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# An Evolutionarily Conserved Clade is Involved in the Detection of Bitter and Sweet Tastants in Insects

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Chapter 1, in part, is a reprint of the material as it appears in: Freeman, E. G., and Dahanukar, A. Molecular neurobiology of Drosophila taste. *Current Opinion Neurobiology* 34:140-8 (2015) Dr. Anupama Dahanukar listed in that publication directed and supervised the research, which forms the basis for this dissertation. We thank C. Scott, A. Lamella, and A. Ganguly for comments on the manuscript and on this chapter.

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ABSTRACT OF THE DISSERTATION

An Evolutionarily Conserved Clade is Involved in the Detection of Bitter and Sweet Tastants in Insects

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The taste system is essential to determine the palatability of potential food sources. Insects use gustatory receptors (Gr) to detect both appetitive and aversive compounds. In *D. melanogaster,* sweet neurons express eight Grs belonging to a highly conserved clade in insects. Currently, it is poorly understood how these receptors detect sweet tastants. A system that can functionally express single Grs to study ligand recognition is necessary to fill a critical gap in the field. Using the $CO_2$-sensing olfactory neuron as a unique *in vivo* decoder, we expressed each receptor of the sweet clade individually and recorded neural activity to a panel of sweet tastants. We also expressed Gr43a, an internal fructose sensor found outside of the sweet clade, and its mosquito ortholog, AgGr 25, in the $CO_2$ neuron. Each receptor conferred sensitivity to two or more sweet tastants and each sweet tastant was detected by more than one sweet Gr, indicating direct roles in ligand detection for all sweet receptors. Moreover, sweet Grs play a role in bitter tastant detection. Bitter compounds can directly suppress sugar induced activity of sweet taste neurons in the absence of

bitter neurons, yet the mechanisms involved are unknown. We found that receptors of the sweet clade can be directly inhibited by bitter tastants and each receptor is inhibited by a unique subset of compounds. This property is a distinguishing feature of sweet clade; neither Gr43a, AgGr25 or Gr21a/Gr63a are inhibited by bitter compounds.

Many features of sweet Grs are evolutionarily conserved in mosquitoes. We discovered that labellar sweet neurons from both *A. gambiae* and *A. aegypti* can detect sweet tastants and be directly inhibited by bitter tastants. Furthermore using the *D. melanogaster* $CO_2$ neuron, we discovered that every receptor of the *A. gambiae* sweet clade was activated by at least one sweet tastant and at least one sweet AgGr can be inhibited by bitter tastants, suggesting the sweet clade has evolved as a dual sensor of sweet and bitter tastants. This work sets the platform for further study of ligand recognition of Grs in other insects, expanding our understanding of insect taste detection.

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## 1.1 Background of taste perception in Drosophila

### 1.1.1 Importance of taste

Taste is one of the main senses responsible for determining the palatability of potential food sources and avoiding ingestion of toxic compounds. Over time, animals have evolved multiple mechanisms to avoid toxic or inadequate compounds as well as find safe high caloric and essential nutrients. Since most potential food sources are mixtures of both appetitive and aversive compounds, animals must determine the risk/reward of potentially consuming a particular source. This is dependent on the state of animal as well as composition of the food. Because taste is important in food choices for all animals, we can use our understanding of taste to create multiple systems to control or eliminate pest populations as well as encourage beneficial insects and animals. Furthermore, taste input from the environment affects feeding behaviors which is useful in studying interactions between an animal and its environment.

### 1.1.2 Fly as a model

*Drosophila melanogaster* is a model organism for studying taste because it displays complex feeding behaviors while having a relatively simple neurophysiology [1]. Taste is used for multiple behaviors including oviposition, complex courting and mating rituals, and aggression, as well as feeding [1-3]. Flies also have a multitude of genetic tools available that make it possible to

manipulate and study multiple mechanisms and genes involved in different neural circuits [3, 4]. Its relatively short breeding and life cycle allows us to make multiple genetic changes and study behaviors in experiments that are normally time prohibitive in more complex organisms such as the mouse. There are also many orthologs found between humans and flies. In fact, 14.9% of the human genome and 40.6% of the fly genome has orthologs found in the fly or human genome, respectively [5]. Furthermore, many of the feeding behaviors found in flies are also found in mammals, pests, and disease vectors [6, 7].

### 1.1.3 Perception of taste in *Drosophila*

One of the main purposes of taste is to determine if an animal should eat or avoid a potential food source. A stimulus is determined to be attractive or aversive depending on if an animal consumes or avoids it, respectively. Based on this paradigm, sugar, carbonation, water, and low concentrations of fatty acids and salt are attractive to flies [6, 8, 9]. Acids, high salt and bitter compounds such as alkaloids are avoided [8, 10, 11]. Amino acids are also detected by taste neurons. Yeast, a major component of the *Drosophila* diet, contains many nutritious amino acids essential for survival and important for female egg development [12]. Some amino acids, such as serine, threonine, and phenyalanine, are attractive to flies [13]. Other amino acids are avoided or flies appear to be indifferent to their presence [13].

