $***p<0.001$. Error bars indicate s.e.m.

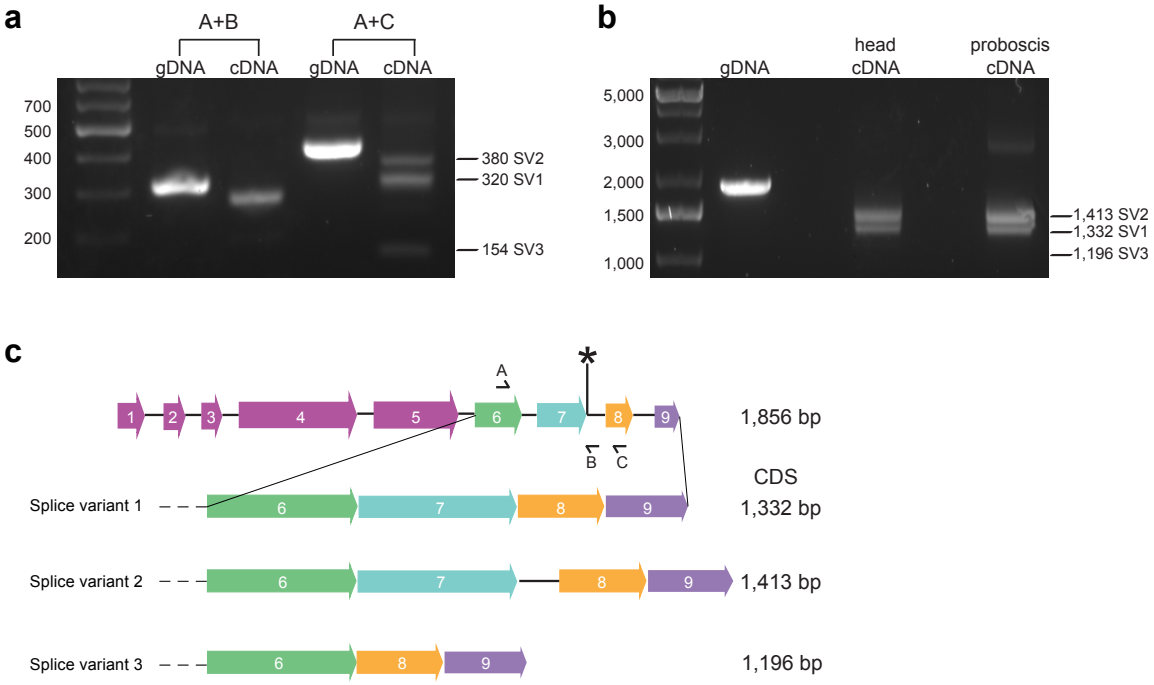

a
A+B
A+C
gDNA
cDNA
gDNA
cDNA
700
500
400
300
200
380 SV2
320 SV1
154 SV3
b
gDNA
head cDNA
proboscis cDNA
5,000
3,000
2,000
1,500
1,000
1,413 SV2
1,332 SV1
1,196 SV3
c
A
B
C
1
2
3
4
5
6
7
8
9
1,856 bp
CDS
Splice variant 1
1,332 bp
Splice variant 2
1,413 bp
Splice variant 3
1,196 bp
d
Protein sequences
0
10
20
30
40
50
60
70
80
90
100
110
Splice variant 1 ATTVDYWMQCRVNFRNLCKLCQVVDDGISTITLLCFSNNLYFICGKILKSMQTKPSASHTMYFWFSLTYLLGRTLVLSLYSSSINDESKRPLRIFRMVPREYWCDE
Splice variant 2 ATTVDYWMQCRVNFRNLCKLCQVVDDGISTITLLCFSNNLYFICGKILKSMQTKPSASHTMYFWFSLTYLLGRTLVLSLYSSSINDESKRPLRIFRMVPREYWCDE
Splice variant 3 ATTVDYWMQCRVNFRNLCKLCQVVDDGISTITLLCFSNNLYFICGKILKSMHSNAFPRRCIWTRWH
120
130
140
150
160
170
180
Splice variant 1 LKRFSEEVHMDTVALTGMKFFRLTRGVVISVAGTIVTYELILLQFNKEETTAFTCENA
Splice variant 2 ESQDRTSILFRKSNLLLFILAQTLFRGGAYGHGGIDGHEVLSSDAWRCHFGGRNYRDLRAYSATVQQGGNDGVYL
Splice variant 3

Figure 2.9: Molecular analysis of *Gr64e* expression in *D. pseudoobscura*. (**a**) RT-PCR analysis of total RNA extracted from heads or proboscis tissue of *D. pseudoobscura*. Primers are indicated above; the location of primer hybridization sites is shown in (**c**). Primer sequences were as follows: A (5'-CGTAGATGATGGCATCTCCA), B (5'-TGGAAGTTCTGTCCTGGCTT), and C (5'-CAGACGAAAGAACTTCATGCC). (**b**) Gel showing multiple products acquired by PCR amplification of "full-length" coding region of *Gr64e* cDNA. In both (**a**) and (**b**), expected sizes for products of splice variants, SV1-SV3 (see also (**c**)), are indicated to the right. SV1 is the transcript that encodes full-length Gr64e protein[74]. (**c**) Schematic illustrating the structure of the *D. pseudoobscura Gr64e* genomic region and the three alternatively spliced transcripts that were identified by sequencing analysis. Colored block arrows indicated exons, and the asterisk shows the location of the splice site polymorphism. (**d**) Sequences of the carboxy terminal ends of the proteins derived from translation of the three alternatively spliced transcripts. Splice variant 2 gives rise to the sequence published in[74].

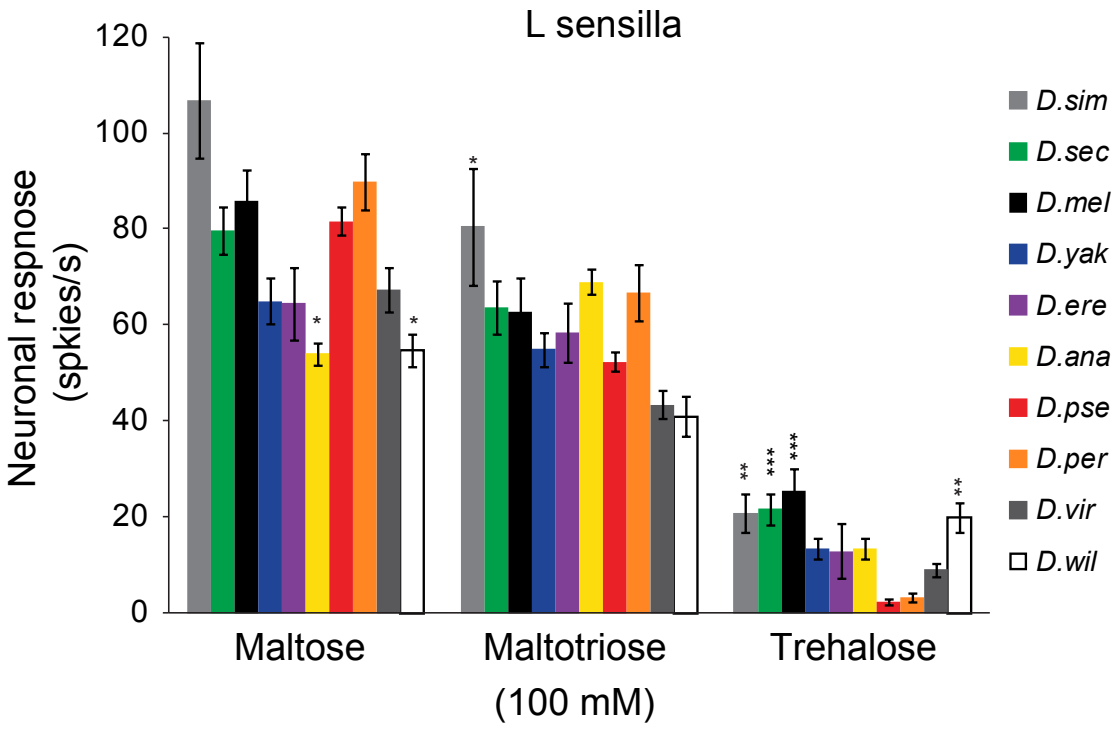

L sensilla
Neuronal respnose (spkies/s)
120
100
80
60
40
20
0
Maltose
Maltotriose
Trehalose
(100 mM)
D.sim
D.sec
D.mel
D.yak
D.ere
D.ana
D.pse
D.per
D.vir
D.wil

Figure 2.10: Comparison of electrophysiological responses to some sugars across *Drosophila* species. Mean responses to sugars were obtained by recordings from L-type sensilla of *Drosophila* species: *D. simulans* (*D.sim*), *D. sechellia* (*D.sec*), *D. melanogaster* (*D.mel*), *D. yakuba* (*D.yak*), *D. erecta* (*D.ere*), *D. ananassae* (*D.ana*), *D. pseudoobscura* (*D.pse*), *D. persimilis* (*D.per*), *D. virilis* (*D.vir*), and *D. willistoni* (*D.wil*). Sugars were tested at 100 mM in 30 mM TCC. $9 \leq n \leq 17$. Asterisks indicate responses that are significantly different from those observed for *D.pse*, one-way ANOVA with Tukey's *post hoc* analysis, $*p<0.05$, $**p<0.01$, $***p<0.001$. Error bars indicate s.e.m.

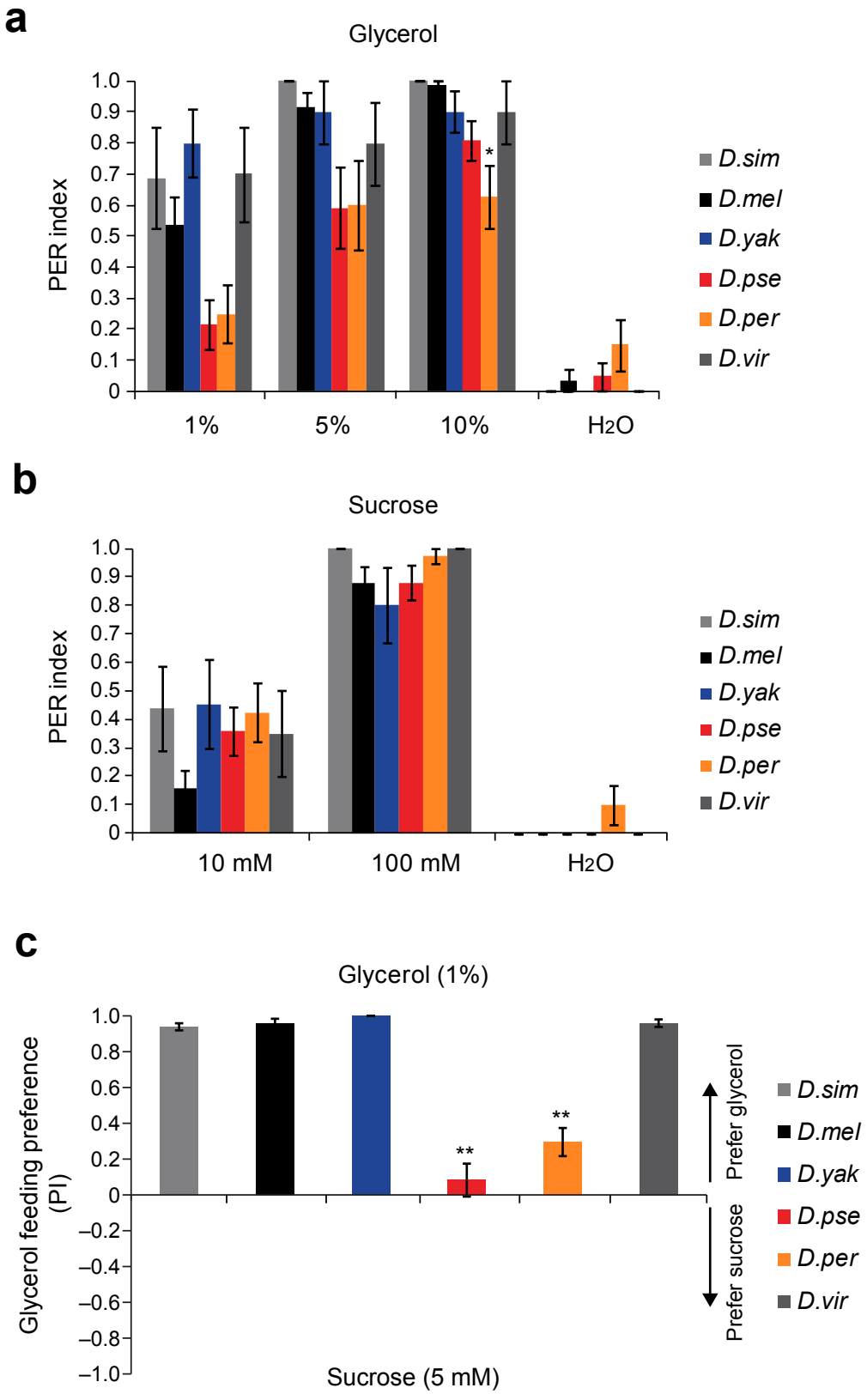

a
Glycerol
PER index
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*
1%
5%
10%
H2O
D.sim
D.mel
D.yak
D.pse
D.per
D.vir
b
Sucrose
PER index
10 mM
100 mM
H2O
D.sim
D.mel
D.yak
D.pse
D.per
D.vir
c
Glycerol (1%)
Glycerol feeding preference (PI)
1.0
0.8
0.6
0.4
0.2
0
−0.2
−0.4
−0.6
−0.8
−1.0
**
**
Prefer glycerol
Prefer sucrose
Sucrose (5 mM)
D.sim
D.mel
D.yak
D.pse
D.per
D.vir

Figure 2.11: *Gr64e* pseudogenization correlates with reduced behavioral response to glycerol. (**a,b**) Proboscis extension responses of indicated species to glycerol (**a**) and sucrose (**b**); the stimuli and water were tested blind. Responses were graded as in Fig. 4. $6 \leq n \leq 10$. (**c**) Glycerol feeding preference in indicated species. $10 \leq n \leq 14$. In all graphs, asterisks indicate responses that are significantly different from those observed for *D.mel*, $*p<0.01$, $**p<0.001$. Error bars indicate s.e.m.

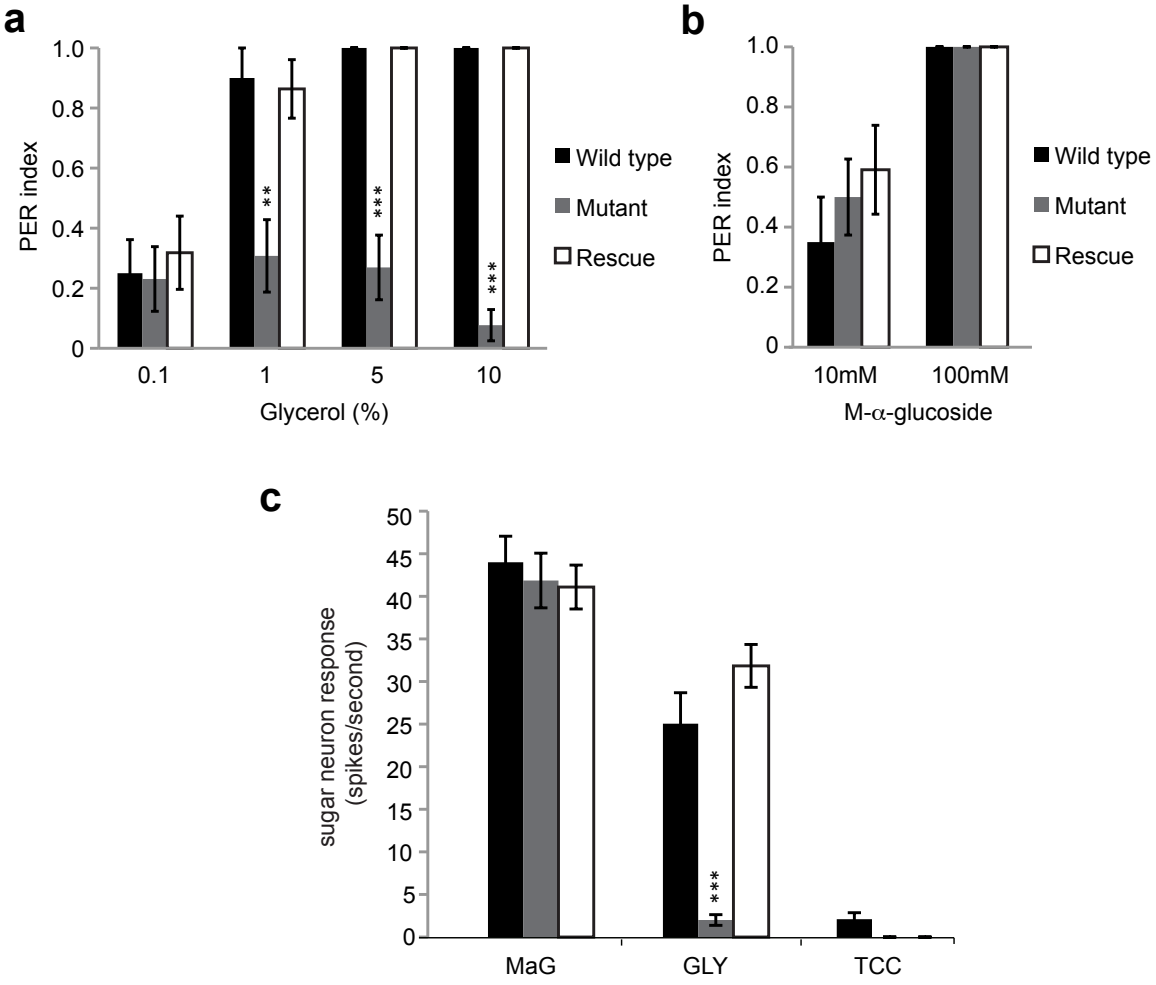

a
PER index
1.0
0.8
0.6
0.4
0.2
0
**
***
***
0.1
1
5
10
Glycerol (%)
Wild type
Mutant
Rescue
b
PER index
1.0
0.8
0.6
0.4
0.2
0
10mM
100mM
M-α-glucoside
Wild type
Mutant
Rescue
c
sugar neuron response
(spikes/second)
50
45
40
35
30
25
20
15
10
5
0
***
MaG
GLY
TCC

Figure 2.12: *Gr64a* is necessary for recognition and behavioral responses to glycerol. (**a**) Proboscis extension responses of wild type (w^{1118}), mutant ($Gr64a^{1}$), and rescue (*Gr5a-GAL4/UAS-Gr64e*; $Gr64a^{1}$) flies to indicated concentrations of glycerol or (**b**) m-α-glucoside. Responses were graded for full extension (1), partial extension (.5), and no movement upon stimulus application (0) ($10 \leq n \leq 13$). (**c**) Mean responses in L-type sensilla. Sweet compounds were tested m-α-glucoside (100mM) and glycerol (10%) in 30 mM TCC ($12 \leq n \leq 22$). In all graphs, asterisks indicate responses that are significantly different from wild type, one-way ANOVA with Tukey's *post hoc* analysis $^{**}p<0.01$, $^{***}p<0.001$. Error bars indicate s.e.m.

Chapter 3:

Characterization of gustatory receptor mutants and feeding state in peripheral sweet gustatory receptor neurons

Abstract:

An insect's ability to taste sweet compounds is critical for food selection and feeding behaviors. Sweet detection in *Drosophila* is mediated by a suite of eight gustatory receptors (Grs) that belong to a highly conserved receptor clade in insects. Despite decoding efforts, how these receptors function in sweet detection is not fully understood. Here, we analyze taste responses in individual *Gr* mutants in order to demonstrate that each Gr is important for normal sweet detection. Gr-ligand interactions can be loosely separated between *Gr5a* and *Gr64a* sugar responses profiles. Ectopic responses are validated by tastant response defects in corresponding *Gr* mutants, when available. We also show that inhibitors previously shown to inhibit glycerol responses are also able to inhibit the response of sucrose. Finally, we demonstrate that peripheral activity of sweet neurons increases with starvation possibly caused by an increase in receptor expression. Our characterization of the sweet Grs and their modulation by external feeding input and internal state lays a foundation for future studies investigating mechanisms of homeostatic-driven changes at peripheral taste neurons.

Introduction:

Peripheral taste detection of compounds is necessary to evaluate the palatability and identify toxic food sources before consumption. Sugar detection is important for feeding on high caloric diets. Mammals contain a heteromeric sweet receptor, Taste receptor 2 (T1R2) functioning with T1R3 exhibiting sweet detection to all sweet compounds tested[39]. However, *Drosophila melanogaster* has 8 gustatory receptors (Grs) in the sweet clade, which raises interesting questions: How are these Grs functioning to respond to sweet compounds? Do flies use all eight receptors for sweet compound detection? Do receptors work together or separately? Gr5a and Gr64a are necessary for most sugar detection[37]. Gr64f is implicated to partner with Gr5a and Gr64a for detection of trehalose[52]. Gr43a, not found in the sugar receptor clade, responds to fructose[49]. Moreover, we have recently shown Gr64e to be a receptor for glycerol, a sweet polyol and found in yeast, a common food source of flies[91].

To investigate how Grs are involved in peripheral detection we performed electrophysiology with a panel of sugars in the labellum of five sweet *Gr* mutants to explore if sweet compound detection profiles were affected. We found all *Gr* mutant genotypes have defects in peripheral sugar detection. Ectopic responses in Freeman et al. 2014 are also verified in corresponding available *Gr* mutants. Additionally, we have discovered that Gr64a along with Gr64e are necessary for peripheral detection and behavioral response to glycerol. Previously described inhibitors of glycerol response[86] in sweet neurons, affect both glycerol and

sucrose but not trehalose detection. If Gr64e and Gr64a work together to produce glycerol response, we posited that inhibitors of glycerol detection might act on one or both of these receptors. In *Gr64e* mutants we have shown that sucrose response is unaffected; however, when sucrose-inhibitor mixtures are tested in Gr64e mutants, sucrose response is still inhibited. Our results suggest that if these inhibitors interfere with Gr64a-detected compounds it is possible these inhibitors may interact with Gr64a directly. Overall our *Gr* mutant analysis of peripheral detection provides evidence that each Gr has a function in sweet detection and supports the model that Grs work as heteromeric partners.

Thus far, we have described how peripheral taste neurons respond to external stimuli, but what about internal feeding state? An animal must be able to detect and feed on substrates that satisfy their caloric needs. When quality food sources are rare, animals require regulatory mechanisms to control food detection and optimize feeding behavior in order to survive. Many studies have investigated central mechanisms, which modulate central feeding circuits during nutrient deprivation or starvation[101,102]. However, very little is known about modulatory effects on peripheral detection. Our current understanding of peripheral detection in the labellum has given us the opportunity to investigate what happens when flies are challenged with food deprivation or starvation. The fly taste system must be adaptive to survive in an ever-changing environment. Modulation of how central processes in the brain perceive food sources is fairly well studied. We know that in response to starvation conditions neuromodulators,

such as dopamine, is released at the presynaptic terminals of primary taste neurons, which increases the activity that contributes to higher order feeding circuits in flies to change. Additionally, there are a handful of studies that have looked at peripheral changes in sweet neuron response to starvation[101,103,104]. However, few studies have looked at the effects of long-term deprivation. One such study found that sugar-feeding behavior is modulated by previous feeding experience and increases in response to deprivation[105]. We wanted to know if these starvation-driven behaviors are manly due to changes in central circuits or if peripheral taste neurons are also “sensitized” during deprivation.