Not all sugars are equally appetitive. When given the choice between two sugars, flies will normally prefer to consume one over the other when they are at the same concentration. In fact, measuring changes in preference between two appetitive stimuli is one way taste discrimination is studied [13, 14]. This is true for aversive compounds as well though the paradigm is slightly altered. Since flies will not consume something that is repulsive, most avoidance experiments involve different concentrations of an aversive compound mixed with sugar [15, 16]. By comparing how much flies eat the mixture versus the sugar alone, one can determine a compound is aversive if flies consume less of the mixture than sugar alone or neutral if flies consume equal amounts of the mixture and sugar alone. Different bitter compounds lead to different degrees of rejection. If one bitter compound requires a lower concentration to lead to the same degree of it avoidance as another, it is said to be more aversive. Concentrations play an important role in the palatability of a stimulus. A compound might be attractive at low concentrations but aversive at high. Salt is a great example where it is increasingly attractive up to 50mM. Then as the salt concentration increases past 100mM, it becomes less appealing until it is completely avoided at 200mM [9, 11, 17]. Fatty acids are a similar example where flie prefer lower concentrations [18].

Preference for specific appetitive compounds is dynamic and dependent on the internal state of the fly. Deprivation of appetitive amino acids, water, or sugar over time can cause flies to exhibit an enhanced preference for them [13, 14, 19, 20]. Furthermore, avoidance of bitter tastants is also dependent on the

internal state of the fly. When flies are starved, they show increased acceptance for mixtures of sucrose and bitter compounds that would normally be avoided when not starved [21, 22].

One of the main goals in studying the taste system in *Drosophila* is to determine how a fly detects nonvolatile compounds found in the environment, evaluates the palatability of a potential source which then leads to specific feeding behaviors. The first step is detection and probing of food sources by taste organs.

### 1.1.2 Taste organs

Multiple taste organs and sensory cells are found over a fly's entire body [3]. The main taste organs are the tarsi, the labellum, and the pharyngeal organs (**Fig. 1.1**). Gustatory neurons have also been discovered in the anterior margins of the wings [23]. The tarsi and the labellum are covered in taste sensilla or hairs that each house multiple taste neurons [24]. These hairs are in the shape of a long shaft with an open pore at the tip. Taste neurons' cell bodies are found at the base with dendrites that extend into the shaft towards the pore. Chemicals come into contact with the pore and then diffuse into the sensillum through the sensillar lymph to receptors on the dendrites that are able to detect nonvolatile compounds. The inner surface of the labellum contains taste sensilla called pegs which come into contact with different substances when the fly begins to feed. Pharyngeal organs are found in pharynx and are believed to be the last taste

organs involved before ingestion. Taste neurons that innervate all of these organs send axons that terminate in the ventral nerve cord or in the subesophageal ganglion (SEZ), where information is processed and relayed to higher order neurons in the brain and leads to either feeding or rejection of stimuli [25].

### 1.1.2 Cellular taste organization

The distribution of taste sensilla across the adult fly is incredibly stereotypical (**Fig. 1.1**). To determine the composition of sensilla and neurons contained with them, scanning electron microscopy were used to image them. Further experiments, involving tip recordings, calcium imaging, genetic silencing, artificial activation, and behavior experiments were used to categorize the neurons found in each taste organ [3, 6, 9, 24, 26, 27].

#### 1.1.2.1 Sensilla on the labellum

The sensilla on the labellum have been heavily characterized. On the labellum, there are about 60 sensilla which contain up to four neurons that are selectively activated by tastants that are either appetitive or repulsive ( **Fig. 1.1 A**) [3, 28]. Sensilla come in three different morphological types: small (S), intermediate (I), and large (L). Previous studies have recorded electrophysiological responses from each sensillum on the labellum to sweet, salt, bitter, water, and sour compounds using extracellular single unit tip

recordings [9, 10, 16, 29, 30]. Each neuron's electrophysiological response has a distinct spike amplitude which allows us determine if responses to different stimuli originated from the same or different neurons. Further experiments, involving genetic ablation or silencing of specific neurons in a sensillum type were used to validate if responses to different stimuli came from the same or different neurons.

Both large and small sensilla were shown to have four chemosensory neurons. In L- type sensilla, separate neurons respond to water, low concentrations of salt, and sweet tastes (**Fig. 1.1 A**) [16, 24]. There is also one neuron in L-type sensilla whose function is unknown. S-type sensilla also house four neurons similar to L-type sensilla with the exception that it contain an aversive neuron responds to bitter compounds as well as high salt. Two of the S-type sensilla are similar to L-type sensilla and do not respond to bitter compounds [16]. I-type sensilla are innervated by two neurons [9]. One responds to low salt and sweet compounds and the other responds to high salt and bitter compounds. Some I-type and S-type sensilla were found to respond to acids, as well [10].

By comparing which neurons respond to which compound, researchers could hypothesize if a specific neuron is appetitive or aversive. Sweet taste neurons on the labellum respond to multiple mono- and oligosaccharides, glucosides, sugar acids, and alcohols [31]. These neurons were shown to be appetitive using behavioral experiments [32]. Sweet taste neurons were either

silenced or ablated which led to starved flies consuming less of the compounds detected by those neurons. Expression of a capsaicin receptor in these neurons led to the flies eating capsaicin which they normally showed no preference for. Every sensillum on the labellum expresses a neuron that responds to sugars and other sweet compounds.

A variety of fatty acids are found in plant sources and are attractive to *Drosophila* at low concentrations [33]. They are detected by the sweet neurons on the labellum [18]. When sweet neurons were killed, the flies did not respond to fatty acids. Furthermore, fatty acid detection is dependent on the Phospholipase C (PLC) pathway. Using *norpA* mutant flies, the PLC pathway was knocked down and fatty acid detection was lost. While fatty acid detection was lost, sugar detection is unaffected suggesting two separate receptors in the same neuron.