Under a period of starvation flies we find an increased firing rate in labellar neurons to sucrose, which is consistent with previous studies[103,104]. Furthermore, we used Gr64f reporter flies to investigate peripheral neuron modulation during feeding deprivation and find increases in GFP reporter levels by imaging. We correlate increased expression of Gr64f using a GAL4/UAS reporter by imaging analysis. It is an intriguing possibility that starvation and deprivation may modulate Gr expression at the periphery and could explain increases in sweet neuron activity[104]. Finally, our data suggests that all sweet Grs are used in a breadth of tuning to compounds detected by the sweet neurons as well as changes in the peripheral neurons due to starvation and possibly from deprivation, which contributes another layer of complexity to peripheral physiological changes during food scarcity.

Materials and Methods:

Fly stocks

Flies were maintained on standard cornmeal-dextrose medium at 25°C. Wild type flies were wCS. Mutants are listed along with reference that they were obtained from: ΔGr5a ΔEP(X)-5 (Dahanukar Nat Neuro 2001) ΔGr64f Gr64f[MB12243], BDSC (#27883), ΔGr64e Gr64e[MB03533], BDSC (#23628)[91], ΔGr61a Gr61a[1], and ΔGr64a Gr64a[1][37]. Reporter flies were two Gal4 lines w; Gr64f-GAL4; UAS-mCD8-GFP and w; UAS-mCD8-GFP; Gr64f-GAL4.

Chemosensory recordings

Extracellular tip recordings from labellar taste sensilla in the fly were performed as described[91] by using 30 mM tricholine citrate as electrolyte; tastants were stored at −20°C, and working aliquots were thawed and kept at 4°C for no more than 1 week. Spikes were counted in the 200- to 700-ms for Gr mutant analysis or 0- to 500ms for starvation condition windows after contact of the stimulus micropipette with the pore of a sensillum.

Behavior

Proboscis extension responses (PER) of male flies were tested in a controlled environment room (22–25^{o}C, 25–45% humidity) as described previously[37]. Flies aged 3–6 days old were transferred to fresh culture vials for at least 24 hours and subsequently starved on water-saturated tissues prior to testing. PER was

performed between 2–6 PM to control for circadian variations in feeding behavior. Deprivation food was made at final concentration of mixtures of sucrose, yeast extract and agar. Final concentrations were: 1% or 10% sucrose, 4.875% yeast extract and 1% agar.

Tastants

All compounds were obtained at the highest available purity from Sigma-Aldrich and were as follows: trehalose (T9531), glucose (G7528), or m-a-glucoside (M9376), melezitose (M5375), sucrose (S7903), maltose (M9171), maltotriose (M8378), fructose (47740), glycerol (G7893), and Inhibitory compounds 2-amino-1,3-propanediol (40362) and 3-amino-1,2-propanediol (a76001).

Imaging

Whole brains were dissected, fixed and stained as described[37]. Antibodies were used at the following concentrations: mouse α-nc82 (1:20), rat α-CD8a (1:100) rabbit α-HA (1:100), Alexa-568 α-mouse (1:150), Alexa-488 α-rat (1:150), and Alexa-647 α-rabbit (1:150). Confocal *z*-stacks were acquired at the same LASER settings using a Zeiss LSM510 and analyzed using ImageJ. Images stacks were merged to create flattened Z-stacks using MAX intensity settings. Total florescence was measured in pharynx, labellum, leg projections, which were separated from a total image to calculated differences contributed form different organs.

Statistics

All statistics were performed by SPSS and are described in each of the legends

Results:

All sweet Gr mutants tested show loss in sugar detection

The sugar clade of receptors was found ~10 years ago[75], but of late we have only characterized around half of the sweet Grs and still do not understand what makes up a functional sugar receptor. The sweet clade of Grs contains 8 receptors, 5 of which (*Gr5a*, *Gr64f*, *Gr64e*, *Gr61a* and *Gr64a*) have verified mutants or loss of responses to sweet compounds[20,37,90,91]. To investigate how other sweet Grs are involved in detecting sweet compounds, we performed electrophysiology recordings on available Gr mutants to a sweet nine compound panel.

Previous observations have found that only a subset of compounds from a large stimulus panel strongly activate sweet taste neurons of L-type labellar sensilla. All of these stimuli responses were either dependent on *Gr5a* or *Gr64a*[37], so we selected a smaller diagnostic panel of nine tastants to characterize the range of responses in taste neurons. We chose four sugars that *Gr5a* is needed for detection in sweet neurons, four sugars that depend on *Gr64a*, and glycerol in which *Gr64e* is necessary for detecting [37,77,91]. We found that all Gr mutants show a loss of detection to sweet compounds in comparison to wild type tested at two separate concentrations (**Figure 3.1**).

We tested all sugars in L type sensilla (**Figure 3.1a**) at 100mM and glycerol at 10% and for higher concentrations sugars were tested at 1M with glycerol at 50% (**Figure 3.1b**). We see a similar pattern of loss of detection between higher and lower concentrations tested (**Figures 3.1c,d**). Our mutant analysis is consistent with previous results and suggests that sweet compounds loosely fit into two major categories of detection, *Gr5a* or *Gr64a* dependent[37].

Ectopic responses are validated by mutant analysis

We observed that expressing single sweet Grs can confer sweet taste responses in ab1C neurons (from Freeman et al 2014) and wanted to validate ab1C response profiles with endogenous neurons activity. We therefore compared the response profiles of ab1C:GrX neurons (from Freeman et al 2014) to those of sweet taste neurons in the corresponding *Gr* mutants (**Figure 3.1c**). We observed complete overlap between Gr5a- and Gr64a-ligand interactions identified by ectopic responses obtained in ab1C:GrX neurons and loss of sensitivity in *Gr5a* and *Gr64a* mutants (**Figure 3.2a**). Although not as complete, there was also substantial overlap between the results of gain-of-function and loss-of-function analyses for Gr61a, Gr64e, and Gr64f (**Figure 3.2a**). Importantly, every single observed ab1C:GrX responses was validated by a significant reduction in sweet taste neuron response in the corresponding *Gr* mutant. We visualized these relationships in a scatter plot of the two data sets (**Figure 3.2b**) and ran a Spearman's correlation to determine the relationship between gain and

loss values, which showed a strong positive correlation (r_s = 0.759, n = 45, P < 0.001). Together, these results verify the functional analysis of single Grs expressed in the ab1C neuron.

Inhibitors block both of glycerol and sucrose, but not trehalose response in sweet taste neurons

Glycerol was shown previously to elicit sugar neuron firing and these authors also found inhibitors that were specific to glycerol detection by out competing a possible glycerol-binding site[86]. We were interested to test our new-found receptors for glycerol Gr64e and Gr64a to see if either Gr64e, Gr64a or both might be the targets of both inhibitors: 2-amino-1,3-propanediol (2AM) and 3-amino-1,2-propanediol (3AM). To investigate whether 2AM or 3AM indeed affect glycerol detection we first verified 2AM and 3AM inhibitory effects on glycerol and tested Gr64a (sucrose) and Gr5a (trehalose) sugars as controls (**Figure 3.3a**). Both 2AM and 3AM inhibited glycerol response as described previously[86], however to our surprise we found that sucrose was also strongly inhibited. Importantly trehalose detection was not affected leading us to suggest that both 2AM and 3AM inhibition profiles were specific to glycerol and sucrose and were not acting as general sugar inhibitors (**Figure 3.3a**). Sucrose response was decreased to ~20 spikes per second, where glycerol was inhibited to ~15 spikes per second with 2AM (200mM) and 3AM (100mM). However, when we stimulated the fly with 2AM or 3AM (~15 spikes per second) alone we also see

activity in the sugar neuron. The levels of activity with 2AM or 3AM alone verses when mixed with sucrose, glycerol or trehalose do not have significantly different ($p > 0.05$ by 2 way ANOVA with pairwise comparison) response levels (**Figure 3.3b**) suggesting that these inhibitors may completely abolish sucrose and glycerol responses. We further investigated the inhibition properties of these compounds and see a dose-dependent inhibition by both 2AM and 3AM (**Figure 3.3c**).

To determine the extent of 2AM and 3AM inhibition on feeding behavior for glycerol, we examined the PER behavior in wild type flies. Wild type flies exhibit a strong dose-dependent proboscis extension upon stimulation by glycerol[86,91]. In contrast we found that glycerol (10%) when mixed with increasing concentrations of either 2AM or 3AM showed a does-dependent inhibition of the flies PER (**Figure 3.3d**). Our results demonstrate that inhibition of sucrose and glycerol detection in the sugar neuron, by 2AM and 3AM, which also correlate to inhibition in feeding behavior for glycerol.

Gr64e mutants do not show loss of sucrose inhibition by 2AM or 3AM inhibitors

Both Gr64e[91] and Gr64a are necessary for glycerol detection, we hypothesized that both of these receptors form a heteromeric receptor. If 2AM and 3AM block glycerol detection it stands to reason that these inhibitors may interfere with either Gr64e or Gr64a function. Gr64a is needed for proper sucrose response while loss of Gr64e shows no loss in sucrose detection. To test this

hypothesis we performed sucrose, or sucrose plus 2AM or 3AM recordings in Gr64e mutants to quantify if sucrose response is still inhibited. We proposed that if Gr64e and Gr64a together form a functional receptor then by testing sucrose response in a Gr64e mutant might alleviate sucrose inhibition by 2AM and 3AM. We find that the loss of Gr64e does not impair sucrose inhibition (**Figure 3.4a**) of 2AM and 3AM. There is not a large difference between wild type and Gr64e mutants in response sucrose and sucrose plus inhibitors (**Figure 3.4b**). Our results suggest that 2AM and 3AM inhibitors may directly interact with Gr64a or other partners of Gr64a to inhibit sucrose response.

Low sugar only diets correlate with increasing Gr64f expression in taste tissues

Recently, Nishimura et al. 2012 demonstrated that some strains of flies increase *Gr64a* expression in the labellum under starvation conditions[104]. However, no studies to date have looked a long-term deprivation and its effect on Gr expression.

To investigate modulation in peripheral sugar neurons we chose to visualize primary neuron projections by employing reporter flies to look at how feeding under different sucrose concentrations affected expression. In the Gr sweet clade there is molecular evidence that all Gr5a, Gr61a, Gr64a and Gr64f are expressed in sweet neurons. Of those receptors, *Gr64f* is the most broadly expressed in sugar neuron tissues[37]. We used *Gr64f-Gal4* to drive the expression of a membrane tethered Green Fluorescent Protein (GFP) under

control of *UAS-mCD8-GFP*. Flies are reared on regular food until eclosion, and subsequently transferred to regular food. Flies are allowed to live on the food for ten days followed by brain staining for visualizing *Gr64f-GAL4* driven GFP signals. We quantified total fluorescence intensity in the pharyngeal, labellar, and leg projections of the sub-esophageal ganglion (SOG) as well as total SOG regions(**Figures 3.5a,b**). When comparing *Gr64f-GAL4* on the second verses the third chromosome driver, there was no significant difference ($p > 0.5$ students *ttest*) between total fluorescence intensity so we pooled the data from both genotypes in subsequent analysis. We next tested how GFP levels would change when flies were subjected to deprivation states at 0.1%, 10% sucrose agar food. The 10% sucrose has comparable sugar content to regular fly food, however missing yeast extract or cornmeal. We found, by imaging analysis, that flies deprived on 0.1% sucrose have increased GFP staining levels in *Gr64f-GAL4* primary neuron projections in the SOG in comparison to flies grown on 10% sucrose or regular food (**Figures 3.5c,d**). To control for variations in fluorescence within fly groups we quantified right (R) and left (L) SOG intensities of all separate tissues (**Figure 3.6a**) and found no significant differences ($p > 0.2$ by students *ttest*) between tissues at any condition (**Figures 3.6b,c,d**). Flies, after eclosion, that were reared in 0.1% sucrose conditions have 2.5-fold greater total intensity than 10% sucrose (**Figure 3.5d**). Our results provide evidence that *Gr64f* expression maybe modulated by external feeding conditions.

Starvation increases peripheral neuron activity

When flies are starved for a period of time they have homeostatic mechanisms that signal to the fly to eat. This mechanism is thought to function in the brain, through central hormone or neuropeptide release so that decreasing threshold for food source detection[101]. However, there is also evidence that peripheral changes occur with starvation[103,104]. We took five male and female wild type flies to seed our vials and collected F1 progeny. F1 flies were separated at 4 days after eclosion and either starved or placed on new regular food. We tested both starved and fed male flies L-type sensilla in the labellum and recorded electrophysiological responses to increasing concentrations of sucrose (1, 10, 100mM). We found that starved flies have increased firing rates in sweet neurons to sucrose at 10mM and 100mM concentrations, but not to 1mM (**Figure 3.7a**). Flies are thought to make a decision within the first 100ms of a neuron firing[85] so that we also counted spike/sec based on the first 50ms (**Figure 3.7b**) and 100ms (**Figure 3.7c**) and see the same results. Our results, consistent with others[103,104,106], showed that starved flies have about a 20% firing-rate increase over fed flies and overall adds evidence that peripheral changes occur under starvation conditions.

Discussion:

In spite of the discovery of a sufficient functional sweet Gr, we have characterized Gr mutant responses and demonstrated each Gr is involved in sweet compound detection. We also found increases in peripheral neuron activity

when flies are starved and correlated increases in Gr64f expression in response to deprived feeding conditions. Our characterization of sweet Gr contribution and evidence that modulatory effects on peripheral neuron detection occurs provide a basis for how insects detect calorically rich food sources and what changes may occur at the peripheral detection level adds complexity to current understanding of modulations of taste circuits.

Previously it was shown that two receptors, *Gr5a* and *Gr64a*, when mutated lose all tested sugar and now glycerol responses[37,90]. When comparing the Grs to the olfactory receptors (Or)s, there does not seem to be an obligate co-receptor like ORCO in the sweet Gr family. However, maybe together *Gr5a* and *Gr64a* would be the closest obligate receptors for sweet detection. The eight sweet Grs are hypothesized to originate from a single ancestral gene that gave rise to two lineages and as both *Gr5a* and *Gr64a* are necessary for sugar detection. Thus, it is not surprising that one family contains *Gr5a* and another includes *Gr64a* following a duplication event[74].

These losses of response in *ΔGr5a* and *ΔGr64a* mutants in comparison with Freeman et al. 2014 directly overlap with their gain of response that is found in the ectopic system. Mutant responses profiles of *ΔGr64e*, *ΔGr64f* and *ΔGr61a* also closely correlate with ectopic expression data. These mutant analyses verify the ectopic expression system. Additionally, *ΔGr64e*, *ΔGr64f* and *ΔGr61a* showed losses of responses that were not seen when these receptors were ectopically expressed and did not have any specific response loss patterns, both

having sugar losses from Gr5a and Gr64a categories. This could lead to the hypothesis that Grs are promiscuous and may be able to partner with multiple sweet Grs.

Previously it was shown that glycerol response was inhibited by 2AM and 3AM compounds[86], however Koseki et al 2004 did not see a significant effect on sucrose (250mM) or trehalose (500mM). Our analysis demonstrates glycerol inhibition by inhibitors, however we found that 2AM (200mM) and 3AM (100mM) additionally interfered with sucrose detection (100mM) at lower concentrations in comparison to Koseki et al 2004. It is possible that sucrose is not as affected at Koseki et al 2004 tested 250mM concentration as well. In addition they used a different electrolyte solution verses our study (tricoline citrate). Furthermore, we did not see a significant loss in trehalose detection when 2AM and 3AM were mixed in, indicating the specificity of 2AM and 3AM inhibitory effects on glycerol and sucrose detection. Our *Gr64e* mutant analysis of sucrose inhibition by 2AM and 3AM suggests that these inhibitors may directly inhibit *Gr64a* by allosteric interaction.

Our results characterize tastant detection of the entire repertoire of sweet taste receptors, which gives great insight into receptor ligand interactions and ultimately insight into the discovery of sufficient functional sweet gustatory receptors. The characterization of how sweet Grs detect attractive tastants allows us to further investigate modulatory effects at the peripheral level. Our understanding of insect feeding behavioral states is shaped by studies

characterizing starvation or sensory deprivation. Modulation of neurons is observed to shift an organism's preference for feeding behavior and neuron sensitivity to food compounds[101,103,107]. Centrally, neuromodulatory peptides have been shown to be involved in increasing external input signals, which in turn relay greater activity to central neurons, inducing stronger feeding behaviors[107,108]. However, the understanding of peripheral neuron modulation during different feeding states is not well understood.

Evidence in other insects such as cricket studies demonstrate that both amino acids and sugar detection changes based on diet. In the absence of amino acids peripheral firing of neurons was higher to amino acids, and conversely in the absence of sugar the peripheral firing of neurons was higher to sugars[109]. Two recent *Drosophila* studies have also shown that peripheral detection increases to sugars with starvation[103,104], but another study claims that peripheral changes are not seen[101].

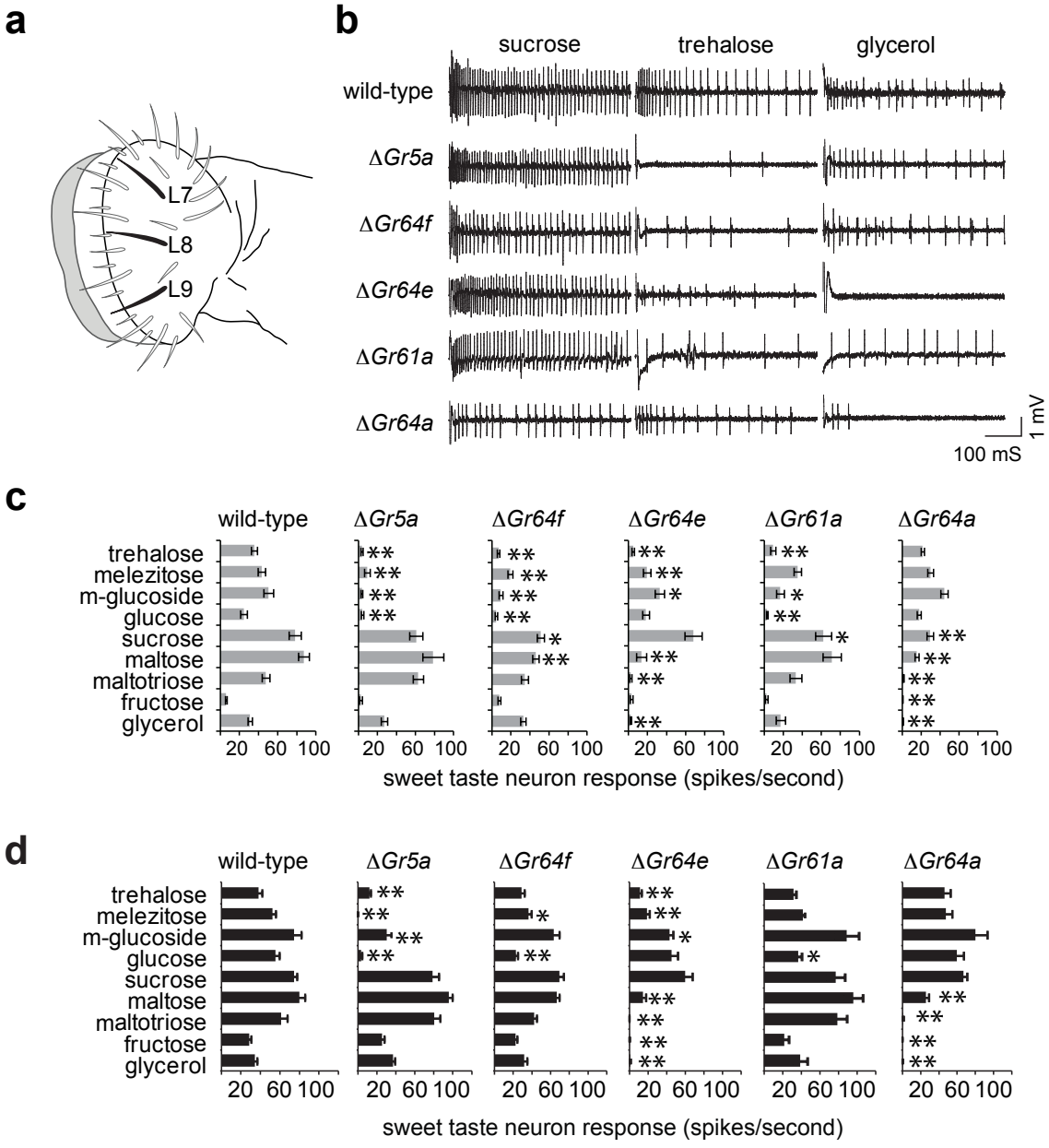
We provide evidence that starved male flies have increased sweet neuron firing as compared to non-starved flies. Does this result extend to other sugars? Previous studies reveal that male flies, when starved, have increases in peripheral sugar neuron responses when tested with sucrose[104] and glucose[103]. Conversely, in starved female flies there was no difference in spike frequency observed compared to the fed condition[101]. This difference in findings could be due to sexual dimorphisms or possibly that starvation period of female flies was insufficient. To fully understand whether starvation state affects peripheral

detection, a dose curve for a comprehensive list of sweet compounds should be performed on both males and females.

The majority of sensory deprivation studies have been performed at acute (1-2 day) time points. Nevertheless, we and other studies have evidence that supports acute peripheral changes[103,104]. One mechanism of peripheral increases in neural activity was purposed by increasing expression of Grs[104]. We wanted to investigate changes in Gr expression upon longer deprivation periods by using a reporter system to quantify *Gr64f* expression.

We find differences in expression when flies were fed at varying concentrations of sucrose. Our data provides evidence that *Drosophila* exposed to low concentrations (0.1%) of sucrose has increases in florescence intensity of axonal projections in the SOG in comparison with flies that are exposed to higher concentrations (10%). The change in fluorescence intensity we observe may be due to lack of sensory input, which may signal to increase Gr expression in the peripheral GRN to allow the fly to detect lower concentrations of sugar. It is entirely possible that central mechanisms could signal peripheral neurons to increase sensitivity to external stimuli. Many questions remain: Is the up regulation of Grs dependent on sugars that they detect? What are the neural signaling pathways leading to increases in peripheral sugar neuron activity? As no carefully constructed experiments to investigate changes in Gr expression during feeding deprivation have been done, this avenue of research could yield novel homeostatic signals occurring in peripheral neurons, contributing to our

understanding of modulatory mechanisms of sugar detection and feeding behaviors.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1 mV
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c
wild-type
ΔGr5a
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sweet taste neuron response (spikes/second)
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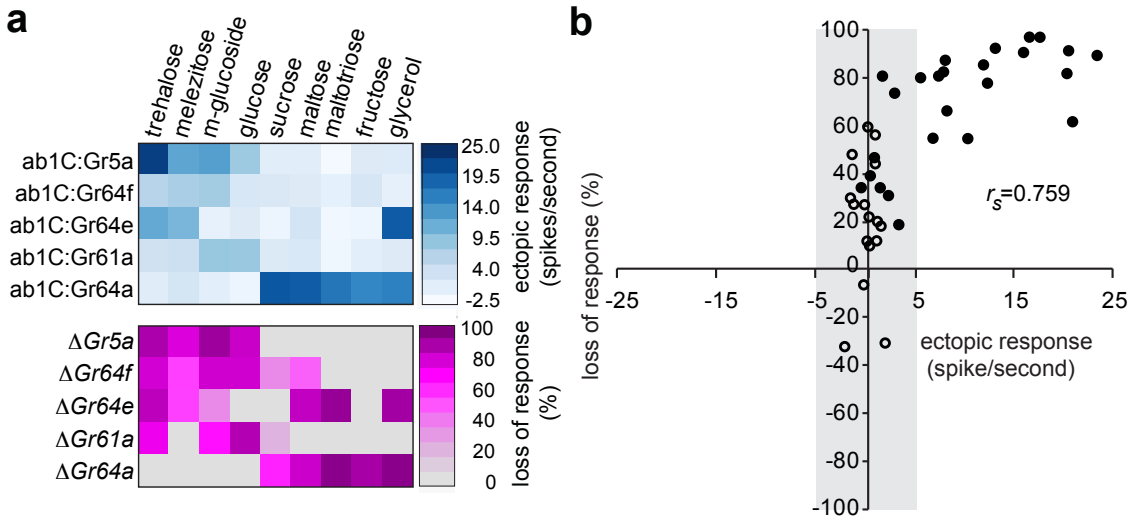
glucose
sucrose
maltose
maltotriose
fructose
glycerol
20 60 100
sweet taste neuron response (spikes/second)

Figure 3.1: Sweet taste responses in *Gr* mutants**.**

(**a**) Schematic of the fly labellum highlighting L-type sensilla that were used for electrophysiological recordings in black (L7, L8 and L9). (**b**) Sample traces of recordings with sweet stimuli obtained from L-type sensilla. Indicated genotypes were: w^{1118} (wild-type), ΔEP(X)-5 (ΔGr5a), Gr64fMB12243 (ΔGr64f), Gr64eMB03533 (ΔGr64e), $Gr61a^{1}$ (ΔGr61a), and $Gr64a^{1}$ (ΔGr64a). (**c,d**) Mean responses of sweet taste neurons in L-type sensilla to indicated tastants (c) 100mM sugars 10% glycerol, (d) 1M sugars 50% glycerol. (c) 100mM sugars traces genotypes shown in (b). *P < 0.05; ***P < 0.001 (one-way ANOVA with one-tailed Dunnett's *ttest* vs. wild-type; (c) *n* = 6–22 (d) *n* = 6-12).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trehalose
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m-glucoside
glucose
sucrose
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fructose
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ab1C:Gr64e
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ΔGr5a
ΔGr64f
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ΔGr64a
25.0
19.5
14.0
9.5
4.0
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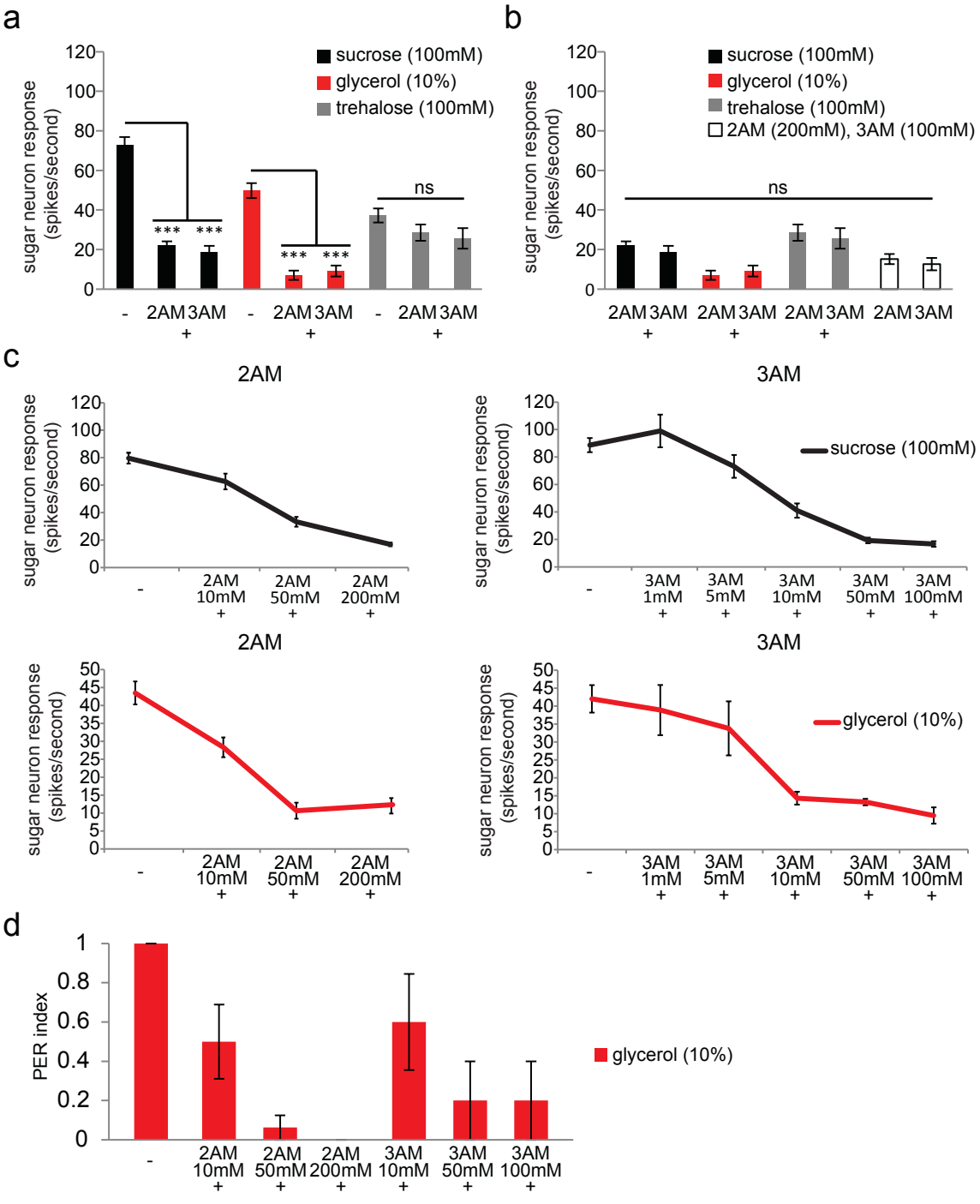
ectopic response (spikes/second)
100
80
60
40
20
0
loss of response (%)
b
loss of response (%)
ectopic response (spike/second)
r_s=0.759
100
80
60
40
20
0
-20
-40
-60
-80
-100
-25
-15
-5
5
15
25

Figure 3.2: Analysis of *Gr* mutants validates identified response spectra. (**a**) Heat maps of stimulus responses of ab1C:GrX neurons (top) and % reduction in taste neuron responses in corresponding mutants (bottom). The heat map on the bottom only includes data for responses that are significantly different from wild-type in (**Figure 3.1**). Percent loss of response was calculated using [(wild-type – mutant)/wild-type]*100. Heat maps were made using JMP 10. (**b**) Scatter plot depicting % loss of response in *Gr* mutant versus ectopic response for each ligand-Gr combination. Closed circles indicate taste neuron responses that are significantly reduced in mutant flies (Δ*GrX*) as compared to wild-type; open circles indicate those that are not. The shaded area indicates ab1C:GrX responses that were not statistically significant.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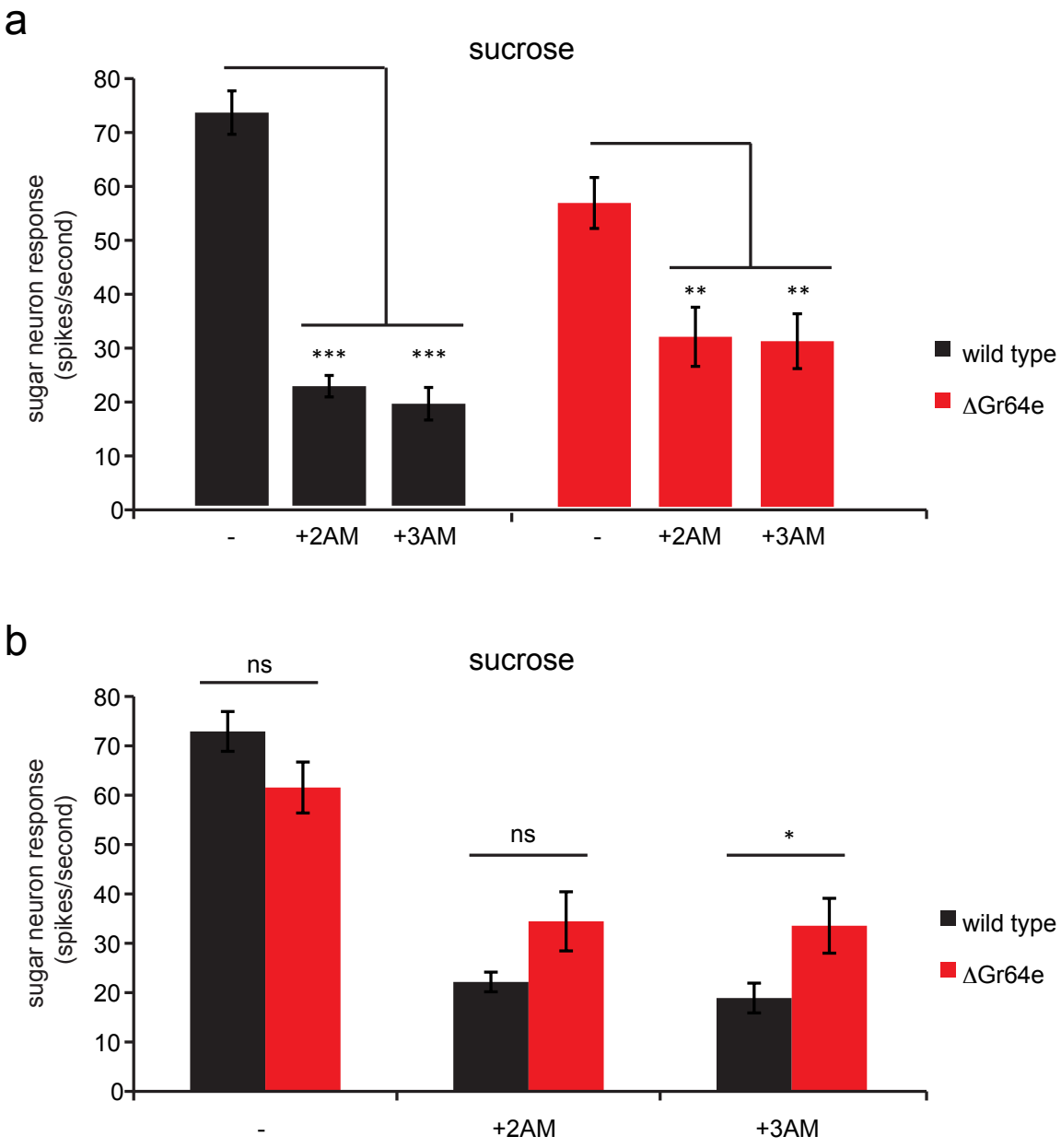
sucrose (100mM)
glycerol (10%)
trehalose (100mM)
sugar neuron response (spikes/second)
ns
***
- 2AM 3AM + - 2AM 3AM + - 2AM 3AM +
b
sucrose (100mM)
glycerol (10%)
trehalose (100mM)
2AM (200mM), 3AM (100mM)
ns
2AM 3AM + 2AM 3AM + 2AM 3AM + 2AM 3AM +
c
2AM
3AM
sucrose (100mM)
- 2AM 10mM + 2AM 50mM + 2AM 200mM +
- 3AM 1mM + 3AM 5mM + 3AM 10mM + 3AM 50mM + 3AM 100mM +
glycerol (10%)
d
PER index
glycerol (10%)
- 2AM 10mM + 2AM 50mM + 2AM 200mM + 3AM 10mM + 3AM 50mM + 3AM 100mM +

Figure 3.3: Inhibitors block glycerol and sucrose, but not trehalose responses in taste sensilla. (**a**) Mean responses of sweet taste neurons in L-type sensilla to indicated tastants. Sucrose (black), trehalose (gray) were tested at 100mM and glycerol (red) was tested at 10%. Sweet compounds were tested alone (-) and mixed with 200 mM 2-amino-1,3-propanediol (+2AM) or 100 mM 3-amino-1,2-propanediol (+3AM). (**b**) Mean responses to sweet compounds (100mM) mixed with 200 mM 2-amino-1,3-propanediol (+2AM) or 100 mM 3-amino-1,2-propanediol (+3AM) compared to 200 mM 2-amino-1,3-propanediol (2AM) or 100 mM 3-amino-1,2-propanediol (3AM) alone (white). (**c**) Dose responses of sucrose (black) or glycerol (red) mixed with indicated concentrations of 2AM or 3AM. (**d**) Proboscis extension response behavior of glycerol (10%) tested alone (-) and mixed with indicated concentrations of 2AM or 3AM. ***$P < 0.001$, 2 way ANOVA with pairwise analysis was performed. n = 6-24. Genotype tested was w^{1118} (wild-type).


a
sucrose
sugar neuron response (spikes/second)
80
70
60
50
40
30
20
10
0
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**
**
-
+2AM
+3AM
-
+2AM
+3AM
wild type
ΔGr64e
b
sucrose
sugar neuron response (spikes/second)
ns
ns
*
-
+2AM
+3AM
wild type
ΔGr64e

Figure 3.4: Sucrose responses are inhibited in *Gr64e* mutants.

(**a**) Mean responses of sweet taste neurons in L-type sensilla to indicated tastants in wild type (w1118) in black and *Gr64e*^{MB03533} (*ΔGr64e*) in red. Sucrose was tested at 100mM tested alone (-) and mixed with 200 mM 2-amino-1,3-propanediol (+2AM) or 100 mM 3-amino-1,2-propanediol (+3AM). (**b**) Same data as in (A) to, but comparing wild type verses *ΔGr64e* responses. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, 2-way ANOVA with pairwise analysis was performed. *n* = 9-24.

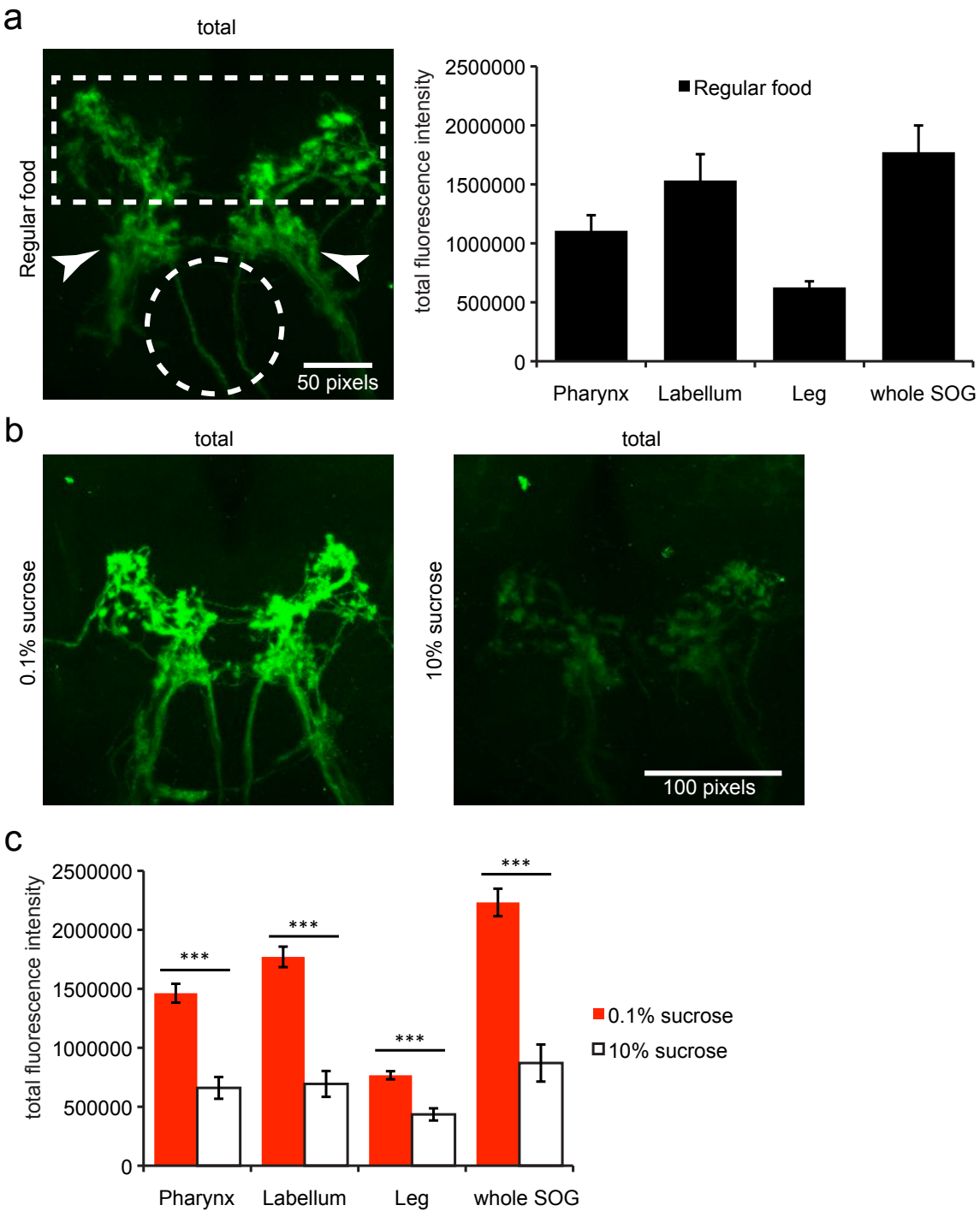

a
total
Regular food
50 pixels
total fluorescence intensity
2500000
2000000
1500000
1000000
500000
0
Regular food
Pharynx
Labellum
Leg
whole SOG
b
total
total
0.1% sucrose
10% sucrose
100 pixels
c
total fluorescence intensity
2500000
2000000
1500000
1000000
500000
0
***
***
***
***
0.1% sucrose
10% sucrose
Pharynx
Labellum
Leg
whole SOG

Figure 3.5: Total fluorescence intensity of *Gr64f-GAL4* expression in the SOG of ten-day-old flies. (**a**) Immunohistological staining of the sub-esophageal ganglion (SOG) in flattened z-stack projection of w; Gr64f-GAL4; UAS-mCD8-GFP flies on regular food. Whole SOG and sub-regions were quantified (right bar graph): pharynx (dotted box), labellum (arrows) and leg (circle) projections. (**b**) Total SOG immunohistological staining of flies on 0.1% or 10% sucrose-agar food and (**c**) quantification of total fluorescence in indicated regions of SOG. ***$P < 0.001$, 1-way ANOVA with Bonferroni post hoc analysis. n = 7-9.

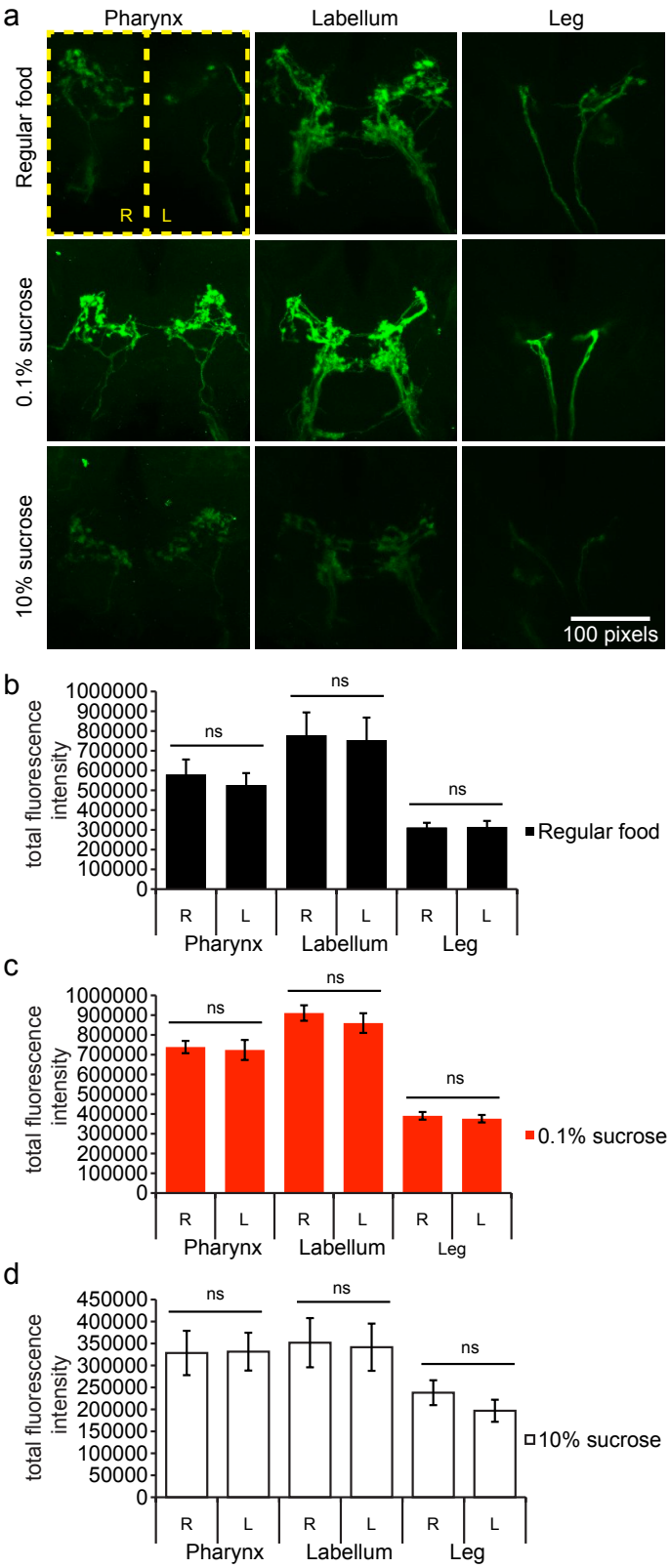

a
Pharynx
Labellum
Leg
Regular food
0.1% sucrose
10% sucrose
R
L
100 pixels
b
total fluorescence intensity
1000000
900000
800000
700000
600000
500000
400000
300000
200000
100000
0
ns
R
L
Pharynx
Labellum
Leg
Regular food
c
0.1% sucrose
d
10% sucrose
450000
400000
350000
300000
250000
200000
150000
100000
50000

Figure 3.6: Total fluorescence intensity right-left controls of *Gr64f-GAL4* expression in the SOG of ten-day-old flies. (**a**) Immunohistological staining of the sub-esophageal ganglion (SOG) in flattened z-stack projection of w; Gr64f-GAL4; UAS-mCD8-GFP flies on regular food, 0.1% and 10% sucrose-agar food. Right (R) and left (L) total fluorescence intensities measurements were obtained (example: yellow dotted box in top left image) to control for overall immunohistochemical staining. Whole SOG and sub-regions were quantified: pharynx, labellum and leg projections. Quantification of total fluorescence of flies on (**b**) regular food (black), (**c**) 0.1% (red) or (**d**) 10% (white) sucrose-agar in indicated regions of SOG. ns = no significance $P > 0.2$, by Students *ttest* analysis. (data analyzed is the same as in **Figure 3.4**) $n = 7\text{-}9$.