Water neurons respond to hypo-osmolarity [34]. If the osmolarity of a potential liquid is high, then the water neuron has low neuronal activity. As osmolarity drops, the neuronal activity increases. The perception of water induces feeding in thirsty flies so they are considered appetitive neurons [26, 34]. The L-type and S-type sensilla have neurons that respond to water while I-type do not.

As previously stated, low concentrations of salt are attractive to flies and flies avoid high concentrations of salt [11, 17, 35]. Two different neurons respond to different concentrations of salt. All sensilla on the labellum have neurons that

respond to low salt stimuli but the most sensitive are in the L-type and S-type sensilla. At 100mM NaCl, the low salt attractive neurons have the highest response but neural response falls as the concentration increases [36]. For concentrations above 10mM, high salt aversive neurons fire in a dose dependent manner. In the I-type and most of the S-type sensilla, these neurons also respond to bitter compounds and a further specialized sub-class of these neurons respond to low pH [10].

To determine if a specific neuron type is required for avoidance behaviors; aversive compounds are typically mixed with an attractive stimulus to determine how they alter feeding behaviors. This means if an aversive neuron class is silenced, a hungry fly will consume more of an attractive compound mixed with an aversive compound when compared to *wild type* flies. Many deterrent compounds are alkaloids, terpenoids, phenolic compounds, and non-organic acids [10, 16, 37-40]. Most bitter compounds are usually harmful to the health of the fly or their presence at high concentrations indicates a poor quality food source. On the labellum, all of the sensilla have been tested by a panel of bitter compounds. Based on this panel, the bitter neurons on the labellum can be classified into multiple groups ( **Fig. 1.1 A**) [16]. The S-A and S-B type bitter neurons respond to the most compounds, and I-A and I-B type bitter neurons respond to fewer compounds. The L-type and S-C type sensilla respond to none of the tested bitter compounds.

Avoidance of non-organic acids seems to be driven by low pH [10]. The anions of some organic acids are appetitive while others are aversive [16, 31]. This means potentially two neuron classes could be activated: a deterrent neuron which responds to low pH or the anion itself and an appetitive neuron that responds to the anion of an organic acid [10]. The S-B and I-B type bitter neurons have the strongest concentration dependent response to pH while the S-A and I-A type bitter neurons have weaker responses.

#### 1.1.2.2 Sensilla on the tarsi

The tarsi are the first to come into contact with a potential food source and are used for probing [41]. In essence, the tarsi are thought to be the first taste organ involved in feeding behaviors. Taste hairs are usually found on the distal segments of the tarsi. Taste sensilla can be found on the foreleg, midleg, and hindleg (**Fig. 1.1B**) Females have about 28, 21, 22 sensilla, respectively. In comparison, male flies have more sensilla on the forelegs, which are hypothesized to be involved in pheromone detection, but relatively similar numbers on the midleg, and foreleg [30, 42]. Many experiments used calcium imaging to record activity to different stimuli [30, 43]. Using specific-GAL4 drivers, it was shown that there are four taste cell types that detect taste compounds on the tarsi. Separate neurons respond to water, low salt, sugar and bitter. Some neurons also respond to amino acids and are thought to be the same neurons that respond to sugar, since calcium imaging experiments found significant

overlap between neurons responding to both sweet compounds and amino acids [13, 44]. There are also specialized taste cells on the tarsi that are thought to be involved in pheromone detection, which are distinct from these neurons and are involved in courtship and sexual behavior [45-47].

#### 1.1.2.3 Taste pegs and pharyngeal organs

On the inner surface of the labellum are about 30 peg sensilla on each side, which come in contact with the food substrate when the fly begins to feed. Not much is known about the function of taste pegs, except that they respond to carbonation [48]. Using a library of enhancer trap Gal4 lines, E409-GAL4 was found to be expressed in some of the peg neuron. Using calcium imaging, these neurons were shown to respond to carbonation but not to any sweet or bitter compounds. After genetically silencing E409 neurons, flies consumed less carbonated water compared to control flies. Furthermore, these neurons were shown to be appetitive by expressing a capsaicin receptor using E409-GAL4. After expression of the capsaicin receptor, the flies consumed higher concentrations of food containing capsaicin.

In the pharynx, there are three internal taste organs called the labral sense organ (LSOs), the ventral cibarial sense organ (VCSO), and the dorsal cibarial sense organ (DCSO) [27]. Only sweet taste neurons have been characterized within internal taste organs (**Fig 1.1C**) [27, 48]. As with the pegs, little is known about what compounds the pharyngeal organs detect, though

sweet and bitter taste receptors have been shown to be expressed in them and some of the neurons in the LSO respond to high salt and lipo polysaccharides [27, 38, 49].

#### 1.1.2.4 Gustatory neurons of the wings

Most of the chemosensory neurons on the wings are found on the anterior margin of the wing[23]. Using calcium imaging, they have been shown to responds to both sugar and bitter tastants. It is believed that chemosensory neurons on the wing margins are used to detect nebulized water droplets that contain sugar and bitter substances. Then, flies would aggregate in the direction of the sugar water vapor.

All taste cells express receptors which are used to detect different taste compounds. The receptors that have been identified so far will be described in the next section.

### 1.1.3 Molecular taste organization

All of the taste organs have taste sensilla or cells that respond to different compounds. As of now, a collection of pickpocket (ppk) receptors [50-52], ionotropic receptors (IR) [35, 53], transient receptor potential channel (Trp) [54, 55], and gustatory receptors (Gr) [56-59] have been identified to be involved in the taste detection.