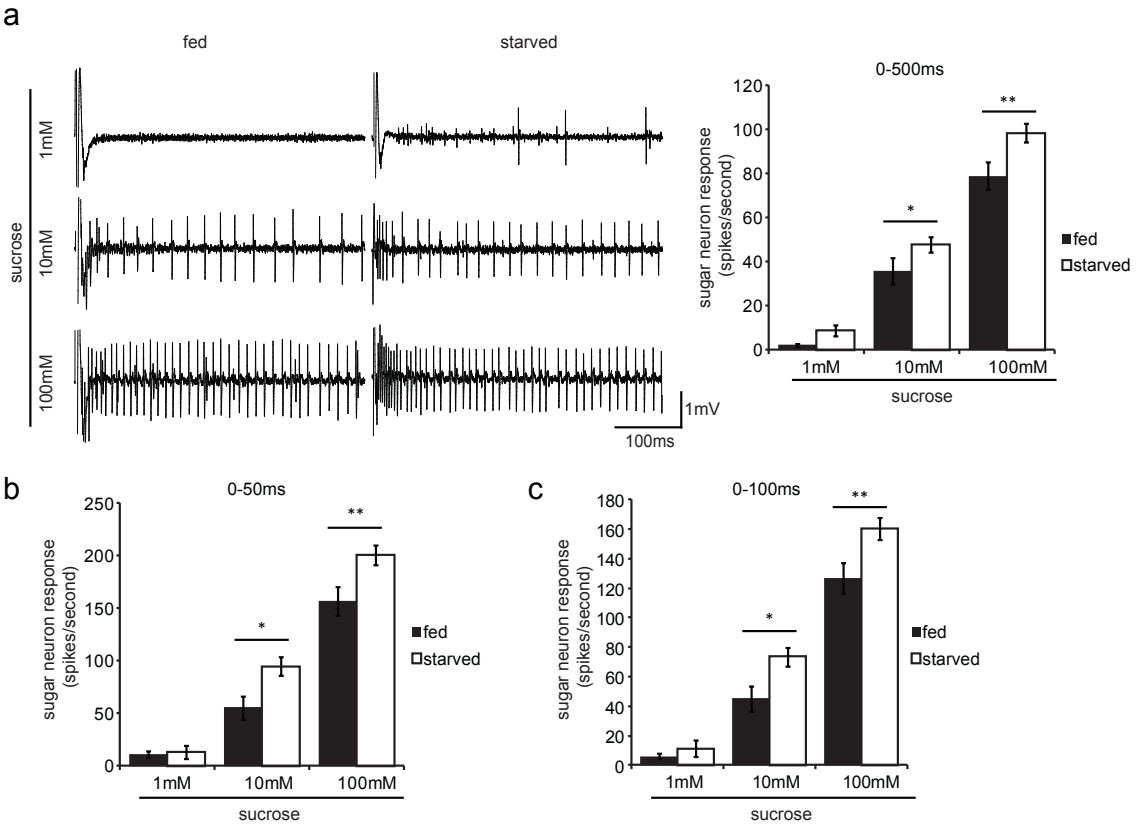

a
fed
starved
sucrose
1mM
10mM
100mM
1mV
100ms
0-500ms
sugar neuron response (spikes/second)
fed
starved
1mM
10mM
100mM
sucrose
b
0-50ms
c
0-100ms

Figure 3.7: Starvation increases firing rates to sucrose in peripheral sugar neurons in male flies. (**a**) Sample traces of recordings with increasing concentrations of sucrose obtained from L-type sensilla in fed or starved wild type (w1118) flies. Mean responses to sucrose at indicated concentrations quantified at 0-500mS (**b**) 0-50mS, and (**c**) 0-100mS. *$P < 0.05$, **$P < 0.01$, 2-way ANOVA with pairwise analysis was performed. n = 25-34.

Chapter 4:

Acid sensing by bitter taste neurons in *Drosophila melanogaster*.

Abstract:

Drosophila can taste various compounds and separate them into few basic categories such as sweet, bitter and salt taste. Here we investigate mechanisms underlying acid detection in *Drosophila* and report that the fly displays strong taste aversion to common carboxylic acids. We find that acid tastants act by the activation of a subset of bitter neurons. Bitter neurons begin to respond at pH 5 and show an increase in spike frequency as the extracellular pH drops, which does not rely on previously identified chemoreceptors. We also see acid-dependent inhibition of a bitter stimulus. This acid-dependent activation of a subset of bitter neurons in the fly plays an important role in the overall evaluation of ingesting acidic foods.

Introduction:

In animals, the taste system is important not only for detecting nutritious foods, but also as a first line of defense against ingesting noxious stimuli[110]. Both mammals and insects are able to detect compounds from both attractive and aversive stimuli. Mammals are able to detect compounds of five basic categories: sweet, umami, salt, bitter, and acid (sour)[39,111-113]. Previous studies have shown that *Drosophila* can taste compounds in sweet, bitter, and salt taste categories[33,37]. Among the aversive stimuli, acid detection (sour) is the least understood, albeit carboxylic acids being widely found in nature. Intriguingly,

carboxylic acids are found in ripe and rotting fruits, common food sources to *Drosophila*, so that acid detection might play an important role in guiding feeding behaviors[112].

Taste sensing in both mammals and flies is organized into a few cell types that are specialized to detect various categories of tastants and convey either acceptance or rejection signals to the brain[112]. In the fly, neurons that detect sweet and bitter stimuli express members of the large, divergent gustatory receptor (Gr) family[33,53]. Individual Grs expressed in these neurons have been linked to detection of sugars and polyols[37,52,76,77,91], or alkaloids and other bitter compounds[29,55-57]. Bitter-sensing neurons also express nociceptors of the transient receptor protein family, such as TrpA1 and painless, which mediate responses to reactive electrophiles[27,114,115]. Previous studies have identified additional classes of taste neurons that detect osmolarity[45,116], salts[41], carbonation[46], and pheromones[28,117-119]. However, receptors for acids have not yet been identified.

Taste receptor cells that are selectively activated by acidic, or sour, stimuli have been found in mammals. These cells are labeled by polycystic kidney disease-like channels, PKD2L1 and PKD1L3, and are necessary for physiological and behavioral responses to acidic stimuli[43,61]. However, mice lacking PKD2L1 and PKD1L3 show only a partial reduction in acid sensitivity[60], indicating the presence of other molecular acid sensors in these taste cells. A more recent study showed that responses to acidic stimuli are mediated by

proton conductance, which is blocked by zinc but not by a range of other agents that are known to block proton channels[120].

Previous studies in the blowfly identified excitatory responses to a panel of carboxylic acids in a neuron that was tentatively identified as a deterrent neuron[66]. Carboxylic acids have also been shown to inhibit responses of sweet and salt sensing taste neurons in larger fly species and in moth larvae[67-69,121], suggesting that this class of chemicals may be sensed by its activity on multiple categories of taste neurons. Acid tastants are found in common food sources of the fly, including over-ripe or rotting fruit[122], but whether or not the *Drosophila* gustatory system detects acids has not yet been investigated.

Here we have revealed the cellular basis of acid taste in *Drosophila*. Common carboxylic acids are detected by bitter taste neurons and have inhibitory affects on feeding behavior in *Drosophila*. Using a panel of fruit carboxylic acids we find that flies reject acidic stimuli in a pH-dependent manner. Electrophysiological analysis reveals that this class of tastants is detected by a subset of bitter taste neurons. Flies in which bitter taste neurons are genetically silenced have a loss of pH detection showing that bitter taste neurons play a role in the rejection of acids. Further characterization with standardized hydrochloric acid solutions suggests that these neurons sense acidic pH via an unidentified receptor. Interestingly, a bitter compound, caffeine is inhibited when mixed with decreasing pH. Acid detection by a subset of bitter neurons contributes a mechanism for sensing acid in foods that may be perceived separately to

canonical bitter compounds to convey the palatability of acidic food sources.

Methods:

Fly Stocks

Flies were raised on standard cornmeal-dextrose-agar diet at 25°C. Wild-type flies were w^{1118}. Mutant fly stocks were obtained from the Bloomington Stock Center: Ir mutant screen: *Ir64a*[MB05283] (BL24610), *Ir25a*[MB09568] (BL27789), *Ir100a*[EP-G19846] (BL31853), *Ir48a*[MB09217] (BL26453), *Ir48b*[MB02315] (BL23473), *Ir51b*[PBc00387] (BL10046), *Ir10a*[MB03273] (BL23842), *Ir31a*[PBf06333] (BL18963), *Ir84a*[GAL4] (BL41750).
Gr, Trp, Task, ppk other candidates: *Gr36c*[MB09703] (BL26496), *CG14605*[MB08700] (BL26399), *Task7*[PBf05437] (BL18864), CG13500[MB03392] (BL23844), *CG2604*[EPey05974] (BL15814), $TrpA1^{1}$ (BL26504), *painless*[EP2621] (BL28799), $Pkd2^{1}$ (BL24495), $Gr33a^{1}$ (BL31427), *Task6*[EPey23668] (BL22651) and *Ork1*[XPd09258] (BL19309).
Pickpocket mutants: *ppk12*[MB11059] (BL29179), *ppk8*[MI01460] (BL34417), *ppk19*[MI02888] (BL36434), *ppk23*[BG01654] (BL12571), *ppk29*[PBf06838] (BL19016), *ppk20*[MB01352] (BL23071), *ppk11*[MB02012] (BL23781), *ppk27*[MB00592] (BL22847), *ppk*[MI04968] (BL38075), *ppk18*[MB07822] (BL25571), *ppk16*[MB11536] (BL27869), *ppk5*[EPey00388] (BL15022), *ppk31*[MI06637] (BL41105) and *ppk28*[EPg981] (BL33559). *UAS-Kir2.1* flies were kindly provided by K. Scott (University of California, Berkeley). DGRP lines were obtained from Bloomington Stock Center #'s tested are shown in (**Figure**

4.6).

Solutions

Tastants were obtained from Sigma Aldrich at the highest purity available. Organic acid-containing solutions were prepared on the day of use. Standardized pH solutions with HCl were stored for one week at room temperature.

Behavior

Feeding preference assays: Adult flies aged 3-7 days were sorted and housed in fresh food vials for 1-2 days; one vial was prepared for every trial and contained 10 males and 10 females. Flies were then starved for 24 hours in vials with water-soaked Kimwipes. Feeding assay plates were prepared a few hours before the experiment and dotted with 9 10μl-spots of each stimulus solution in 0.75% agarose[91]. Tastants were mixed in with melted agarose and dispensed immediately. Flies were anesthetized momentarily with CO_2, transferred to feeding plates, and allowed to feed for 2 hours in a dark, humidified chamber, after which they were frozen and scored within 48 hours for the color of their abdomens.

Preference index was calculated as:

$$\frac{\#pink - \#blue}{\#pink + \#blue + \#purple}$$

Participation was calculated as:

$$\frac{\#pink + \#blue + \#purple}{\#pink + \#blue + \#purple + \#uncolored}$$

Electrophysiology

Single sensillum recordings were performed using the tip-recording method [123]using 30 mM tricholine citrate as the electrolyte[85]. Recordings were obtained from male flies aged 3-10 days. In every case, a positive control of caffeine or lobeline, as appropriate, was tested before and after recordings with acid tastants. For recordings with standardized pH solutions, each sensillum was first tested with electrolyte alone as a control (pH 6.65), followed by HCl solutions of pH 2–6 in descending order. Neuronal responses were quantified to spikes/second by doubling the number of spikes in the 0-500 ms window upon contact with the stimulus.

Statistical analysis

Unless otherwise indicated, for behavior experiments arcsine-transformed data were analyzed using 2-way ANOVA and Tukey's *post hoc* analysis or pairwise comparisons. Electrophysiology data were analyzed with 2-way ANOVA followed by pairwise comparisons to determine significance at single concentrations. For all graphs, error bars indicate s.e.m.

Results:

Flies reject carboxylic acids in taste behavior assays

To identify taste responses to acids, we examined behaviors to four carboxylic acids that are found in fruit and vinegar. As acids were previously shown to inhibit sugar and salts responses when mixed[67] we hypothesized that acids would have an inhibitory behavioral affect when mixed with sugars in *Drosophila*. We therefore tested if the presence of acids could suppress acceptance of sucrose. We found that all four acids were able confer taste aversion when mixed with sucrose using a feeding preference assay. In a series of binary choice experiments in which flies were tested for preference between 1 mM sucrose and mixtures of 5 mM sucrose with acids, we found that sucrose-acid mixtures were rejected as the concentration of acid was increased (**Figure 4.1a**). Interestingly, feeding preference strongly correlated with pH of the tastant mixtures (**Figure 4.1b**), raising the possibility that the fly gustatory system may sense free proton concentration, as is observed for mammalian sour taste cells[120].

Carboxylic acids activate bitter taste neurons

We next sought to identify if there were taste neurons responsible for acid recognition. Although taste neurons are located in a number of external and internal taste organs, we focused on the labellum, which is the best-characterized taste organ with respect to identification of individual taste hairs[124],

and their molecular and functional properties[13,53]. Each taste hair in the labellum can contain up to four gustatory neurons, of which one is tuned to sweet compounds and a second to bitter compounds[37,53,103]. Previous studies have shown that activation of bitter neurons drives behavioral taste aversion[33,38,73]. Given that acids are rejected in feeding choice assays, we tested the possibility that they directly activate bitter neurons. We surveyed responses of previously defined bitter sensilla of the labellum[53] to each of the four acids, which were tested at three different concentrations: 0.1%, 1%, and 10%. We also tested 10 mM caffeine (S-a, S-b, and I-b classes) or lobeline (I-a class) as positive controls for bitter neuron activation. We observed robust, concentration-dependent responses to carboxylic acids in S-b and I-b sensilla, which represent two of the four classes of labellar sensilla that house bitter-sensing taste neurons[53]. Our recordings revealed a neuron that fired in response to acids with spike amplitudes comparable to that seen in response to caffeine (**Figure 4.2a**). Importantly, acid application did not significantly affect subsequent responsiveness to caffeine, indicating that acidic tastants were not damaging taste neurons in the sensillum (**Figure 4.2b**). Stronger responses were elicited in the S-b class as compared to the I-b class, but in both cases the responses increased with higher acid concentrations (**Figure 4.2c**). Consistent with the results of the feeding choice experiments, the neuronal firing rates in S-b and I-b sensilla were inversely correlated with pH of carboxylic acid tastants (**Figure 4.2d**). Responses were also observed from the other bitter sensilla, S-a (**Figure**

4.2e) and I-a (**Figure 4.2f**), but they were generally weaker and did not exhibit consistent concentration dependence or correlation with acidic pH (**Figure 4.2e,f**), suggesting that they play little if any role in acid detection.

Subsets of bitter neurons are pH sensors

Given the relationship of taste neuron responses to pH of carboxylic acids, we wanted to determine whether S-b and I-b sensilla could sense low pH. We therefore performed recordings using standardized hydrochloric acid (HCl) solutions at pH 2–6, as well as control 30 mM tricholine citrate electrolyte alone, which read at about pH 6.65 (**Figure 4.3**). We also tested the responses of S-a and I-a sensilla, which did not show strong responses to carboxylic acids, as well as L-type sensilla that lack canonical bitter neurons[53] to verify lack of pH activation in them. Overall, our results reveal a defined sub-population of bitter taste neurons that detect acidic pH.

First, our recordings confirmed that neurons in S-b and I-b sensilla were activated by acidic pH (**Figure 4.3a,b**). Second, our analysis showed that S-a and I-a sensilla were unresponsive to acidic pH, corroborating heterogeneity in pH sensitivity across sensillar classes (**Figure 4.3a,b**), as has been observed for bitter compounds[53]. Third, we found that acidic pH solutions were not able to activate neurons in L-type sensilla (**Figure 4.3b**), supporting the involvement of bitter taste neurons that are absent in these sensilla. Indeed, genetic silencing of all labellar bitter taste neurons by expressing an inwardly rectifying potassium

channel, Kir2.1, under the control of *Gr89a-GAL4*[53] resulted in loss of pH responses in S-b and I-b sensilla ($P < 0.001$; **Figure 4.3c**), but had no effect on the weak pH response in S-a ($P = 0.126$; **Figure 4.3c**). Thus, acid taste appears to be encoded by a subset of bitter neurons in the periphery.

Caffeine response is inhibited by acidic pH

We next investigated if there were differences in the mechanism of detection between canonical bitter compounds verses pH detection. To test this hypothesis we analyzed bitter neuron activity during caffeine alone verses caffeine mixtures with decreasing pH. We find that pH inhibits caffeine detection in both S-b and S-a bitter neurons (**Figure 4.4a**). Caffeine (10mM) elicits ~75 spikes/second in S-b sensilla and pH 2 also activates this neuron, but to about half as much ~35 spikes/second. We observed that caffeine-pH mixtures in S-b bitter neuron approach pH 2 responses alone as we decreased pH (**Figure 4.4b**). We hypothesize that the remaining response at caffeine at pH 2 is primarily due to pH 2 alone, however it is possible that this bitter neuron is firing to both caffeine and pH 2. S-a bitter neurons respond to caffeine (~30 spikes/second), however have little to no response to pH alone (**Figure 4.3**). Moreover, when we mixed decreasing pH with caffeine and assayed bitter neuron activity in S-a sensilla, we observed a dramatic decrease of caffeine response (**Figure 4.4b**). Our data suggests that acidic pH inhibits bitter neuron activity in the labellum, which has also been observed in sugar and salt taste

modalities[67-69,121].

Acidic pH responses in candidate taste receptor mutants

To investigate the molecular mechanism of pH detection in bitter taste neurons, we examined pH responses of S-b sensilla in mutant flies lacking candidate receptor genes. We set out to test receptor families that are associated with aversive compound detection or that lead to aversive behavior.

Notably receptors in the ionotropic receptor (Ir) family were tested as *Ir64a* mutants, in the olfactory system, is necessary for acid detection. *Ir64a* may be necessary for acid sensing in olfactory neurons[22], but we found no reduction in the sensitivity of their taste neurons to acidic pH (**Figure 4.5a**); in fact there was a small but significant increase in the level of acidic pH response, which may be attributable to differences in the genetic background. We also investigated genes in the Gr family as bitter receptors are involved with aversive behaviors. Notably, similar results were obtained for mutants lacking Gr33a, a receptor that is broadly required for responses to various noxious tastants[29]. Genes from the transient potential receptor (Trp) family were tested as *painless*, a cation channel is involved in aversion to wasabi[114]. Another potential pH receptor candidate from the Trp family was TrpA1[125], which is expressed in bitter taste neurons in *Drosophila*[27,115]. However pH responses of *TrpA1* mutants were also not significantly different from those of wild type controls (**Figure 4.5b**). Recordings from flies lacking a PKD-like gene *Pkd2*, related to PKD1L3 and PKD2L1[43,61]

channels that are expressed in mammalian sour taste cells showed that it is not necessary for pH-dependent activity in S-b sensilla (**Figure 4.5b**). We also tested a family of pickpocket genes, which are implicated in water taste detection[45] and shown to be pheromone receptors in male-male avoidance behavior[28] (**Figure 4.5c**). Various other acid detecting receptors were tested for their involvement of acidic pH detection with no overt differences in acidic pH detection. TWIK-related acid-sensitive K^+ channels (Task) 6 and 7 and Open rectifier K^+ channel 1, (Ork1) were evaluated (**Figure 4.5b)**. Task genes, which by name have been shown to be involved in sensing pH changes[126] and it is not known whether pH effects Ork1 function. Our mutant analysis did not yield a receptor(s) that mediate acidic pH responses.

A recent study showed that acid response in mammalian sour taste cells is inhibited by zinc chloride but not by amiloride hydrochloride[120]. However, we found that pH-evoked response in *Drosophila* S-b sensilla was refractory to both zinc ion (P = 0.074 for 1 mM, P = 0.267 for 10 mM, Student's t-test) and amiloride (P = 0.948, ANOVA) (**Appendix Figure A1**). Together with our mutant analyses, these results suggest that acidic pH is detected by a novel receptor that is expressed in a subset of bitter taste neurons.

Drosophila Genetic Reference Panel (DGRP) exhibits variation in acidic pH bitter neuron responses

A Drosophila Genetic Reference Panel (DGRP) screen was undertaken to

search for loss of function to acidic pH detection in male *Drosophila melanogaster* S-b bitter neurons. DGRP fly lines were developed to screen few fly lines, but over large regions of genome to connect genetic changes with variation in behavioral output of interest[127,128]. We decided to use this screening strategy to measure the activity of the acid-activated S-b neurons. Each DGRP fly line contains many single nucleotide polymorphisms, which can affect many genes throughout the whole genome[127,128]. We screened thirty of the forty core lines, which contain the most DGRP variation to screen for fly lines that have loss in acidic pH detection. Each of the DGRP fly lines assayed was first tested with caffeine, bitter neuron positive control for bitter neurons, followed by pH 4, 3, and 2 and lastly a second caffeine response to confirm normal bitter neuron activity after possible acid insult. Our results showed a variation in acidic pH detection, in comparison to wild type responses (**Figure 4.6a**). We observed both high responders and low responders, shown in red and yellow to acidic pH with wild type-like response shown in black. To our surprise we did identify a candidate DGRP candidate #25181, in red, that had a wild type caffeine response, but a loss in acidic pH detection (**Figure 4.6b**). This result suggests there are genes that can be discovered that are possibly responsible for acidic pH detection. Overall this finding demonstrates the separation between acid and caffeine detection and therefore strongly suggests different molecular mechanisms for bitter versus acid activation in S-b aversive neurons.

Discussion:

We provide evidence of a cellular detection mechanism of acids in *Drosophila*. Acids are detected in a subset of bitter neurons in the labellem and detection is pH dependent. Unlike in mammals, where a separate cell type[43] from bitter detecting cells responds to sour/acids, *Drosophila*, commandeers bitter neurons for acid detection. Our data demonstrates that acid detection in bitter neurons contributes to acid avoidance in feeding preference assays[129]; however, Charlu et al. 2013 also shows that sugar neurons are inhibited by acids[67,129], which also leads to avoidance of acid-laced foods[129]. We propose a model that includes both acid activation of bitter neurons and inhibition of sugar neurons, allowing flies to assess and modulate the risk-to-reward of ingesting acidic food sources.

We provide evidence that flies have different mechanisms to detect acids verses general bitter compounds, such as caffeine. Caffeine when mixed with decreasing pH inhibits bitter neuron responses. Chen et al 2014 also observes similar acid-bitter inhibition in the legs of *Drosophila*. Their observation of acidic inhibition of bitter suppression of sugar response in sugar-acid-bitter mixtures, simulating natural food sources, provides a hypothesis that acids allow flies to eat bitter-laced foods.

Inhibitory mechanisms of acid inhibition of sugar and activation of bitter GRNs are detected and signal appropriate feeding behaviors[129]. A study by Chen et al. 2014 proposes a separate mechanism of acid detection in the tarsal GRNs.

They show by calcium imaging techniques that there is no activation of bitter GRNs by acids in the legs[130]. It could be possible that bitter GRNs in the leg are non-acid-activating neurons similar to what we observe in S-a and I-a type sensilla in the labellum[129]. Additionally Chen et al. 2014, demonstrated acids inhibit bitter GRN activation in tarsi. We also observe this phenomenon in the labellum (**Figure 4.4**), suggesting similar inhibitory mechanism of bitter compounds by acids in both labellar and tarsal bitter neurons. However, Chen et al. 2014 did not observe sugars to be inhibited by acids at tested pH 3. We believe these results to be explained by the concentration of acids that were tested, at pH 3 we also observe ~60% of flies preferring acid-laced 5mM sucrose concentration[129]. Interestingly, Chen et al. 2014 also observes that acids inhibit bitter suppression of sugar attraction behavior, which could provide evidence for a critical acidic range. This range could account for fly's ability to feed on sugar-bitter-acidic fermenting foods, like beer, which might otherwise be avoided[130]. However, to test this hypothesis we would need to preform recordings and quantify sugar-bitter-acid mixtures in labellum GRNs.

Is the inhibitory action of acidic pH a general mechanism to neurons or cells? Chen et al. 2014 predicts acidic pH affects odor binding protein conformation, causing loss of bitter suppression. However, because acidic pH inhibition is seen in sweet, bitter, and salt activated neurons, we postulate that acidic pH may act in a more general mechanism as OBP49a was only seen to affect inhibition of sugar responses. Overall these results indicate flies use fine

scale control from bitter/acid inhibition of sugar neuron activity at the periphery. This meticulous control of sugar neuron firing suggests an overall activity "threshold" that would need to be met for feeding. Further fine scale characterization of how overall sugar input leads to feeding behavior[150] would be necessary to test how possible inhibitory mechanisms affect feeding.

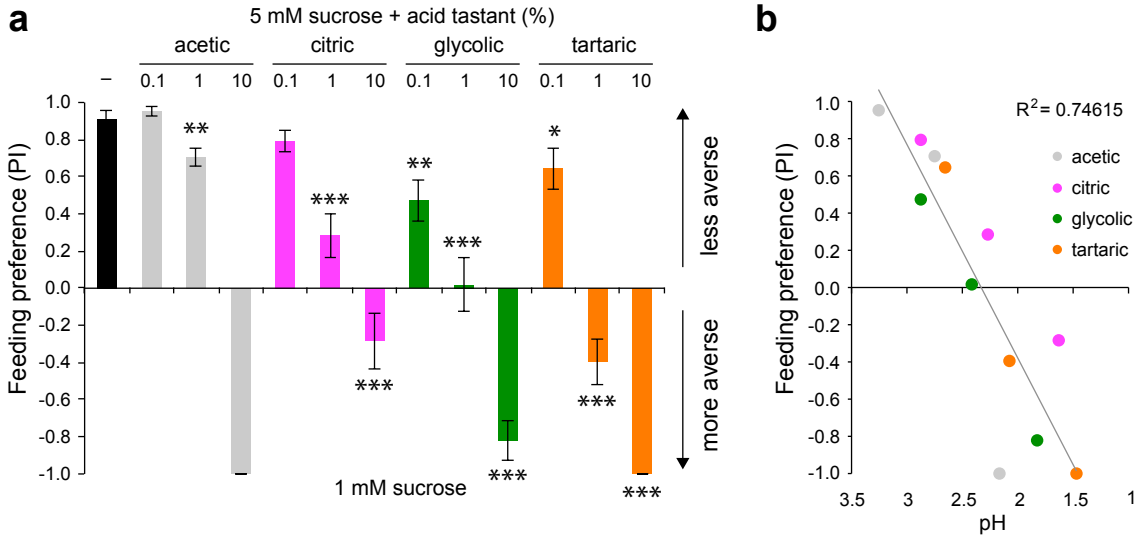
Additionally our DGRP analysis also provides evidence that pH detection can be separated from bitter activity in bitter neurons. This data suggests that there is a separate molecular mechanism for the detection of acids and bitter compounds, which raises the possibility that flies may be able to distinguish between acids and bitter compounds.

Sour taste receptors have not been characterized in any organism. Mammalian PKD2L1 and PKD1L3 have been associated with acid responsive cells, however neither of these receptors is necessary for acid detection[43,61]. We investigated different receptor families in *Drosophila* proposed from previous studies to be involved in bitter and nociceptive detection, or pH sensors in other tissues. A member of the transient receptor potential (Trp) family, TrpA1, is expressed in acid-responsive trigeminal nociceptive neurons. In this instance, the relevant excitatory cue for TrpA1 appears to be intracellular acidification caused by weak acids such as acetic acid[125]. A more recent pharmacological analysis implicates the presence of Zn^{2+}-sensitive receptor that is activated by free protons in sour taste cells (**Appendix Figure A1a**)[120], but a candidate receptor protein has not been found. Our results support the view that the fly taste

receptor for acidic pH is not among those with previously identified roles in detecting noxious tastants, which include the Gr33a, TrpA1, and painless receptors. Moreover, the activity of the fly pH receptor is not blocked by amiloride (**Appendix Figure A1b**), an inhibitor that broadly affects acid-sensing ion channels (ASICs)[131], or Zn^{2+} (**Appendix Figure A1a**). Our finding that low pH response is specific to a sub-population of bitter neurons, which offers the means to identify novel receptors that are restricted to those cells.

Recent studies expose an increasingly sophisticated role of sensory neuron function and communication in encoding the context of chemical stimuli. Such mechanisms break the neat compartmentalization of sensory cells at the periphery and have been found to act in both cell-autonomous and non-autonomous ways. For example, the response of the cell to one stimulus can be modified by the presence of other stimuli[132]. Also, an activated cell can influence the firing frequency of other cells in the same grouped unit by non-synaptic communication[133]. Charlu et al 2013 reports that sweet neuron activity reflects the concentrations of both sugar and acid tastants, independent of acid-evoked excitation of bitter neurons. Such integration of information about different taste categories at the periphery may allow the fly to quickly calibrate the risk of ingesting nutritious foods that contain potentially harmful substances without the involvement of central processing mechanisms. Whether the inhibition of sweet neurons by acid tastants occurs by direct action on sweet taste receptors or via more general mechanisms remains to be determined. It is possible that

carboxylic acids also act on other classes of taste neurons such as the salt- or water-sensing neurons. Nonetheless, an independent role for an acceptance taste neuron in sensing acid tastants suggests a mechanism for facilitating precise evaluation of mixed stimuli within the constraints of a labeled-line coding system[129]. The fly might use such mechanisms to better calculate the risk of consuming tainted food sources, which might otherwise be avoided irrespective of their nutritious value.


a
5 mM sucrose + acid tastant (%)
acetic
citric
glycolic
tartaric
–
0.1
1
10
Feeding preference (PI)
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
**
***
***
**
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*
***
***
1 mM sucrose
less averse
more averse
b
R2= 0.74615
acetic
citric
glycolic
tartaric
Feeding preference (PI)
pH
3.5
3
2.5
2
1.5
1

Figure 4.1: Common fruit acids inhibit Drosophila taste behaviors.

(**a**) Results of binary choice assays using indicated mixed stimuli tested against 1 mM sucrose. n = 10 (sucrose control), n = 6–10 (acetic), n = 16–17 (citric), n = 7 (glycolic), n = 7 (tartaric). *P < 0.05, **P < 0.01, ***P < 0.001, versus 5 mM sucrose, Student's t-test. For each acid tastant series independent trials were performed on 2–5 days. (**b**) Scatter plot of behavioral data in (**a**) against pH of the stimulus mixtures. Two pH measurements were taken and averaged for each stimulus solution. Linear best fit and R^2 value were calculated in Excel. Error bars = s.e.m.

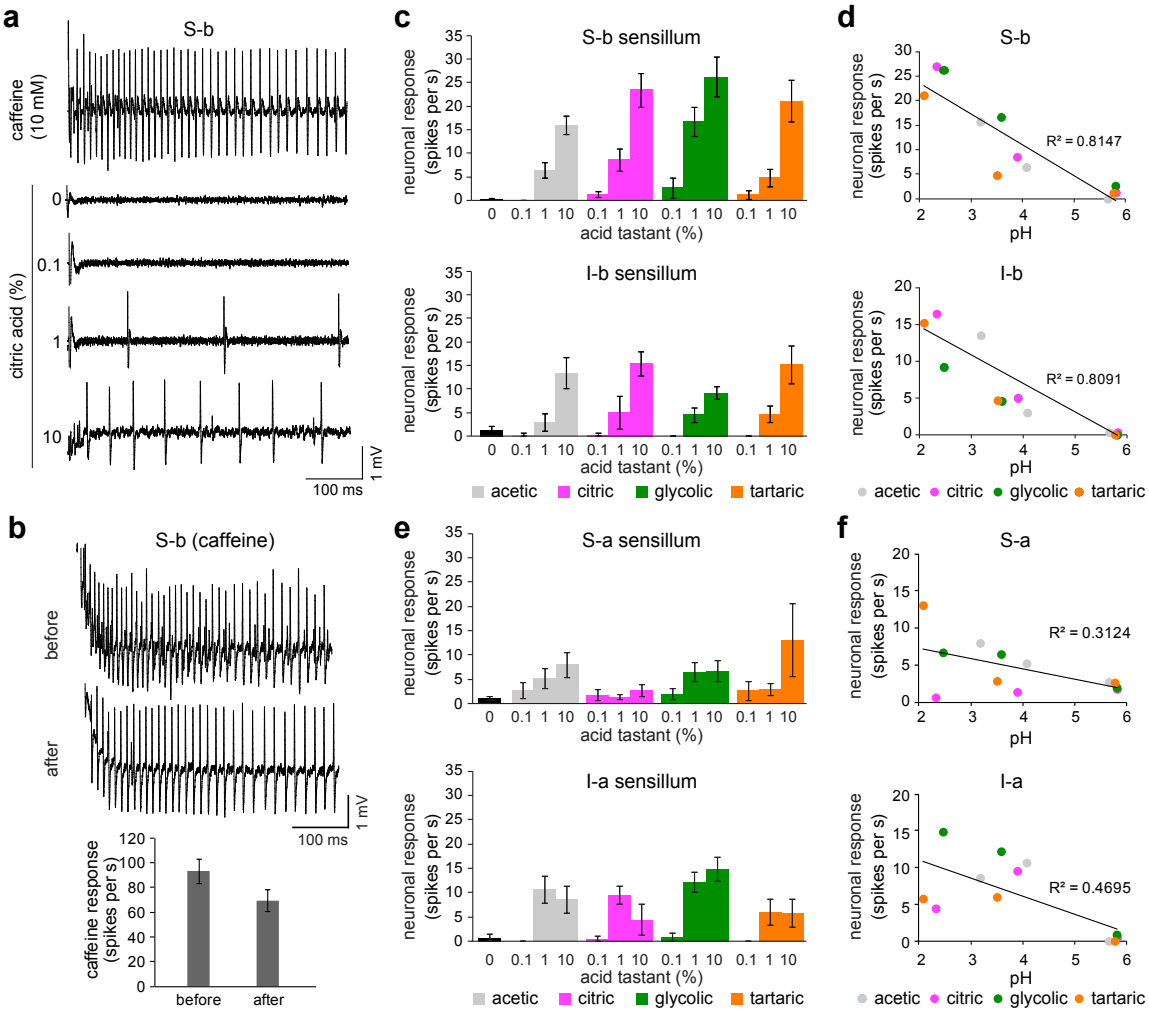

a
S-b
caffeine (10 mM)
citric acid (%)
0
0.1
1
10
100 ms
1 mV
b
S-b (caffeine)
before
after
100 ms
1 mV
caffeine response (spikes per s)
before
after
c
S-b sensillum
I-b sensillum
neuronal response (spikes per s)
acid tastant (%)
acetic
citric
glycolic
tartaric
d
S-b
I-b
neuronal response (spikes per s)
pH
R² = 0.8147
R² = 0.8091
e
S-a sensillum
I-a sensillum
f
S-a
I-a
R² = 0.3124
R² = 0.4695

Figure 4.2: Fruit acid tastants activate a subset of bitter neurons. **a.** Representative traces of recordings obtained from S-b labellar sensilla of wild type flies. (**b**) Representative 10 mM caffeine traces and mean firing rates of the bitter neuron obtained before and after stimulation with acid tastants. n = 9. P = 0.305, Student's *t*-test. (**c**) Mean responses of S-b and I-b classes of bitter-responsive sensilla to indicated acid tastants and 30 mM tricholine citrate electrolyte control (0). S-b: n = 30 ('0' control), n = 9–14 (acetic), n = 10–11 (citric), n = 8–9 (glycolic) and n = 10–11 (tartaric). I-b: n = 22 ('0' control), n = 6–12 (acetic), n = 6–10 (citric), n = 7–10 (glycolic) and n = 9–10 (tartaric). For each sensillum-stimulus series, independent recordings were acquired over 2–6 days. (**d**) Scatter plots of mean responses of indicated sensilla to acid tastants tested at 0.1%, 1% and 10% against pH of the stimulus mixtures. (**e**) Mean responses of wild-type S-a and I-a classes of bitter-responsive sensilla to indicated acid tastants and 30 mM tricholine citrate electrolyte alone (control). S-a: n = 29 ('0' control), n = 8–14 (acetic), n = 10–14 (citric), n = 8–11 (glycolic) and n = 9–11 (tartaric); I-a: n = 7 ('0' control), n = 9–10 (acetic), n = 8–9 (citric), n = 9 (glycolic) and n = 9 (tartaric). For each sensillum-stimulus series, independent recordings were acquired over 2–6 days. (**f**) Scatter plots of mean responses of indicated sensilla to acid tastants tested at 0.1, 1 and 10% against pH of the stimulus mixtures. Three independent pH measurements were taken and averaged for each stimulus solution for all correlations. All best fit lines and R^2 values were calculated in Excel. Error bars = s.e.m.

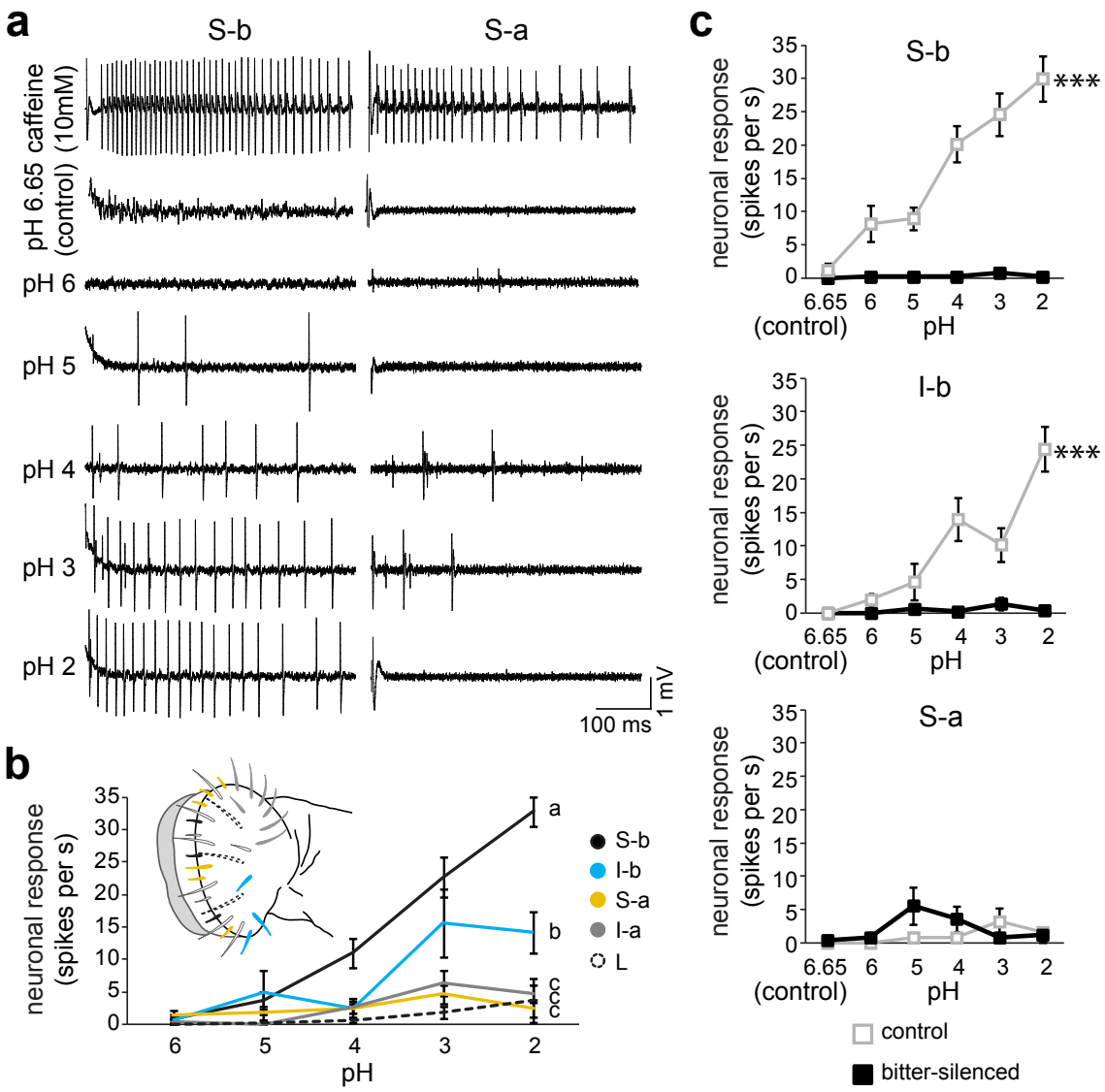

a
S-b
S-a
pH 6.65 caffeine (10mM)
(control)
pH 6
pH 5
pH 4
pH 3
pH 2
1 mV
100 ms
b
neuronal response (spikes per s)
pH
S-b
I-b
S-a
I-a
L
a
b
c
c
c
c
S-b
I-b
S-a
***
***
(control)
control
bitter-silenced

Figure 4.3: Bitter taste neurons are activated by acidic pH. (**a**) Representative traces of HCl responses obtained from S-b and S-a sensilla of wild-type flies. (**b**) Mean responses of five classes of labellar sensilla to solutions of HCl at indicated pH; corresponding sensillar classes are indicated in the schematic of the labellum. n = 12 (S-b), n = 9 (I-b), n = 9 (S-a), n = 5–10 (I-a) and n = 10 (L). Lines with different letters are significantly different, $P < 0.001$ (S-b), $P < 0.001$ (I-b), $P = 0.861$ (S-a), $P = 0.478$ (I-a) versus L-type, two-way ANOVA with Tukey's post hoc analysis. (**c**) Mean responses to 30 mM tricholine citrate electrolyte alone (pH 6.65, control) and standardized HCl solutions in electrolyte (pH 2–6) of indicated sensilla in control (*Gr89a-GAL4/ + ; + /TM3*) and bitter-silenced (*Gr89a-GAL4/ + ;UAS-Kir2.1/TM3*) flies. Control: n = 12 (S-b), n = 9 (I-b) and n = 5 (S-a); bitter-silenced: n = 10 (S-b), n = 10 (I-b) and n = 5 (S-a). ***$P < 0.001$, two-way ANOVA with pairwise comparisons. Error bars = s.e.m.

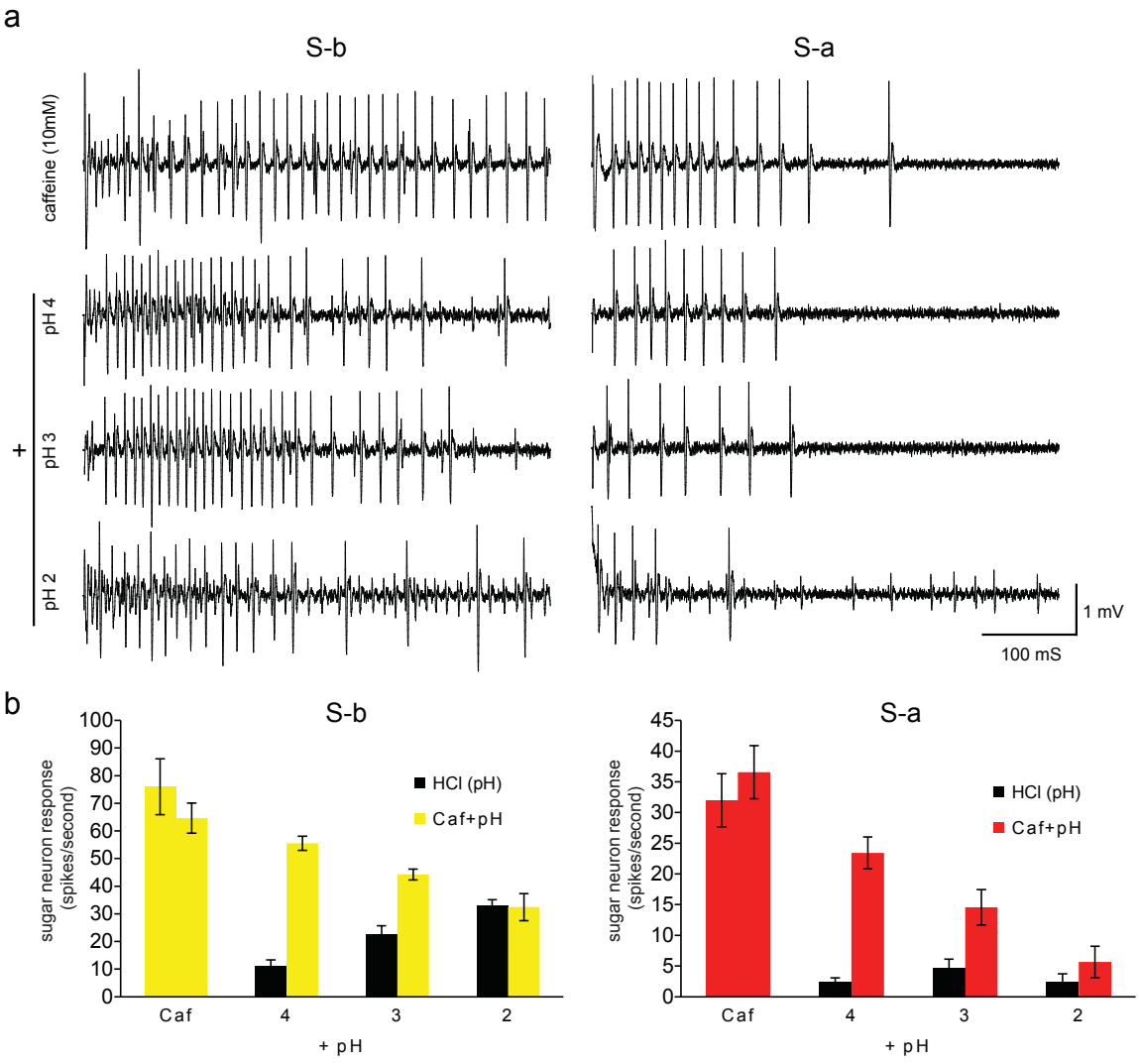

a
S-b
S-a
caffeine (10mM)
+
pH 4
pH 3
pH 2
1 mV
100 mS
b
S-b
S-a
sugar neuron response (spikes/second)
HCl (pH)
Caf+pH
Caf
4
3
2
+ pH

Figure 4.4: pH inhibits caffeine response in acid activated and non-acid activated bitter neurons. (**a**) Representative traces for caffeine alone and mixed with decreasing pH in S-b (acid activated) and S-a (non-acid activated) bitter neurons. (**b**) Mean responses of S-b (yellow) and S-a (red) bitter neurons to caffeine alone (Caf) or with decreasing pH with pH alone (black) for comparison. Error bars = s.e.m.