#### 1.1.3.1 Ppk genes are involved in the detection of water and high salt

Ppk genes encode degerin/epithelial sodium channels (Deg/ENaC) which are non-voltage gated, amiloride sensitive cation channels. Ppk receptors are important for multiple mechanisms including: pheromone detection, mechanical nociception, and liquid clearance from the trachea as well as detection of taste compounds [52, 60, 61]. Both water and salt detection are dependent on the expression of ppk genes. Ppk 28 is involved in water detection and is responsible for osmolarity discrimination [51]. Using tip recording, a knockout of ppk 28 led to a reduction in neural response of the water neuron to low osmolarity. Furthermore, heterologous expression of ppk 28 in bitter neurons and HEK cells conferred water sensitivity.

Another ppk gene, ppk 19, and the gene *Serrano* are necessary for high salt behavior in larvae [11]. Knockouts of both genes cause a loss of avoidance to high salt in larvae. At this point, the receptor for high salt detection in adults is unknown. In larva, ppk19 and ppk11 are necessary for attractive salt detection [62] An IR is necessary for low salt attraction.

#### 1.1.3.2 IRs are involved in the detection of salt and amino acids

Ir genes are expressed in all taste organs but their functions are not well understood though many steps have been taken to deorphanize them. They are involved in detection of low salt, polyamines and amino acids [13, 35, 63]. In the

olfactory system, Irs are involved in the detection of some odors, acids and pheromones [53, 64, 65].

Ir76b is one of the most broadly expressed Irs and is found in every taste organ (**Fig. 1.1**). In the adult fly, Ir76b is involved in low salt taste, polyamines, and amino acid detection [35, 63]. Ir76b is a semipermeable $Na^+$ channel that is sensitive to changes in concentration of sodium ions in the sensillar lymph. Flies without Ir76b will reject low concentrations of salt. Since they still reject high concentration of salts, it indicates that only aversive salt neurons are functioning and Ir76b is not necessary for high salt detection. Ir76b is also expressed in gustatory neurons that do not respond to salt and is important for amino acid detection in these neurons [65]. Loss of Ir76b and other Ir genes leads to reduction in feeding behaviors to appetitive amino acids [13]. When Ir76b was coexpressed with Ir20a in a sweet taste neuron, it conferred detection of a three amino acid mixture containing serine, threonine, and phenylalanine. This suggests that on its own Ir76b is a low salt detector but when expressed with other Ir genes it is an co-receptor to detect amino acids

#### 1.1.3.3 Trp channels

Trp channels are some of the most evolutionarily conserved receptors with orthologs found in humans and mammals [66, 67]. There are 13 members in the *Drosophila* Trp family. Nine Trp channels are involved in mechanosensing, hearing, phototransduction and temperature sensitivity [67-69]. Two Trp

channels are involved in gustatory detection of deterrents. Trpl is involved in camphor detection in labellar taste hairs [55]. TrpA1 is required for the avoidance of aristolochic acid and bacterial lipopolysaccharides [38, 70]. Both are expressed in aversive neurons in S-type sensilla. Considering that TrpA1 is also involved in thermal sensing, it is hypothesized that TrpA1 is involved in integrating both temperature and chemical information in gustatory neurons.

#### 1.1.3.4 Gr

Gr receptors are the receptors responsible for the detection of sweet and bitter compounds. In 2001, a family of 68 Gr genes was discovered using a BLAST algorithm to locate sequences encoding multi-transmembrane receptors in *Drosophila* [56] (**Fig. 1.2**). Since then, Grs have also been discovered in all insects and arthropods and even Gr-like homologs are found in *C. elegans* [71]. The Gr family in flies is highly divergent with as little as 8% sequence identity between receptors [72]. The closest related receptors are Gr5a and Gr64f with 40% amino acid identity [31]. A common feature of all *Drosophila* Gr proteins is a highly conserved C-terminus domain [56].

Grs have been linked to the detection of sweet and bitter compounds, pheromones, odors, and internal nutrients [31, 37, 39, 73-77]. Recent studies also suggest they play a role in photoreception and thermosensation [78, 79]. They have been found in multiple locations including sweet and bitter taste neurons, in the reproductive system, neuroendocrine cells, the central nervous

system, and in the olfactory system [58, 75, 80, 81]. Many Grs are also coexpressed in the same neurons as IRs [35]. This is true for both appetitive and aversive neurons.

There are still questions about whether Grs are G-protein coupled receptors (GPCR) or ligand gated ion channels. Gustatory receptors are unique in that they have an inverted topology compared to traditional GPCRs and other multi-transmembrane proteins [82]. Olfactory receptors (Ors) which are closely related to the Gr family have an inverted topology as well [83]. Previously, Ors were shown to be ligand gated ion channels so it was hypothesized that Grs may function similarly as well [84, 85]. Furthermore, Gr43a and its *Bombyx mori* ortholog, BmGr9, both function in cell culture and BmGr9 was to function as a cation channel [86]. When BmGr9 is expressed in cell culture, the cells respond to fructose with an influx of extracellular calcium when performing whole cell recordings. This suggests that Grs are cation channels but there is strong evidence of GPCR involvement as well.