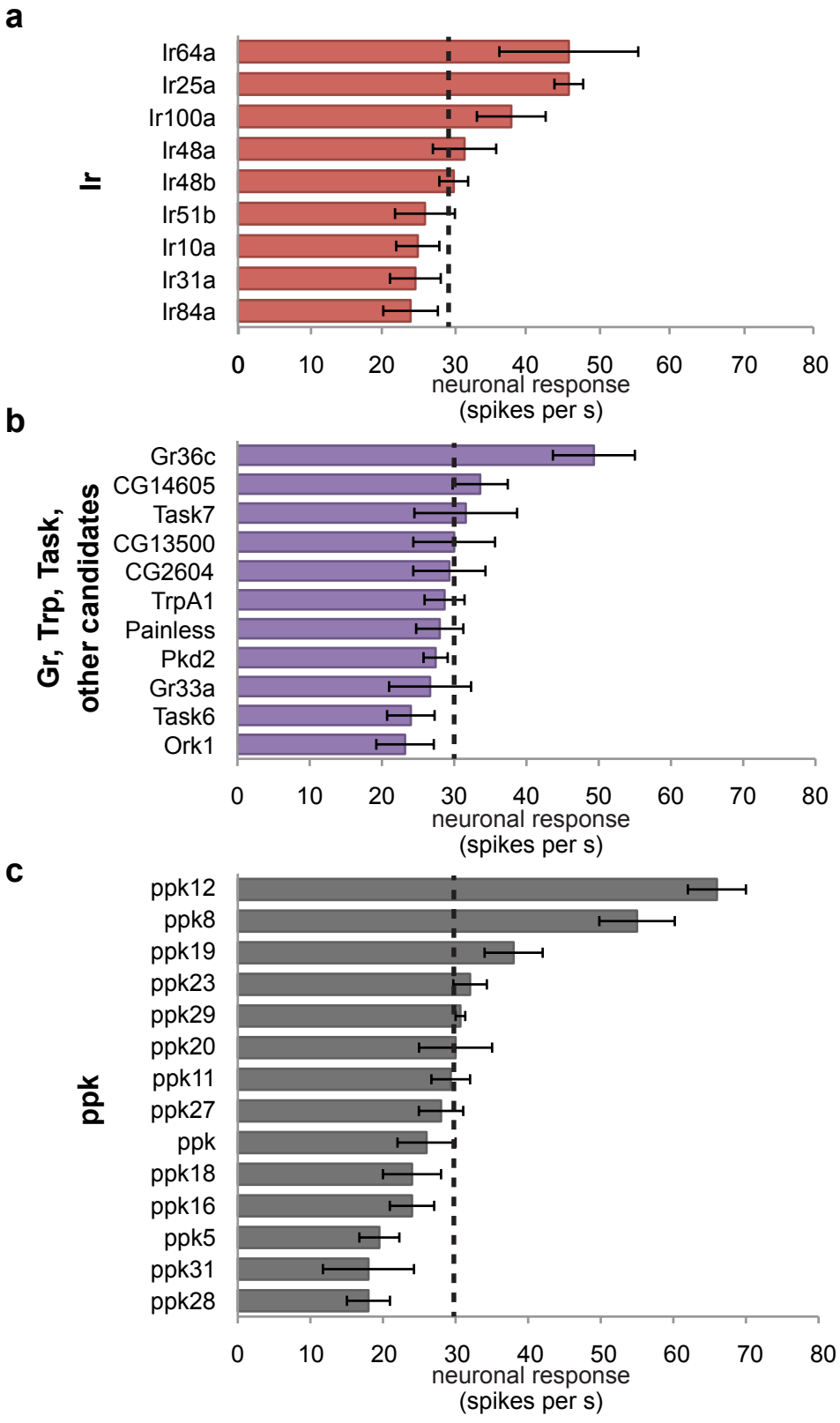

a
Ir
Ir64a
Ir25a
Ir100a
Ir48a
Ir48b
Ir51b
Ir10a
Ir31a
Ir84a
0 10 20 30 40 50 60 70 80
neuronal response
(spikes per s)
b
Gr, Trp, Task, other candidates
Gr36c
CG14605
Task7
CG13500
CG2604
TrpA1
Painless
Pkd2
Gr33a
Task6
Ork1
0 10 20 30 40 50 60 70 80
neuronal response
(spikes per s)
c
ppk
ppk12
ppk8
ppk19
ppk23
ppk29
ppk20
ppk11
ppk27
ppk
ppk18
ppk16
ppk5
ppk31
ppk28
0 10 20 30 40 50 60 70 80
neuronal response
(spikes per s)

Figure 4.5: Response to low pH is not affected in a subset of receptor mutants **a.** Mean pH responses of S-b sensilla in (**a**) Ir mutants (red), (**b**) Grs, Trp, channel proteins (purple), and (**c**) pickpocket mutants (grey). Genotypes are further described in methods. The black dotted line indicates wild-type (w^{1118}) No significant differences were observed ($P > 0.05$). One-way ANOVA and Dunnett's *t* tests were performed comparing all responses to wild type.

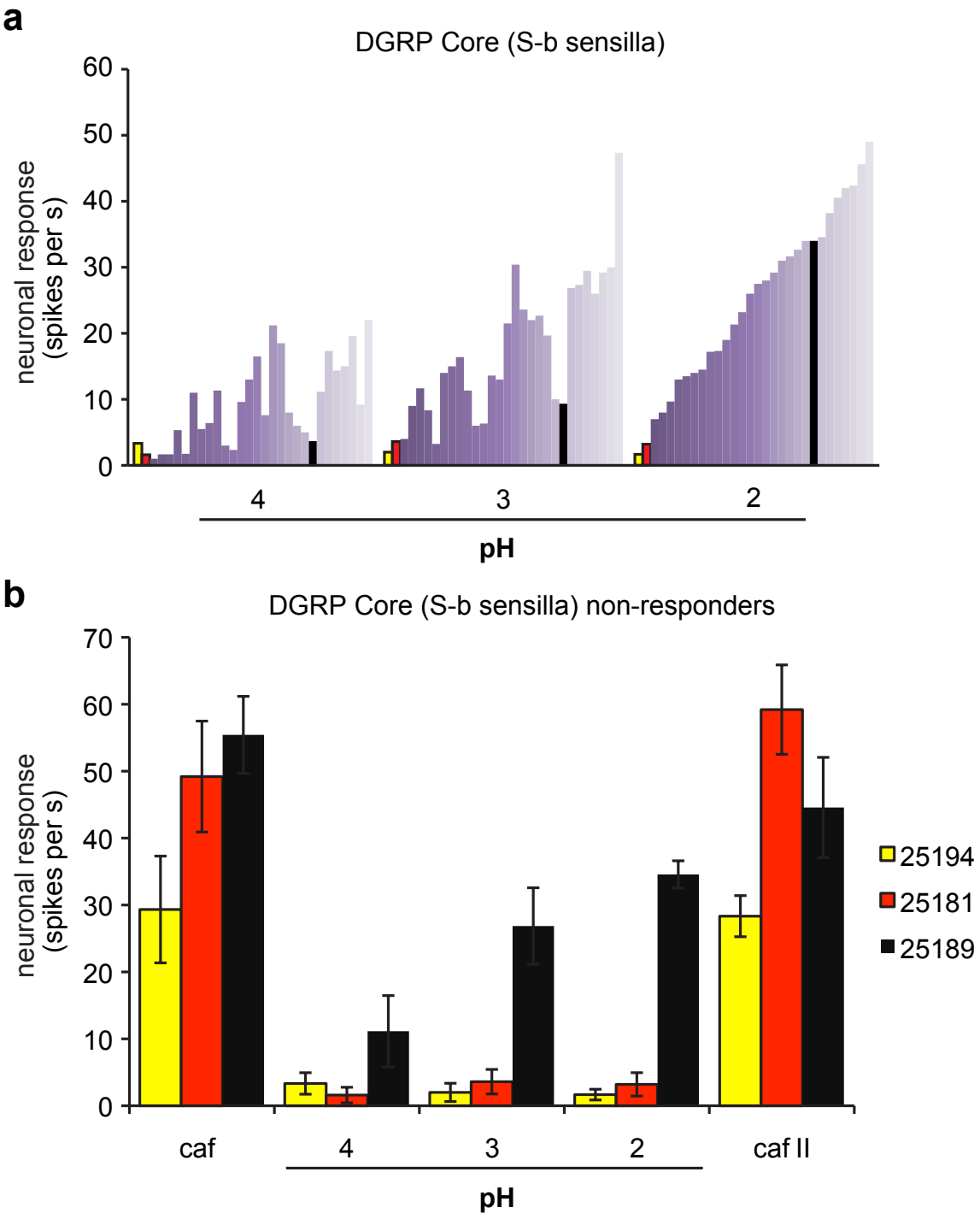

a
DGRP Core (S-b sensilla)
neuronal response
(spikes per s)
60
50
40
30
20
10
0
4
3
2
pH
b
DGRP Core (S-b sensilla) non-responders
neuronal response
(spikes per s)
70
60
50
40
30
20
10
0
caf
4
3
2
caf II
pH
25194
25181
25189

Figure 4.6: Drosophila Genetic Reference Panel (DGRP) pH screen. (**a**) Thirty core lines (represented by each bar) were tested for caffeine control (not shown), followed by pH 4, 3, and 2, n = 3-11 and n > 2 flies per genotype. Black shows wild type-like responder DGRP #25189 (control), red and yellow show non-responders lines DGRP #25194 and #25181 respectively. (**b**) Same data shown in (**a)** with responder (black) and non-responder (#25194, yellow and #25181, red) lines responses to caffeine controls (before and after pH stimulation) pH 4, 3, and 2. Error bars = s.e.m.

Chapter 5:

General Discussion

The taste system from mammals to insects is vital for ingestion of nutritious foods and avoidance of harmful or toxic compounds. Flies are commonly seen feeding among yeast and bacteria-ridden ripe and rotten fruits. Fermented foods are staple for flies, in which they feed, find mates and oviposit, thus completing their life cycle. However, decomposing and fermenting fruit is a complex mixture of both attractive and aversive compounds. How do flies detect peripheral responses to natural food sources, which contain both attractive and aversive components? This dissertation examines the feeding behavior to attractive and aversive components of fermented mixtures e.g. beer and investigates how peripheral taste receptors are involved in detection and feeding behaviors.

Sugars, salts, amino acids, acids and bitter compounds are found in beer and so it is interesting that flies do not reject beer, but actually prefer it to sugar[91,134]. It stands to reason that detection of both attractive and aversive signals combine to elicit overall attraction to beer. Therefore we set out to investigate the neural basis for this sensory and behavioral response. As flies use various chemosensory senses to find and detect food sources (olfactory and gustatory), we proposed to test the hypothesis that *Drosophila* have attractive feeding behaviors to beer.

Attractive detection and behavior to compounds in the complex stimuli, beer

Sugars, salts, amino acids and carbonation are compounds found to be attractive to fly feeding behavior and in beer. Primarily, glucose, maltose and melizitose sugars are detected in beers, however the concentration are very low in pale ale beers 0.0008-0.002% (w/v)[79] such as Bass Pale Ale tested in our study. Additionally, salts and amino acids, found in beer, are both attractive to *Drosophila*[24,58]. Bass pale ale contains 24 mg of sodium per 12 oz. bottle, which when calculated comes to ~3 mM concentration. Flies are primarily attracted to sodium chloride[24], so it stands to reason that salt detection may play a role in attraction to beer. Amino acid concentrations are found to be ~ 27.4 nM in pale ale beer[135]. Even though there is no evidence for amino acid-activated neurons, behavioral evidence suggests that amino acids could influence positive feeding behavior for beer. Additionally, CO_2 elicits positive feeding responses in flies and interestingly to *E409-GAL4*[46] and *Gr64e-GAL4* labeled cells from our study overlap in the peg neurons of the labellum. It is entirely possible that CO_2 from beer could activate peg neurons, but it is also possible that glycerol in beer may also activated peg neurons[91].

Our study provides strong evidence in support of our hypothesis and demonstrates that *Drosophila melanogaster* does in fact have an attractive feeding behavior towards the fermented mixture, beer. We find this preference for beer based on a gustatory receptor for glycerol, Gr64e, which contributes to

differences in *Drosophila* species-specific feeding behaviors towards natural yeast food sources[91].

Aversive detection and behavior to compounds in fermented foods

Beer can taste bitter because it contains organic, alpha and beta acids, which are produced from hop resins that taste bitter to humans[80]. It has yet to be shown that flies detect alpha or beta acids. Additionally, organic acids are a common component in fruits and are also found in beer. Moreover, beer has an acidic pH ranging from 3.5-5[136]. Currently there is no molecular mechanism for acid detection. We hypothesized that flies are able to detect acids and this detection can modulate feeding behaviors.

We find that two mechanisms of detection, activation of bitter and inhibition of sweet neurons, can give flies the ability to assess whether to eat or reject acid-laced foods. Unlike mammals, which have a separate cell type for sour detection, acid detection in *Drosophila* is mediated by a subset of bitter neurons. This raises an important distinction between labeled-line coding for acid detection in mammals, but not in flies. Can *Drosophila* taste acids? Yes, but can they tell the difference between acidic and bitter compounds? Possibly, but our data suggest that flies do not contain an exclusive neuron for acid at the level of the peripheral neurons. However, it is possible that there could be differences in feedback mechanisms between consumption of bitters verses acids, which might signal separately in the brain similarly to how non-nutritive verses nutritive sugars

can be detected over time in feeding assays[137,138]. These hypotheses however have yet to be tested.

Mechanisms of signaling by aversive compounds

As *Drosophila* commonly feed on fermenting foods, such as beer,[139] they are exposed to sugar-acidic mixtures. We find that acids inhibit sugar detection along with activating bitter neurons[129]. Furthermore, among the rotting and fermenting fruits, bacteria and fungi colonize and grow producing bitter compounds that deter flies from feeding[140]. Bitter compounds have many mechanisms that inhibit sweet neurons and affect attractive feeding behaviors[103,139,141]. Recently it was demonstrated that odorant-binding protein 49a (OBP49a) is needed for bitter inhibition of sugar neurons. OBPs, found to be expressed in the support cells surrounding taste neurons, act via sugar neurons, as bitter neuron activity is not affected in OBP49a mutants[139]. Additionally, circuit control is also observed as bitter neurons activate GABAergic interneurons in the SOG that presynaptically inhibit sugar neurons[141]. These studies confirm bitter compound inhibition of sugar neuron activity and our findings of acid inhibition of sugar neuron firing may involve these mechanisms.

Acid detection is also an important cue for egg laying behavior[142] in which females will preferentially lay eggs in acetic acid or in acidic pH 3-2 ranges. This egg laying behavior seems to be partially mediated by gustatory input as mutants of poxneuro, where all external taste sensilla develop into mechanosensory

hairs, lose acetic acid egg laying preference[142]. Additionally, mutants for Gr66a, a bitter Gr, lose preference for egg-laying behavior on bitter compounds[143]. It is interesting to speculate that acid-activated bitter neurons could be involved in this behavior. Flies are observed to lay eggs in generally feeding-avoidance environments that contain acids[142], bitters [143], and ethanol as it is predicted to protect progeny from predation[144], bacterial and mold growth[140].

We performed a mutant screen to investigate involvement of aversive behavior related genes in acid detection, but this analysis has yet to yield any genes involved in acid detection (**Figure 4.5**). Further exploration has identified two fly lines that have wild-type bitter neuron responses to caffeine detection, but a dramatic loss in acidic pH detection. One candidate we have tested further is DGRP #25181 (**Figure 4.6**). We find the loss of pH response in DGRP #25181 F1 progeny to be X-linked (**Appendix Figure A3**). Additionally, a separate candidate, Sh^5 third chromosome mutant (111^3), has a large loss in pH detection and wild type caffeine response. Despite identifying these two mutants, we have yet to discover genes associated with acid detection and further experimentation is needed.

Evolution of peripheral neuron detection leads to changes in behavior

A large question in evolutionary biology is “do genes affect behavior?” To approach this question, studies focus on sensory systems and investigate the genetic mutations (ex: receptors) that underlie changes in peripheral neuron

detection, which leads to overt shifts in behavior. Sensory neurobiologists are only beginning to understand how peripheral neural circuitry plays a role in the evolution of behavior.

Feeding is essential for survival and as the majority of animals utilize their taste system to detect essential nutrients in food, in which the hypothesis could be made that diet and niche preference is largely influenced by taste receptor breadth of detection. The summary of sweet and umami detection in mammalian taste cells requires two heteromeric receptors: T1R2+T1R3 for sweet and T1R1+T1R3 for umami detection. Many studies performed in mammals have demonstrated that the loss of one of two taste receptors T1R1 or T1R2 when pseudogenized or lost leads to the loss of attractive detection to sweet or umami compounds. Cats have the ability to taste umami (savory) requiring T1R1 and T1R3 receptors, however, they lack the ability to taste sweet compounds due to a pseudogenization of the taste receptor *T1R2* gene[145]. Interestingly, other non-feline mammalian carnivores also contain pseudogenization of the*T1R2* gene, providing strong support in the notion that loss of *T1R2* function has played a role in shifting from an omnivorous diet to carnivorous diet[146]. However, the Giant Panda in clade *Carnivora*, feeds primarily on bamboo. This result is correlated to pseudogenization of *T1R1*, responsible for umami taste, in Pandas[147]. Further studies have correlated these results finding that of all major lineages of bats only vampire bat species have a pseudogenized *T1R2* gene[148]. These results support the hypothesis that taste receptors influence peripheral detection of

foods, which in turn leads to feeding behavior and finally niche specification. However, these hypotheses cannot confirm that loss of taste receptor genes cause diet or niche evolution. If that hypothesis were true all carnivores might have a loss of sweet detection, which is not observed. Another example predicts that if the *T1R1* gene has shifted the diets of Pandas from meat to bamboo then why do other herbivores (cows and horses) retain the function of *T1R1*[147,148]? These data indicate strong correlations between gain and loss of taste receptors to diet evolution, however in addition other mechanisms exist, which influence the evolution taste receptors and diet choice.

The fact that all bird species that have been sequenced lack the *Tas1r2* gene cannot explain why hummingbirds among other nectar feeders are able to sense sweet compounds in nectar. Recently investigation by Baldwin et al. 2014 has revealed that hummingbirds have evolved sweet detection by the changes to the ancestral umami taste receptor function and therefore have evolved to feed on nectars[149]. This study demonstrates that mutation of a taste receptor has caused radiation in sugar and nectar feeding in birds.

Additionally, in insects a recently study provided evidence that cockroaches are able to sense glucose with aversive neurons as an evolutionary adaptive result of pest control mechanisms. Toxic baits “fool” cockroaches to feed on glucose laced with toxins that are fatal. In response to this strong artificial selection pressure, cockroaches have rapidly evolved a detection mechanism to avoid glucose[150]. The authors hypothesize that bitter Grs have evolved

recognition sites for glucose and therefore activates bitter neurons and sends feeding inhibitory signals. This study provides insights into evolution of taste receptors during extreme selective pressures, while educating about the efficacy of pest control strategies.

Characterization of sugar receptors

While mammals contain only one broad sugar receptor (T1R2+T1R3), flies have eight sweet Grs. Why is this? *Drosophila* may have two possible obligate Grs for sugar detection; mutants of *Gr5a* and *Gr64a* have no measured sugar response[37]. Our findings demonstrate that mutants for all sweet Grs show losses in sweet detection[90]. This strongly suggests heteromeric Grs complexes with possible obligate receptors Gr5a and Gr64a.

However, it is still unclear why *Drosophila* has evolved eight receptors to encode sugar detection. One hypothesis is that combinations of Grs may encode for different affinities toward sweet compounds as we have observed changes in sweet detection by electrophysiology at different concentrations (**Figure 3.1c,d**). Additionally, another possibility is that sugar receptors have overlapping response profiles but may have different inhibitory profiles. *Gr64e* and *Gr64a* are both necessary for glycerol detection (**Figures 2.4, 2.5 and 2.12**)[90]. We see that glycerol inhibitors block both sucrose and glycerol detection (**Figure 3.3**). It is interesting to postulate that if *Gr64e* and *Gr64a* form the glycerol receptor and *Gr64e* mutants do not affect sucrose inhibition by inhibitors may suggest that

Gr64a is the target of inhibition. Finally, plants that produce bitter compounds inhibit sugar neuron firing and inhibit insect feeding[103]. Redundancy in sugar detection by numerous sweet Grs could potentially overcome aversive compound inhibition and allow for feeding. Insects are in competition for food and the idea that Grs may have redundant functions may be important if a niche is limited and foraging for new foods sources is necessary. Overall, our findings strongly suggest the notion of sweet detection by heteromeric Gr partners.

Possible mechanism of starvation and deprivation on taste behavior

To survive animals must be able to adapt to the external environment. Thus taste behaviors in animals must be "plastic" and process internal hunger and external environmental cues. Central mechanisms modulate behaviors during sugar sensory deprivation, however mechanisms in peripheral neurons are far less understood. Both mammals and *Drosophila* modulate feeding behaviors by neuropeptides e.g. neuropeptide Y in mammals and neuropeptide F in *Drosophila* are able to enhance or inhibit the activity of neural circuits involved in feeding[102,151]. Dopamine regulation was recently demonstrated to affect feeding homeostasis. When the dopamine receptor is misregulated in the brain it causes fed flies to act starved and starved flies to act fed[101].

One study proposes a genetic mechanism affecting peripheral neuron activity due to starvation. Meunier et al. 2007 showed that starved male flies demonstrate a 20% increase in peripheral neuronal firing to 100mM glucose after

one day of starvation. However, in takeout (TO) mutants, a gene involved in nutrient homeostasis, peripheral firing due to starvation does not occur[103]. Interestingly, *Inagaki* et. al 2012 report that starved female flies do not exhibit peripheral taste differences to sucrose in any hair (L, I, and S) types after 1 day of starvation[101]. This discrepancy within the literature may be due to male and female firing differences. However the effects of peripheral modulation in taste neuron activity in male versus female flies is unknown and warrants further investigation.

We find that starvation increases peripheral activity in sugar neurons to sucrose in male flies. Additionally, using a *Gr64f-GAL4* reporter fly we observe that deprivation may cause an increase in Gr expression. Our data supports the hypothesis that the lack of sugar neuron activity during starvation or deprivation may leads to increases in Gr expression and overall sugar neuron activity. Potentially, this peripheral modulation may allow flies to feed in unfavorable conditions for survival. How this signaling occurs, either through peripheral expression of Grs[104] or feedback from presynaptic control of central neurons[141], will be goals of future studies.

Final comments

We still do not know what makes up a functional Gr in Drosophila, which will be vital to fully understand taste signaling in GRNs. Freeman et al. 2014 expressed Grs ectopically in the olfactory ab1C neuron and with further

characterization of this system will eventually lead to decoding Grs as was done for Ors[18]. We would also use this system to uncover possible inhibitory compounds on that inhibit Grs directly and could lead to novel compound screening to find inhibitors of pest and vector species feeding behaviors.

Feeding behavior experiments are a staple when linking gene function to behavioral output, however until recently there have been quantifiable issues with how feeding is measured. Itskov et al. 2014 and Ro et al 2014 have created high throughput automated capacitive measuring devices to quantify various aspects of fly feeding behaviors. Advantages of using these tools will allow experiments to follow free moving fly feeding behavior over time as well as testing many feeding paradigms on the same fly[152,153]. These next steps in fine-scale measurements of feeding combined with molecular and genetic tools for flies will allow us to explore how molecular and cellular mechanisms of peripheral neuron circuits control feeding, nutrient homeostasis and change over time.

Both mammals and insects share fundamental taste circuitry organization and feeding behaviors toward similar taste modalities. Studying *Drosophila*'s peripheral taste detection will allow for new discoveries of general mechanisms that modify taste circuits in both mammals and insects. Additionally, learning how to exploit peripheral taste detection in flies, will allow for screening and the creation of better and safer pest and vector control compounds, which is being done utilizing the olfactory system[154]. Overall, utilizing *Drosophila melanogaster* as a model organism to study peripheral mechanisms of taste detection has laid

the foundation to understand how the evolution of gain or loss of taste receptors leads to feeding behavior changes, and niche specification.

Appendix

Introduction:

We have yet to discover the receptor or genes involved in acidic pH detection in *Drosophila*. However, we find that two previous inhibitors of acid detection, amiloride and Zn^{2+}, in mammals did not affect acidic pH responses in *Drosophila*[120,131]. We undertook two studies that are ongoing. DGRP analysis has shown that BL#25181, a non-responder line which has a loss of pH detection. Complementation crosses between BL#25181 with BL#25189, a responder line suggest that mutations causing acidic pH detection loss map to X-chromosome. Electrophysiological results of F2 progeny from crosses BL#25181/BL#25189 heterozygous flies show a bimodal distribution of “responding” or “non-responding” flies suggesting a single locus on the X-chromosome responsible for loss of acidic pH detection. Sequencing of F2 lines will be able map genes that correlate to non-responders verses responders and generate a candidate gene list to further screen.

As we discovered BL#25181 mutant was X-linked we concurrently started screening of X-chromosome trasmembrane proteins, which revealed a mutant Shaker 5 (Sh^5). However, further analysis demonstrated that Sh^5 mutant on the X-chromosome does not correlate with loss in acid response. If Sh^5 wasn’t acting through the X-chromosome we wondered if either the second or third chromosome was involved. We further mapped Sh^5 genotype and found that the third chromosome of Sh^5 contains a recessive mutation responsible for loss of

acidic pH detection. A third chromosome deficiency screen was undertaken to map the mutation observed on Sh^5 third chromosome. Overall both the DGRP analysis and Sh^5 third chromosome screen will create new candidate gene lists to be tested by mutant or RNAi analysis to uncover genes involved in acidic pH detection.

Methods:

Flies

Flies were raised on standard cornmeal-dextrose-agar diet at 25°C. Wild-type flies were w^{1118}. Mutant fly stocks were obtained from the Bloomington Stock Center: X-Chromosome screen: Sh^5 (BL111), Sh^{14} (BL3563), EAG1 (BL3561), Sh^{MNS} (BL24194), *CG42340*[PBf00393] (BL18325), *CG42594*[KG03323] (BL12878), *inc*[PBf00285] (BL18307), $ShakB^{25}$ (BL4769), *EAG*[EPey00714] (BL15038), *SK*[MB03486] (BL24653), *Clic*[EPey01352] (BL20116), iav^{3621} (BL24768), *Tom40*[PLg0216] (BL11859), *Inx7*[EP1641] (BL11251), *ppk8*[KG10039] (BL16498) and *Nmdar2*[MB09441] (BL29884).

Electrophysiology

Acid tastant recordings: Single sensillum recordings were performed using the tip-recording method[123] using 30 mM tricholine citrate as the electrolyte[88]. Recordings were obtained from male flies aged 3-10 days. In every case, a positive control of caffeine or lobeline, as appropriate, was tested before and after recordings with acid tastants. For recordings with standardized pH

solutions, each sensillum was first tested with electrolyte alone as a control (pH 6.65), followed by HCl solutions of pH 2–6 in descending order. Neuronal responses were quantified by doubling the number of spikes in the 0-500 ms window upon contact with the stimulus.

Immunohistochemistry

Whole brains were dissected, fixed and stained as described[37]. Antibodies were used at the following concentrations: mouse α-nc82 (1:20), rat α-CD8a (1:100), Alexa-488 α-rat (1:150), and Alexa-568 α-mouse (1:150). Confocal *z*-stacks were acquired using a Zeiss LSM510 and analyzed using ImageJ.

Results:

Receptor blocker of pH in mammals does not affect acidic pH responses in Drosophila

Pharmacological analysis has observed a Zn^{2+} inhibition of an unknown receptor that is activated by free protons in sour taste cells[120]. Additionally, the inhibitor amiloride blocks acid-sensing ion channels (ASICs)[131]. We set out to test these inhibitors on acid sensing bitter neurons. Our observation of both mixtures shows not significance in firing of acid sensing neurons, Zn^{2+} with pH (P = 0.074 for 1 mM, P = 0.267 for 10 mM, Student's *t*-test) (**Appendix Figure A1a**) or amiloride with pH (P = 0.948, ANOVA) (**Appendix Figure A1b**). These results suggest alternative mechanisms of receptor activation in acid responsive neurons.

S-b verses S-a subsets bitter neuron projections compared to all bitter neuron projection patterns in the SOG

Acid detection is mediated by a subset of bitter S-b and I-b, but not S-a and I-a neurons[129]. We wanted to know if there were differences in projection patterns of acid-responsive (S-b) verses acid non-responsive (S-a) neurons. We examined the expression of *Gr22f*, *Gr36b*, and *Gr89a* in taste neuron projections in the SOG using the bipartite *GAL4/UAS* system (**Appendix Figure A2a**), which was previously demonstrated[53]. We observed that qualitatively *Gr22f-GAL4* densities of axons and GFP signal is less than *Gr36b-GAL4*. We measured width and depth of labellar and found that *Gr36b-GAL4* may have deeper of projections in the SOG verses *Gr22f-GAL4* (**Appendix Figure A2b**). Overall it appears that *Gr22f-* and *Gr36b- GAL4* are a subset of *Gr89a-GAL4* bitter neuron projections in the SOG. However, additional fine-scale imaging analysis is needed to confirm our analysis.

Drosophila Genome Reference Panel (DGRP-BL#25181) has loss in acidic pH response

From our DGRP screen (**Figure 4.6**) we further tested BL#25181 non-responder and crossed males and females with BL#25189 responder male and females accordingly. We tested pH responses in male BL#25181/BL#25189 flies in both crosses and found that female BL#25181 crossed with male BL#25189 had a dramatic loss in acidic pH where the reciprocal cross had wild type pH

responses (**Appendix Figure A3**). These results suggested that the X-chromosome is involved in the loss of acidic pH phenotype. These results lead us to perform another round of electrophysiology on unique F2 lines created from the F1 male and female BL#25181/BL#25189 heterozygotes. F2 flies were assessed for caffeine (positive control) pH 4, 3, 2, and TCC (negative control) responses (**Appendix Figure A4**). We compared acidic pH detection with caffeine response (**Appendix Figure A5a**) as well as TCC response (**Appendix Figure A5b**) to control for general bitter neuron fluctuations in activity and we see no correlation over all F2 pH responses that were obtained ($R^2 < 0.2$). We further analyzed F2 response profiles of pH 4, 3 and 2 responses by cluster analysis we find there is about a 50% 14/30 F2 lines that are in the responder lineage (**Appendix Figure A6**). These data suggest an acidic pH loss of response may map to a single locus on the X-chromosome.

X-chromosome mutant screen

We hypothesize that a transmembrane protein mediates acid detection. Our results for the DGRP analysis lead us to concurrently test transmembrane mutants on the X-chromosome. We tested many transmembrane proteins and stumbled upon Shaker gene Sh^5 mutation that had a large decrease in response to acidic pH, but relatively wild type caffeine response (**Appendix Figure A7a**). Our hypothesis was that Shaker, a potassium channel, fit our criteria for a possible acid receptor. However, when verified Sh^5 mutation by out-crossing

female Sh^5 (as this mutation is on the X) with male wild type flies and tested male F1 progeny (obtaining Sh^5 X chromosome) we observed wild type acidic pH responses (**Appendix Figure A7b**). This indicates that the Sh^5 mutation does not directly contribute to the loss in response to acidic pH.

Our results were unsatisfactory as Sh^5 mutants have a clear loss in acidic pH phenotype. We hypothesized that this phenotype was due to alternate chromosomes and out crossed Sh^5 mutants creating a line that contained the Sh^5 chromosome on the second and third. Our electrophysiological assessment of Sh^5 on the second and third revealed that Sh^5 on the third (111^3) showed the loss of acidic pH phenotype. The location of the mutation of 111^3 is unknown so we undertook a deficiency screen to uncover genes involved in acidic pH detection. So far fifty-six F1 111^3/Df lines have been tested, which is about 30% of all of the deficiency lines that cover the third chromosome. We assayed both S-b and S-a classes of sensilla for caffeine, pH2 and TCC. We find two possible regions (BL#8965 and #1842) of the third chromosome that may contain genes, which do not rescue 111^3 loss of acidic pH phenotype. Further screening and analysis of BL#8965 and #1842 regions of third chromosome will need to be performed to uncover genes involved in acidic pH detection.

Discussion:

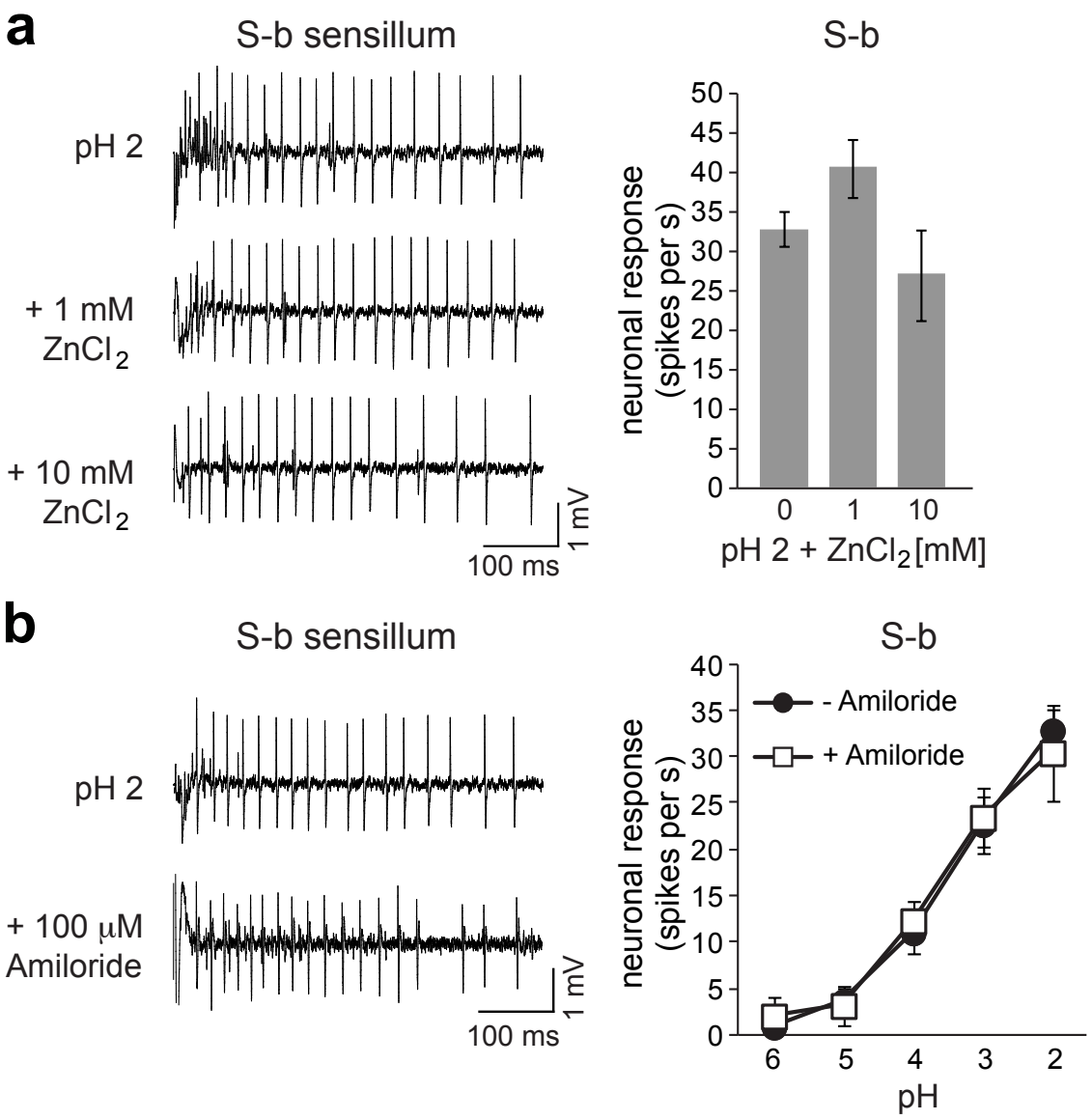
We investigated differences in brain projections of acid-activated (Gr22f) verses non-acid-activated bitter neurons (Gr36b) and found that projection patterns in the SOG look similar (**Appendix Figure A2**). Without a more

sophisticated brain registration comparison it remains unclear whether acid-activated compared to non-acid-activated neurons project to separate bitter neuron locations in the SOG.

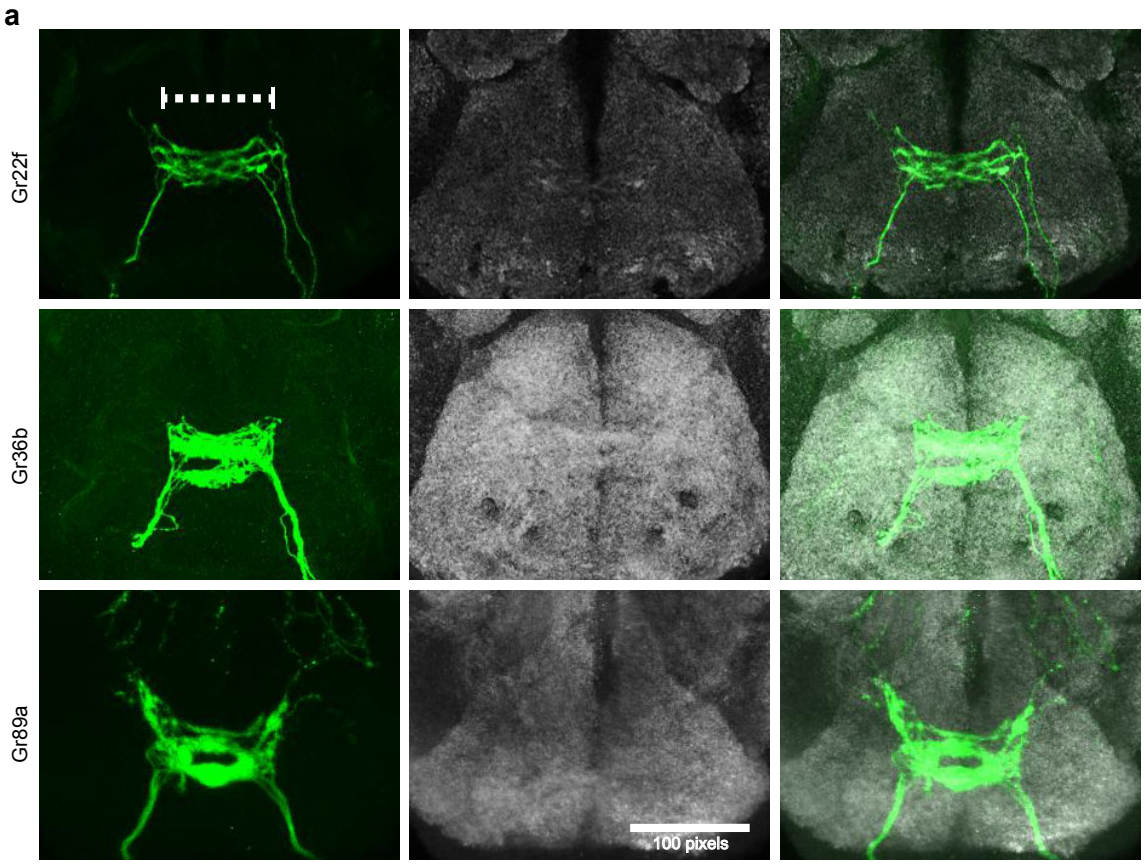
Our analysis of DGRP responder and non-responder fly lines suggest the possibility that there is a single locus and possibly a single gene on the X chromosome (**Appendix Figure A6**) that is involved in acidic pH detection. We hypothesize that trasmembrane protein (ex: channels or receptors) mediate the detection of pH and so we performed an X-chromosome screen (from our DGRP analysis) for transmembrane mutants. Our X chromosome mutant screen uncovered Shaker mutant 5 (Sh^5) to have little to no pH response (**Appendix Figure A7a**). The *Shaker* gene is a potassium ion channel that is important for general cell function and could fit our criteria for acidic pH detection. However, upon further investigation we discovered that Sh^5 mutation was not linked to loss of pH phenotype (**Appendix Figure A7b**). We predicted that a mutation on chromosome II or III was responsible for this loss of acidic pH detection. To find what chromosome the potential mutation was on we outcrossed Sh^5 mutant and identified that a recessive mutation mapped to the third chromosome (**Appendix Figure A8a**).

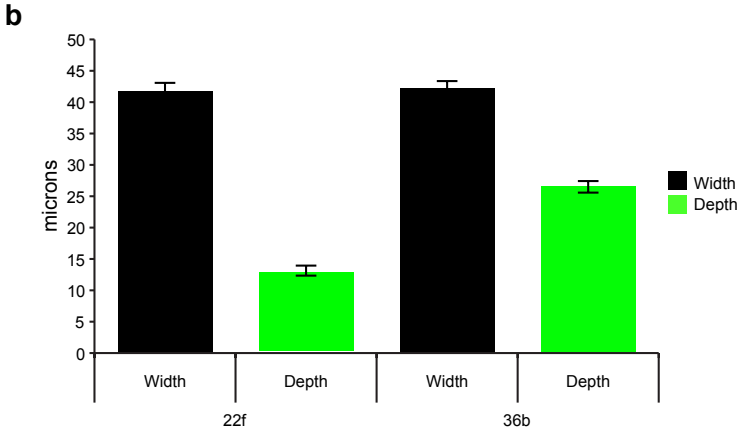
Furthermore, loss of acidic pH candidate, Sh^5 third chromosome mutant (111^3), was used to map the location of the loss of acidic pH phenotype. 111^3 was crossed to different hemizygous large deficiencies that span third chromosome in which F1 progeny were screened with electrophysiology to

uncover genes involved in acidic pH detection (**Appendix Figure A8b**). The third chromosome screen is ongoing, however so far we have discovered 111^3 possibly maps to two regions on chromosome III that have a loss in acidic pH (**Appendix Figure A8b**). However, further screening will have to be performed to uncover all possible genes that are involved in acidic pH detection. We have yet to elucidate a molecular mechanism for acidic pH detection in *Drosophila*, however our results lay the foundation to discover genes involved in acid detection.

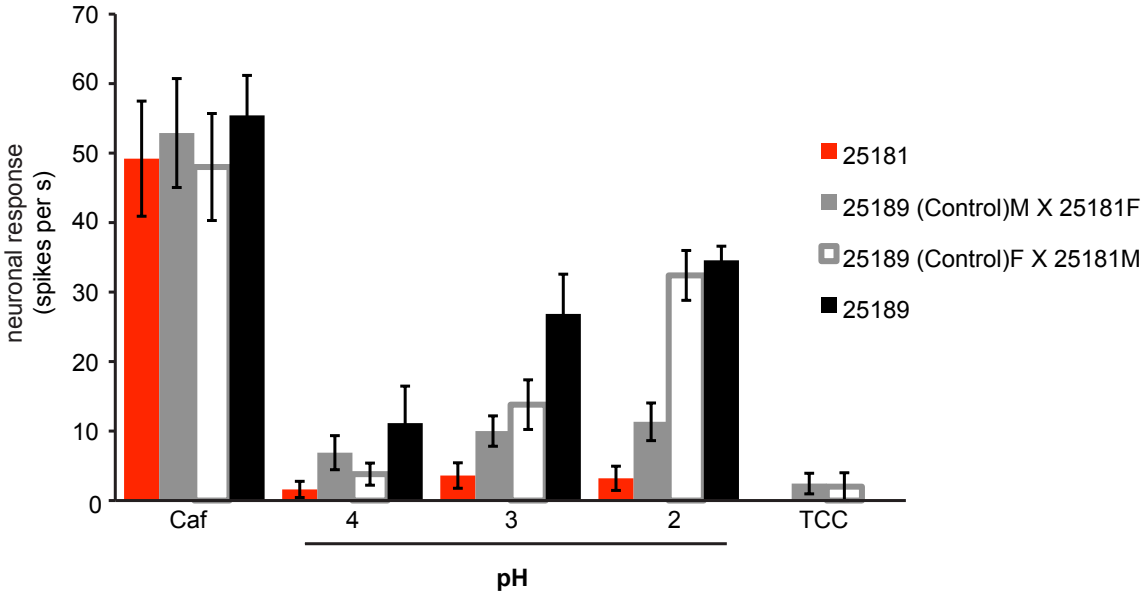

a
S-b sensillum
pH 2
+ 1 mM ZnCl2
+ 10 mM ZnCl2
1 mV
100 ms
S-b
neuronal response (spikes per s)
50
45
40
35
30
25
20
15
10
5
0
0
1
10
pH 2 + ZnCl2 [mM]
b
S-b sensillum
pH 2
+ 100 μM Amiloride
1 mV
100 ms
S-b
- Amiloride
+ Amiloride
neuronal response (spikes per s)
40
35
30
25
20
15
10
5
0
6
5
4
3
2
pH

Appendix Figure A1: Response to low pH is not affected in receptor blockers. (**a**) Representative traces and mean responses of wild-type S-b labellar sensilla to standardized HCl solutions at indicated pH alone *n*=12 (0 or –) or in mixtures with $ZnCl_2$ (*n*=10 for 1mM $ZnCl_2$ and *n*=4 for 10mM $ZnCl_2$) or (**b**) amiloride hydrochloride *n*=5-7. Neither $ZnCl_2$ (Student's *t*-test) nor amiloride hydrochloride (2-way ANOVA with univariate analysis) caused any change in pH response. Error bars = s.e.m.

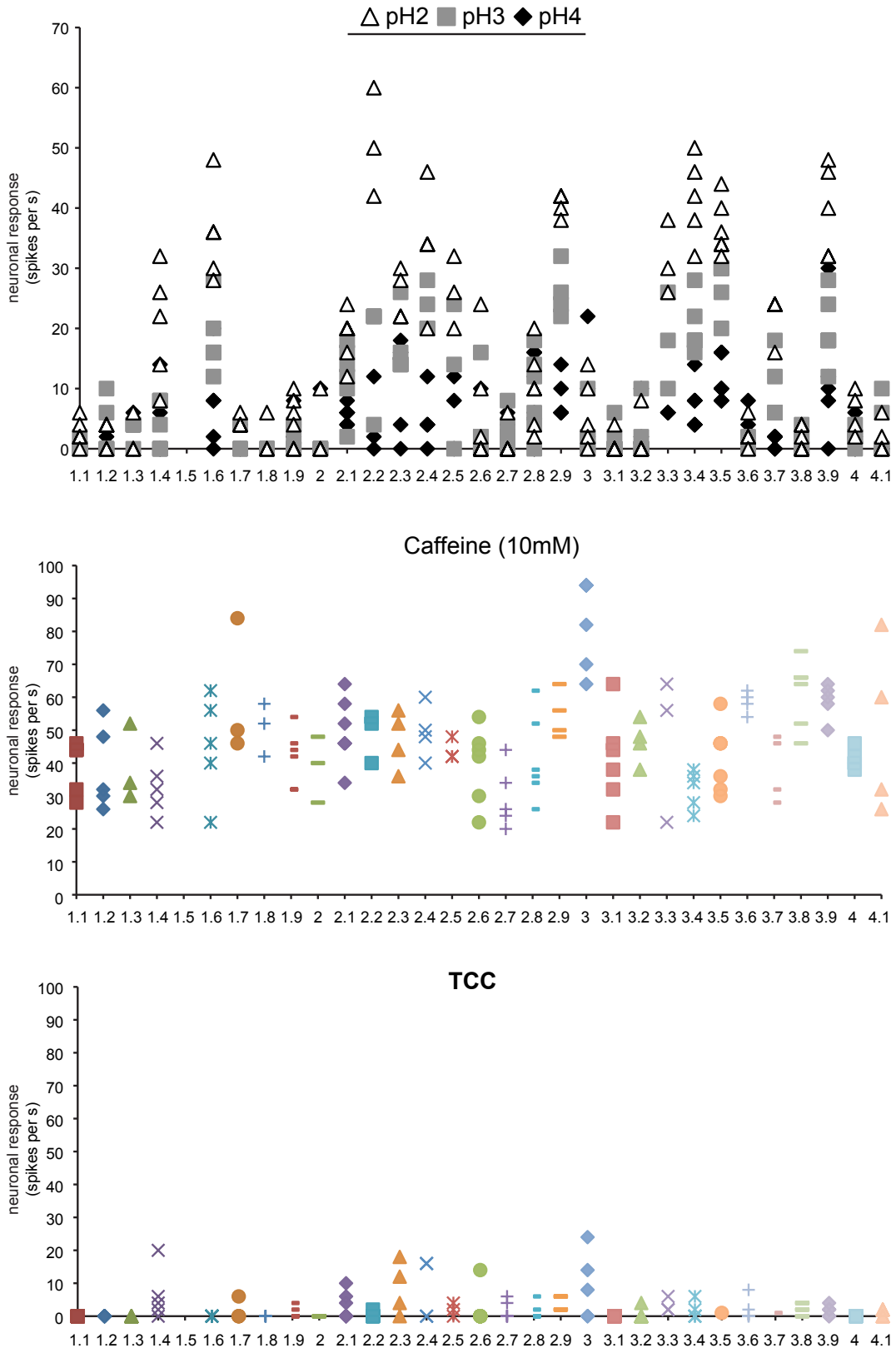

a
Gr22f
Gr36b
Gr89a
100 pixels


b
microns
50
45
40
35
30
25
20
15
10
5
0
Width
Depth
Width
Depth
22f
36b
Width
Depth