Since GPCR are important for development and multiple functions, a mutant that disrupts all GPCR signaling would cause multiple physiological problems and could cause lethality. Due to this problem, previous labs used RNAi to knockdown expression of multiple G-proteins involved for GPCR transduction pathway. Chemicals that block specific sections of the GPCR pathway were implemented as well. RNAi for Goα subunits causes a modest reduction to neuronal responses to sucrose in sweet taste neurons and reduces

feeding behaviors for sucrose [87]. PTX which inhibits Goα and Giα significantly reduces responses to sucrose especially at lower concentrations. Expression of Gsα RNAi or Gγ1 RNAi also reduces responses to sucrose and other sugars [88]. Another G protein of interest is Gqα. Two Gr are necessary for $CO_2$ detection, Gr21a and Gr63a, in the olfactory system. Knock down of Gqα using RNAi, significantly reduces neuronal responses to $CO_2$ [89]. Furthermore when Gr21a and Gr63a are misexpressed in another olfactory neuron, expression of Gqα dramatically increases the neuron's responses to carbon dioxide [90]. Based on these studies, it is suggested that g-proteins play a role in signal transduction from Grs. None of these knockdown experiments cause a complete loss of neuronal responses in taste neurons which leaves the possibility of another mechanism open.

As of now, it is not completely known how these receptors function. There are examples of Grs functioning alone, in pairs, or in triplets [91-93]. Interestingly, there is a complete separation and no overlap between receptors that are expressed in $CO_2$ olfactory neuron, bitter taste neurons, or sweet taste neurons[16]. This means that Grs do not have a common co-receptor involved for all signal transduction as seen for Ors in the olfactory system. All Ors are expressed with OrCo which is necessary for signal transduction [94]. Due to this, Grs can be divided based on where they are expressed ( **Fig. 1.2**). The next two sections will focus on bitter and sweet Grs.

#### 1.1.3.4.1 Bitter Grs

Most members of the Gr family were discovered to be expressed in bitter neurons via GAL4 analysis and are hypothesized to be involved in the detection of bitter compounds (**Fig 1.2**). The sheer number of bitter Grs expressed in a single neuron makes studying bitter taste detection more difficult. These receptors have been found to be expressed singly like Gr66a in peg bitter neurons to as many as twenty eight in a single neuron on the labellum [16, 27]. It is unknown how bitter Grs function together to detect bitter compounds

On the labellum, each bitter neuron expresses five putative co-receptors, known as the Commonly Expressed Receptors (CERs), Gr33a, Gr32a, Gr66a, Gr89a, and Gr39a.a (**Fig 1.1A**) [16]. These receptors are believed to play an important role. Previous experiments have shown that loss of Gr32a, Gr33a, or Gr66a, causes a dramatic reduction in avoidance behaviors and neuronal response in deterrent neurons to bitter stimuli [39, 76, 77, 95]. The S-type bitter neurons express the most receptors and have the broadest neuronal response to bitter compounds [16]. The I-type bitter neurons express fewer receptors and respond to only a handful of compounds. I-a bitter neurons have the fewest bitter receptor with only a single bitter receptor, Gr59c, being expressed with the five CERs. When Gr59c is expressed in another bitter neuron, it confers new ligand sensitivity similar to that of I-a neurons. Gr59c may also play a different role in its endogenous neuron. Previously, Delventhal and Carlson (2016) expressed bitter Grs in I-a neurons with and without Gr59c and recorded the neural activity using

tip recordings [81]. When bitter Grs are expressed in I-a bitter neurons without Gr59c, there are new ligand sensitivities to multiple compounds. Surprisingly, expression with Gr59c drastically inhibits the responses to some of the compounds but not others. Furthermore, deletion of Gr59c leads to I-a bitter neurons having a similar response profile as I-b bitter neurons. This suggests the Gr59c and potentially other Grs can play both an inhibitory role as well as an excitatory one. It also suggests that some of the CERs may be involved in ligand detection since Gr59c is not necessary for some bitter responses in I-a bitter neurons.

While it is still unknown how bitter Grs function together, both mutant and misexpression experiments have provided some clues. In flies that do not express Gr47a, S-b bitter neurons completely lose responses to strychnine and avoidance behavior is greatly reduced [37]. Interestingly, single mutants of some CERS, Gr32a, Gr33a, and Gr66a, do not have a loss in avoidance to strychnine but they do have a loss of strychnine induced action potentials in S-type bitter neurons. S-type bitter neurons are the only bitter neurons on the labellum to respond to strychnine. Furthermore, misexpression of Gr47a in I-type bitter neurons does not confer strychnine sensitivity. This suggests that another Gr may be involved for strychnine activity and that Gr47a may be expressed in bitter neurons on other taste organs without Gr32a, Gr33a, or Gr66a.

Multiple receptors including members of the CERs seem to be necessary for responses to bitter compounds. Single mutants of Gr93a, Gr66a, and Gr33a

have defects in caffeine driven neural activity and avoidance behaviors but misexpression of all three receptors cannot confer caffeine detection [39, 95]. This leads to questions of how many Grs are necessary for responses. So far only one receptor complex for a bitter compound, L-canavanine, has been found [93]. Loss of Gr8a, Gr98b, or Gr66a each causes a loss of neuronal and behavioral responses to L-canavanine. When the three are expressed together in either S2 cell or sweet taste neurons, they confer L-canavanine sensitivity [77, 93, 96]. This suggests that at least three Grs maybenecessary for responses to different ligands. It also implies CERs are necessary for responses to be bitter stimuli but all five CERs are not necessary for responses to all bitter compounds.

#### 1.1.3.4.1 Sweet Grs

Within the Gr family, there is an evolutionarily conserved clade of receptors that is expressed in sweet taste neurons (**Fig. 1.2**). The sweet receptor clade is unique in that members have been found in multiple insects and arthropods including the ancient crustacean, *Daphnia pulex* [97]. In comparison to the 44 bitter Grs, there are only nine Grs that encompass sweet taste. Eight of these are part of the sweet clade, plus Gr43a which is found outside of the clade. Within the twelve other *Drosophila* species, one to one orthologs can be found in each of the genomes except for some cases [71]. Gr64e is pseudogenized in *Drosophila pseudoobscura* and *Drosophila persimmons*. Gr5a is missing in both these species as well as being pseudogenized in *Drosophila grimshawi* [71].

Gr43a, the one sweet receptor found outside of the clade, is highly conserved with orthologs found in all insects [71]. Gr43a is unique in that it is found in the brain where it measures fructose levels in the hemolymph and functions as an internal nutrient detector [75].

Sweet Grs are found in all sweet taste neurons in the fly and many are coexpressed together. This has been shown using GAL4 analysis and functional studies [16, 58]. They have also been found in multiple locations outside of canonical sweet taste neurons including neuroendocrine cells, and in the brain [80, 98]. There is controversy in the field over which sweet receptors are expressed in labellar sweet taste neurons, specifically the Gr64 cluster. In the labellum, all receptors of the sweet clade have been shown to be expressed together using GAL4 analysis with the exception of Gr64a which has only been shown using functional studies [16, 31] Studies show that flies lacking Gr64a appear to have defects in sugar detection when recording from labellar sweet taste neurons, a deficit that can be rescued by artificially expressing Gr64a [31, 99, 100]. As such, there are still some questions that need resolving.

It is unknown how these receptors function together to detect sweet compounds or which ones are involved in ligand recognition. Compared to mammals' two receptors necessary for sweet tastant detection, *Drosophila* have nine [6, 99, 101]. One interesting question is why do insects have so many sweet receptors. Single Gr mutants of two receptors, Gr5a and Gr64a, have multiple defects for two large non-overlapping subsets of sugars when recording from

labellar L-type sensilla [31]. Both Gr5a and Gr64f are necessary for trehalose detection in sweet taste neurons and for appetitive behaviors for trehalose [100]. Gr5a is also involved glucose detection with Gr61a [31, 102]. Wisotsky *et al.* (2011) showed that flies lacking Gr64e have a defect for glycerol in both labellar tip recordings and behavior [57]. Loss of a single sweet receptor does not cause a complete loss of sugar detection in labellar taste neurons which argues against one obligate co-receptor for all receptor complexes

Since sweet receptors are expressed in multiple combinations and single mutants of any sweet Grs can reduce neural responses to sweet tastants, the composition of an endogenous sweet receptor is unknown (**Fig.1.1**) [31, 58, 81, 99, 100, 102, 103]. Only two examples are known where labellar L-type sweet neurons lose all responses to sweet compounds due to loss of sweet receptors. Those include flies lacking both Gr5a and Gr64a or where the entire Gr64 cluster is gone [31, 100]. In both cases, there is still not a complete loss of sugar detection by flies, since Gr43a when expressed alone in tarsi still responds to sucrose and fructose [92]. There are multiple examples where heterologous expression of a single receptor in cell culture or misexpression of multiple receptors in neurons devoid of sweet Grs can confer or rescue sugar detection. Both Gr5a and Gr43a can function independent of other Grs in cell culture [86, 91]. Pairs of sweet Grs can function in a “empty” sweet taste neuron that is devoid of all other sweet receptors [92]. When Gr64b and Gr64e are expressed in an “empty” sweet taste neuron, the pair responds glycerol. Coexpression of

Gr64a and Gr64e in this same empty taste neuron rescues sucrose and maltose detection. Gr64a and Gr64f in labellar L-type sweet neuron in *ΔGr64* flies also rescued sucrose detection [100]. In this case, Gr5a and Gr61a are endogenously expressed while Gr64a and Gr64f are artificially expressed. Artificial expression of single sweet Grs with the endogenously expressed Gr43a in a tarsal neuron can confer ligand sensitivity to multiple sweet tastants in *ΔGr5a; ΔGr1a, ΔGr64* flies. Overall, it appears there may be no common core receptor for sweet Grs and that multiple combinations of sweet Grs may be able to respond to sweet compounds. There are three combinations that have been found that can rescue or maintain sucrose detection: Gr43a [92], Gr64a+Gr64e [92], and Gr5a+Gr61a+Gr64a+Gr64f [100]. Because of the degree of overlap in sucrose detection it suggests that multiple receptors are capable of ligand recognition of sucrose and sweet Grs may be able to function in multiple overlapping combinations.

### 1.1.4 Taste modulation at the periphery

Many potential food sources are complex and contain both appetitive and aversive stimuli. It is essential to avoid the ingestion of food sources that can be toxic to the fly. A highly adaptive taste system is required to quickly respond to new stimuli. Flies have multiple mechanisms to avoid toxins. First, there are deterrent neurons expressed in taste organs and are activated by aversive stimuli such as bitter compounds, high salt, and low pH [9, 10, 16, 30]. Secondly,

there are other mechanisms that are capable of modulating taste perception. Bitter tastants can directly inhibit sweet neurons and activation of bitter or mechanosensory neurons can suppress sugar induced responses via GABAergic interneurons [15, 22, 104, 105]. Others are nutrient based and depend on previous experience. [20, 106-108].