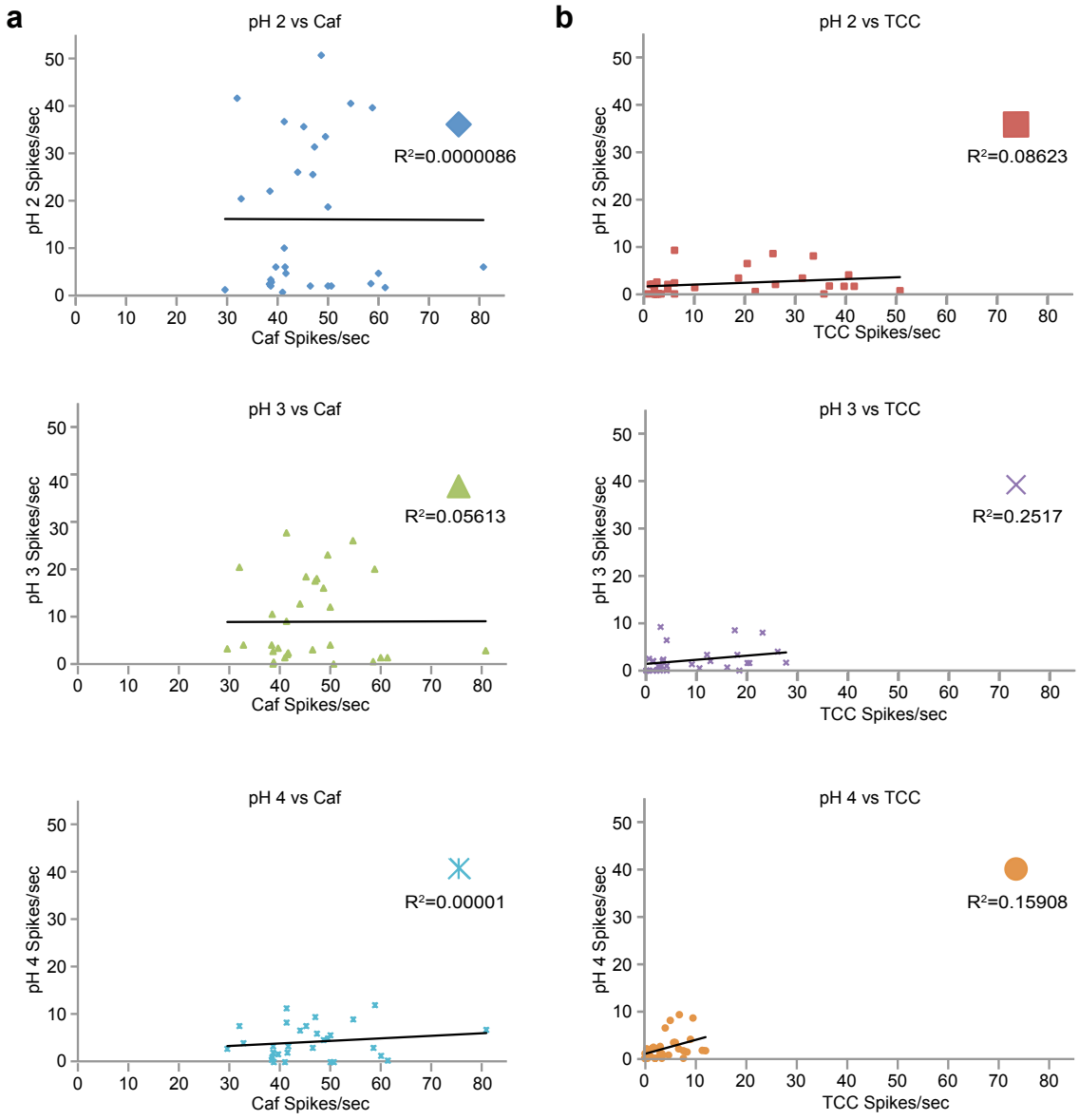
Appendix Figure A2: *Gr22f*, *Gr36b* and *Gr89a-GAL4* expression in the SOG. (**a**) Representative SOG projection patterns by GAL4 expression from Gr22f (top row), Gr36b (middle row), and Gr89a (bottom row) bitter neuron projections alone (Left column), SOG (middle column) and merged (Right column) obtained from Z-stacks. (**b**) Measurements of projection patterns in X (width, black bars) and Z (depth, green bars) coordinates white bar indicates X coordinates measured and quantified in top left panel in (**a**) and Z coordinates were measured by onset and offset of GFP signal in Z-stacks. (n = 6-7) Gr89a is shown for comparison only. Error bars = s.e.m.


70
60
50
40
30
20
10
0
neuronal response
(spikes per s)
Caf
4
3
2
TCC
pH
25181
25189 (Control)M X 25181F
25189 (Control)F X 25181M
25189

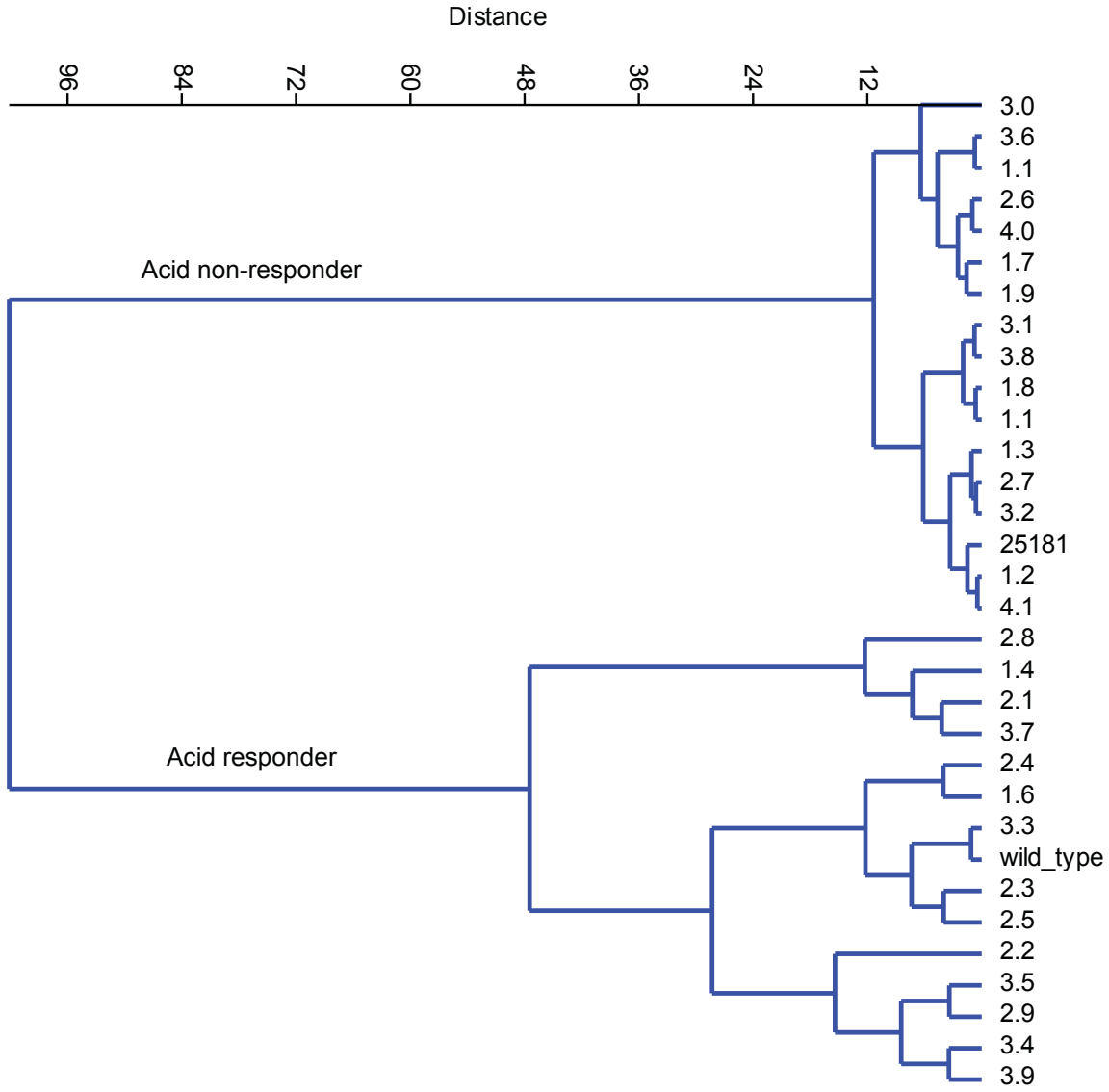
Appendix Figure A3: Loss of acidic pH phenotype is X-linked demonstrated by DGRP complementation. Single-unit extracellular electrophysiology mean responses of S-b type sensilla from male flies tested 3-8 days old. Stimuli were tested in the order shown above, left to right. Caffeine (positive control) is tested to assure that the bitter neuron is responding and then pH 4, 3, 2 and TCC (negative control) electrolyte alone to assure no aberrant firing to electrolyte. n = 3-10. Error bars = s.e.m.


△ pH2 ■ pH3 ◆ pH4
neuronal response (spikes per s)
Caffeine (10mM)
TCC
1.1 1.2 1.3 1.4 1.5 1.6 1.7 1.8 1.9 2 2.1 2.2 2.3 2.4 2.5 2.6 2.7 2.8 2.9 3 3.1 3.2 3.3 3.4 3.5 3.6 3.7 3.8 3.9 4 4.1

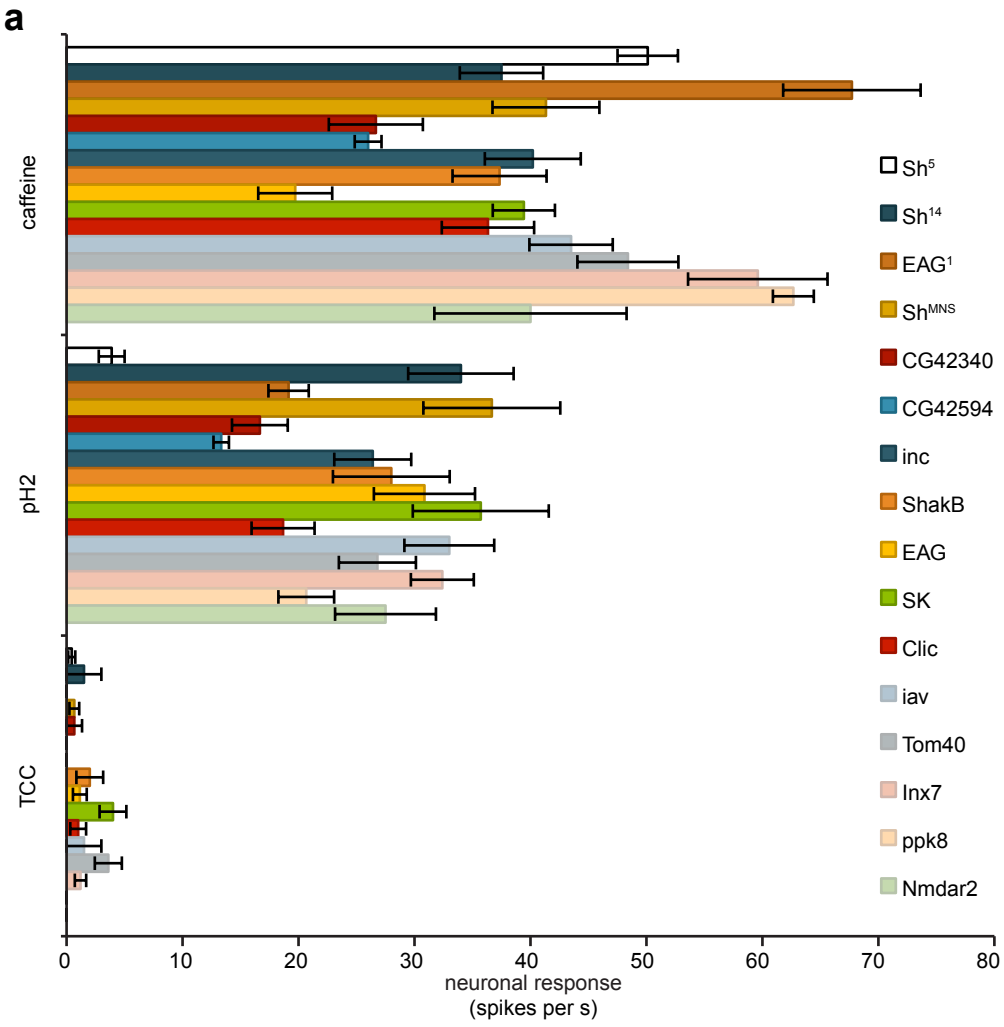
Appendix Figure A4: Variation of pH responses in BL#25181/BL#25189 F2 generation lines. Electrophysiological responses of unique F2 lines (ex: 1.1) were tested on S-b sensilla for pH 4, 3, 2 (top graph), caffeine (10mM, middle graph), and TCC (bottom graph). Each point is representative of a single S-b sensilla response, which is clustered per fly for pH 4 (black diamonds), pH 3 (grey squares) and pH 2 (white triangles). Caffeine and TCC are also represented as single S-b sensilla response, which is clustered per fly by unique shape and color shown. n = 3-11.

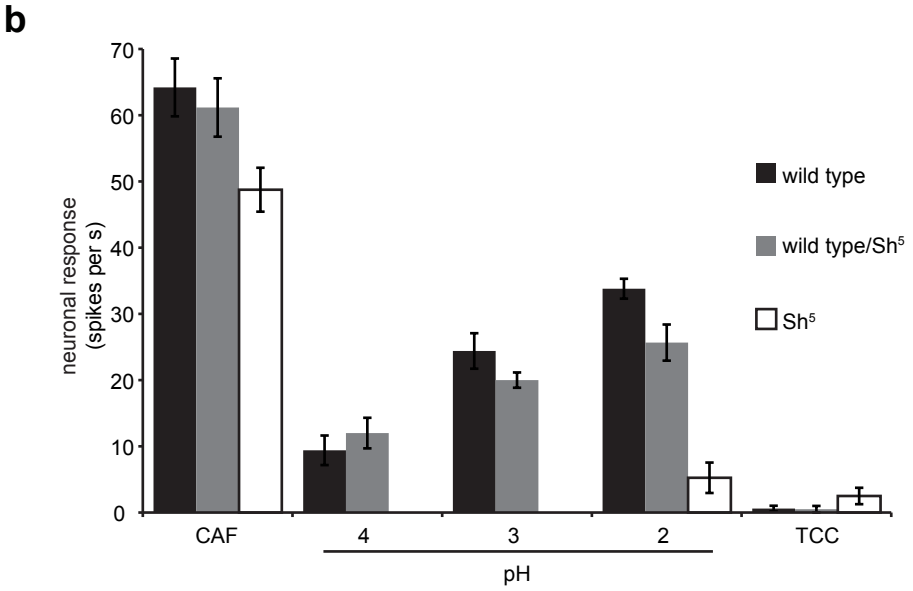

a
pH 2 vs Caf
pH 2 Spikes/sec
Caf Spikes/sec
R²=0.0000086
pH 3 vs Caf
pH 3 Spikes/sec
Caf Spikes/sec
R²=0.05613
pH 4 vs Caf
pH 4 Spikes/sec
Caf Spikes/sec
R²=0.00001
b
pH 2 vs TCC
pH 2 Spikes/sec
TCC Spikes/sec
R²=0.08623
pH 3 vs TCC
pH 3 Spikes/sec
TCC Spikes/sec
R²=0.2517
pH 4 vs TCC
pH 4 Spikes/sec
TCC Spikes/sec
R²=0.15908

Appendix Figure A5: Correlation of pH responses verses caffeine or TCC responses in BL#25181/BL#25189 F2 generation lines. Electrophysiological responses of unique F2 lines (ex: 1.1) were correlated from data shown in (**Appendix Figure A4**). (**a**) pH 2 (top, blue diamond), 3 (middle, green triangle), 4 (bottom, light blue star), were graphed verses corresponding caffeine (10mM) positive control response were compared by scatter plot analysis and R2 values were generated: pH 2 (R2 = 8.6x10-6), pH 3 (R2 = 5.613x10-2), and pH 4 (R2 = 1x10-5). (**b**) pH 2 (top, red square), 3 (middle, purple x), 4 (bottom, orange circle), were graphed verses corresponding TCC negative control response were compared by scatter plot analysis and R2 values were generated: pH 2 (R2 = 8.623x10-2), pH 3 (R2 = 2.517x10-1), and pH 4 (R2 = 1.5908-1). Each data point represents corresponding single S-b sensilla responses to both pH verses caffeine (a) or TCC (b), which are clustered for all F2 flies tested.

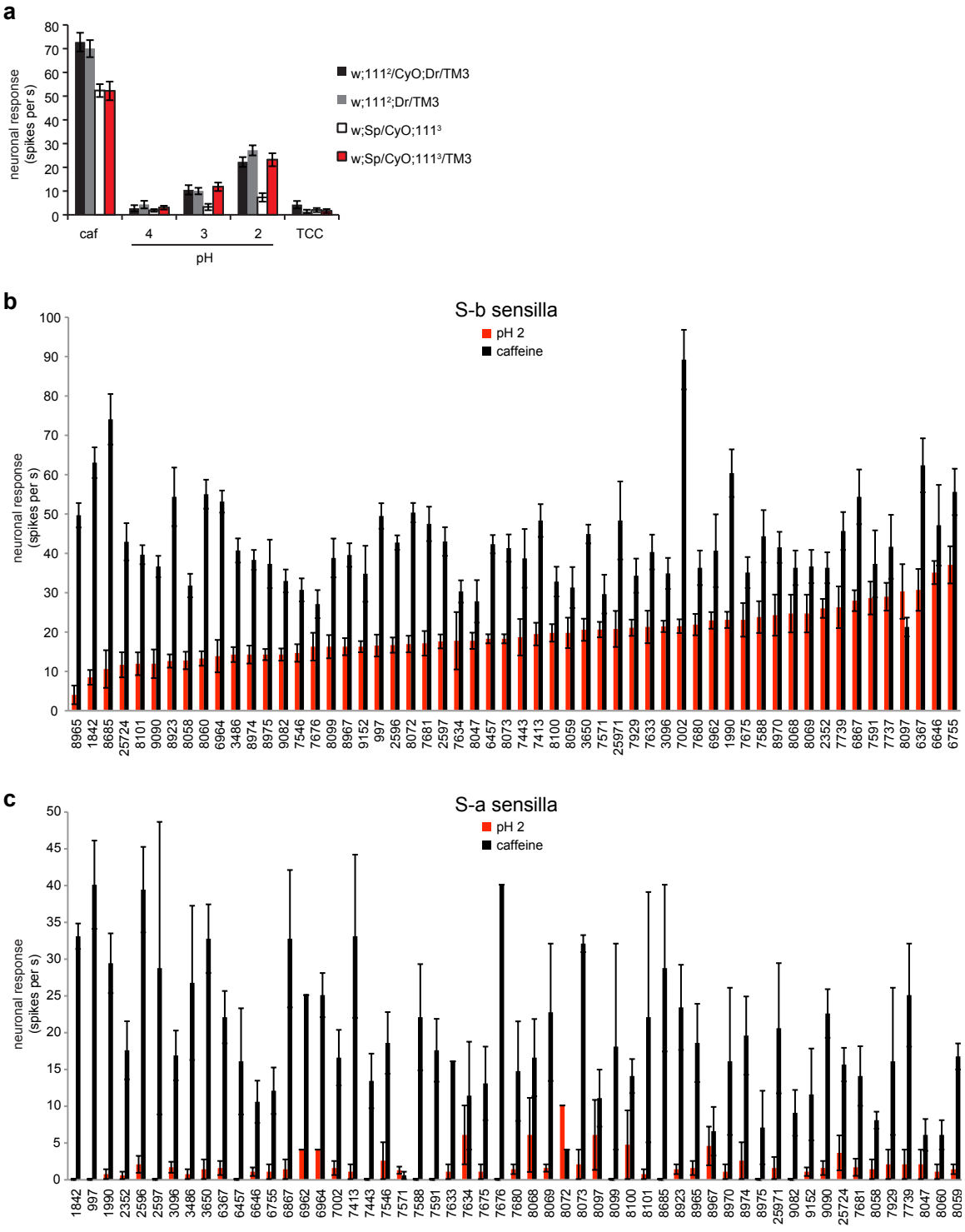

Distance
96
84
72
60
48
36
24
12
3.0
3.6
1.1
2.6
4.0
1.7
1.9
3.1
3.8
1.8
1.1
1.3
2.7
3.2
25181
1.2
4.1
2.8
1.4
2.1
3.7
2.4
1.6
3.3
wild_type
2.3
2.5
2.2
3.5
2.9
3.4
3.9
Acid non-responder
Acid responder

Appendix Figure A6: Cluster analysis reveals two phenotypic response groups. Cluster analysis of pH 4, 3, and 2 responses of DGRP F2s using Ward's method with Euclidean distance. Each number (ex: 1.1) corresponds to a unique F2 line (right), wild type and BL#25181 non-responder are there for comparison. Data used to create dendrogram was from averaged pH responses (pH 4, 3, and 2) combined in analysis from same data in (**Appendix Figure A4**). Data suggests a clear distinction for two groups, which we denoted as "responder" and "non-responder" phenotypes; further there are possibly two sub groups within responder and non-responder groups.


a
caffeine
pH2
TCC
Sh5
Sh14
EAG1
ShMNS
CG42340
CG42594
inc
ShakB
EAG
SK
Clic
iav
Tom40
Inx7
ppk8
Nmdar2
0
10
20
30
40
50
60
70
80
neuronal response
(spikes per s)


b
neuronal response
(spikes per s)
0
10
20
30
40
50
60
70
wild type
wild type/Sh5
Sh5
CAF
4
3
2
TCC
pH

Appendix Figure A7: X-chromosome screen reveals Shaker as loss of acidic pH candidate. (**a**) Single-unit extracellular electrophysiology mean responses of indicated genotype. (**b**) Mean responses to wild type (w^{1118}), Shaker^{5} (Sh^{5}), and outcross between Sh^{5} female and w^{1118} male F1s. S-b type sensilla from male F1s (from Sh^{5} crossed w^{1118}) flies were tested with stimuli: caffeine (10mM, positive control), tested to assure that the bitter neuron is responding, pH 2 and TCC (negative control) electrolyte alone to assure no aberrant firing to electrolyte. n = 2-18. Error bars = s.e.m.


a
neuronal response (spikes per s)
w;111²/CyO;Dr/TM3
w;111²;Dr/TM3
w;Sp/CyO;111³
w;Sp/CyO;111³/TM3
caf
4
3
2
TCC
pH
b
S-b sensilla
pH 2
caffeine
neuronal response (spikes per s)
8965 1842 8685 25724 8101 9090 8923 8058 8060 6964 3486 8974 8975 9082 7546 7676 8099 8967 9152 997 2596 8072 7681 2597 7634 8047 6457 8073 7443 7413 8100 8059 3650 7571 25971 7929 7633 3096 7002 7680 6962 1990 7675 7588 8970 8068 8069 2352 7739 6867 7591 7737 8097 6367 6646 6755
c
S-a sensilla
pH 2
caffeine
neuronal response (spikes per s)
1842 997 1990 2352 2596 2597 3096 3486 3650 6367 6457 6646 6755 6867 6962 6964 7002 7413 7443 7546 7571 7588 7591 7633 7634 7675 7676 7680 8068 8069 8072 8073 8097 8099 8100 8101 8685 8923 8965 8967 8970 8974 8975 25971 9082 9152 9090 25724 7681 8058 7929 7739 8047 8060 8059

Appendix Figure A8: Shaker^{5} loss of acidic pH recessive mutation maps to the third chromosome and the third chromosome deficiency screen. (**a**) Mean responses of S-b sensilla to caffeine (10mM), pH 4, 3, and 2 followed by TCC in Shaker^{5} out crosses on second (111^{2}) in (black and grey) and third (111^{3}) (white and red) chromosomes. Mean responses of (**b**) S-b and (**c**) S-a sensilla indicated to caffeine (black), pH 2 (red) in F1 males of parental lines 111^{3} crossed to deficiency lines (indicated by Bloomington Stock Center number). n = 1-11. Error bars = s.e.m.

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