#### 1.1.4.1 Taste modulation via direct inhibition

Bitter compounds are usually harmful even at low concentrations. While activation of bitter neurons leads to avoidance of potential toxins, other mechanisms involve inhibiting the sweet neuron neural response. By increasing the threshold required to activate sweet neurons, it allows flies to avoid poor quality foods. When recording from L-type labellar taste hairs using mixtures of sucrose and bitter tastants, the neural activity of sweet neurons is reduced when compared to sucrose alone (**Fig.1.3 A**) [104]. Since L-type taste hairs do not express bitter Grs or have a canonical bitter neuron, inhibition of the sweet neuron is in the absence of any bitter neuron activation. An odorant binding protein, OBP49a, is involved in inhibiting the sweet neuron by bitter compounds [104]. When recording from L-type sensilla using mixtures of sucrose and bitter compounds in flies lacking obp49a, inhibition by bitter compounds is reduced and the mixture induces the same level of neural activity as sucrose alone. OBP49a is produced by the support cells in labellar taste hairs and is found in the sensillar lymph. Many bitter compounds are found in low doses and are usually

hydrophobic. It is thought that OBP49a may ferry the bitter compounds to sweet neurons and either OBP49a or the bitter compound inhibits the sweet neuron. One of the advantages of this mechanism is that it increases sensitivity to bitter compounds when mixed in food sources with low sugar concentrations [15, 104].

Beside bitter compounds, acids can inhibit labellar sweet neurons. When performing tip recordings on L-type sensilla, low pH compounds mixed with sugar inhibit the neural response of sweet neurons in a pH dependent manner. [10]. In tarsal taste neurons, both sweet and bitter neurons are affected by acids [106]. Both activation of bitter neurons by bitter compounds and bitter tastant mediated inhibition of sweet neuron are reduced by acids with pH between 3.2 and 4. It is possible sweet taste neurons on the tarsi may be inhibited by acids if a lower pH was used [106]. pH levels between 3-1 showed inhibition of labellar sweet taste neuron [10]. Similar concentrations would need to be used to see if tarsal and labellar taste neurons respond differently to mixed stimuli.

#### 1.1.4.2 Taste modulation via GABAergic interneurons

Sweet taste neurons can be inhibited by another mechanism besides direct inhibition. Bitter or mechanosensory neurons' activation can inhibit the sweet neuron's sucrose response via GABAergic interneuron presynaptic inhibition (**Fig. 1.3 A**) [22, 109]. Using GFP reconstitution across synaptic partners (GRASPs), GABA interneurons were shown to have multiple interconnections with bitter and sweet taste neurons in the SEZ. $GABA_bR2$ is

thought to be the receptor for GABA since it is coexpressed with sweet Grs in sweet taste neurons. Moreover in $GABA_bR2$-RNAi expressing flies, there was a significant reduction in inhibition of sweet taste neurons by bitter neuron activation.

Sweet taste neurons are inhibited by mechanosensory neurons as well [109]. In terms of palatability, food texture plays an important role in feeding behaviors. When choosing between two food sources with the same sucrose content, flies prefer softer food texture. In fact, flies will choose a softer food over higher sucrose content if the texture is too hard. As with bitter neuron activity, mechanosensory neurons inhibit sweet taste neurons via GABAergic interneurons. Inhibition is also dependent on $GABA_bR2$. When mechanosensory neurons are silenced, flies will pick higher sucrose concentrations independent of food texture.

#### 1.1.4.3 Taste modulation by previous experience and starvation

Previous dietary experience modulates taste neuron sensitivity [55] (**Fig. 1.3 C**). Trpl is required for detection of camphor, a non-toxic bitter compound. When camphor is placed in foods for long periods of time, bitter neurons have a reduced response to camphor (**Fig. 1.3 B**). Changes in sensitivity are due to E3 ubiquitin ligase-regulated decline which reduces Trpl levels in bitter neurons. This suggests that the previous exposure and lack of toxicity led to changes in the

bitter neuron for higher acceptance of camphor. When flies are returned to their normal diet, camphor induced activity returns to normal levels.

As previously mentioned when flies are deprived of amino acids, water, or high calorie sugars, there is an increase in preference for these stimuli [13, 19, 110]. Flies that are starved with access to only water have higher sensitivity to sweet compounds and responses to bitter compounds are subdued (**Fig. 1.3 C**). This enhanced sensitivity for sugar is driven by the release of dopamine and dependent on dNPF signaling [19, 21]. When flies are fed L-dopa or dopaminergic neurons are activated using TrpA1, there is an increase in sensitivity to sucrose. This enhancement by starvation is thought to be driven by an increase in intracellular free $Ca^{2+}$ for both baseline and sugar induced activity downstream of the sweet receptors on the neuron. Moreover in the wild derived strain, TW1, starvation was shown to induce upregulation of Gr64a expression in sweet taste neuron, which increases sensitivity to sucrose especially at low concentrations [108].

Independent of changes in sweet neuron sensitivity, bitter neurons have reduced sensitivity to bitter tastants when flies are starved for a long period. Using calcium imaging in the axons of bitter neurons in the SEZ, bitter neurons show a reduction in bitter neural activity in starved flies compared to fed flies [21]. Inhibition of bitter neurons is not dependent on dopamine but instead on a neuropeptide, sNPF. Flies lacking sNPF have higher sensitivity to bitter compounds compared to wild type flies when starved. Starvation cues are

thought to cause the lateral neurosecretory cells to secrete sNPF which is detected by GABA interneurons that inhibit bitter neurons. Moreover, there is a small cluster of neurons that also modulate bitter neurons output during starvation. The ventrallateral cluster octopaminergic neurons (OA-VL) produce octopamine and tyramine which directly depress bitter taste neurons' neural output [107]. Using GRASP, the OA-VL were shown to be in close proximity to the bitter neurons axons terminals in the SEZ. When flies are starved, the neural activity is reduced in OA-VL neurons. This leads to the release of octopamine and tyramine which inhibits the synaptic output of bitter neurons. Both of these neurotransmitters are thought to be detected by OC-TyR on bitter neurons.

### 1.1.5 Conclusion

Recent studies have revealed that ppk, Grs, IRs, and Trp receptors are responsible for the detection of multiple taste modalities. Still, there remain critical gaps in the field. Many members of the IR, and Gr families have not been deorphanized. Little is known about the actual composition of a sweet taste receptor complex, if all the sweet receptors respond to sweet compounds or if there is an obligate coreceptor.

The development of new ectopic expression tools could lead to further analysis of taste receptors. Many members of these classes of receptors are expressed across all insect species. Insight into the properties of individual receptors would broaden the understanding of taste across all insect species, including disease vectors.

## 1.2 Goals of this Study

In this study, we set out to design a heterologous expression system to express individual members of the Gr family. Using the $CO_2$ neuron of the olfactory system, we express both sweet and bitter Grs individually and apply different stimuli to the sensillar lymph using an electrode to determine which Gr confers ligand sensitivity (**Chapter 2**). Using this system, we successfully expressed receptors of the sweet clade in *D. melanogaster,* Gr43a, bitter receptors, and putative sweet receptors from the malaria vector, *A. gambiae*. We demonstrate that all the receptors of the sweet clade are capable of detecting sweet compounds and each receptor can detect a unique subset of sugars (**Chapter 3**). Furthermore, the sweet receptor clade can be loosely separated into two groups: one that detects Gr5a dependent sugars and one that detects Gr64a dependent sugars.

Using a panel of 20 diverse bitter compounds, we also determine that the sweet taste neuron response to multiple sugars can be inhibited by bitter tastants even in the absence of OBP49a at higher but environmentally relevant concentrations (**Chapter 4**). Multiple bitter compounds can inhibit labellar sweet taste neurons with different degrees of inhibition suggesting specificity. Surprisingly, we discover that individual sweet receptor's sugar responses can be directly inhibited by bitter compounds in a dose dependent manner. Each receptor of the sweet clade is inhibited by a unique panel of bitter compounds with different levels of sensitivities supporting the theory that bitter inhibition is

dependent on receptor specificity. Gr43a and its mosquito ortholog, AgGr25, are immune to this inhibition and it seems that bitter tastant mediated inhibition is unique to the sweet clade.

In Chapter 5, we set out to study sweet tastant detection in the malaria vector, *A. gambiae* (**Chapter 5**). Their sweet labellar neurons are capable of detecting multiple sweet compounds. We then expressed every member of the *A. gambiae* sweet Gr family individually in the *D. melanogaster* $CO_2$ sensing neuron. All the receptors function and each receptor responds to a unique panel of sweet compounds. The inhibition of labellar sweet taste neurons by bitter compounds is conserved between flies and mosquitoes, as revealed by recordings from two different mosquito species. Furthermore, we demonstrate that a receptor of the mosquito sweet clade can be directly inhibited by bitter tastants suggesting bitter tastant mediated inhibition of sweet receptors is evolutionarily conserved in the sweet clade of insects.

### 1.3 Relevance

This study fills in a critical gap in the field. We created a system that can ligand detection properties of individual sweet and bitter receptors and showed that all the sweet Grs are involved in ligand detection. Before this, there were only two examples of receptors involved in ligand detection, Gr5a and Gr43a. Furthermore, we discovered that the sweet Grs are dual sensors of both sweet and bitter compounds. Bitter tastant mediated inhibition of sweet Grs may be

evolutionarily conserved since a sweet *A. gambiae* Gr response to sugar can be inhibited by bitter compounds. Understanding the mechanisms for bitter and sweet detection will allow us to build better techniques to control insect populations and understand mechanisms involved in sugar detection in insects.

### 1.4 Abbreviations

Subesophageal ganglion (SEZ) Pg. 4

Long type sensilla (L-type sensilla) Pg.5

Intermediate type sensilla (I-type sensilla) Pg.5

Small type sensilla (S-type sensilla) Pg.5

Labral sense organ (LSO) Pg.10

Ventral cibarial sense organ (VCSO) Pg. 10

Dorsal cibarial sense organ (DCSO) Pg 10

Pickpocket (ppk) receptor Pg.11

Ionotropic receptors (IR) Pg.11

Transient receptor potential channel (Trp) Pg.11

Gustatory receptors (Gr) Pg.11

Degerin/epithelial sodium channels (Deg/ENaC) Pg.11

G-protein coupled receptors (GPCR) Pg. 14

Olfactory receptors (Ors) Pg. 14

Ventrallateral cluster octopaminergic neurons (OA-VL) Pg. 27

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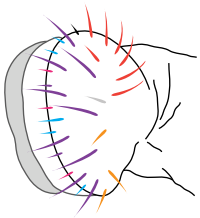
# Figure 1.1

**A**

Labellum



- L
- S-a
- S-b
- I-a
- I-b

Sweet
Bitter
Salt (appetitive)
Water
Deterrent (uncharacterized)
Uncharacterized
Pheromone

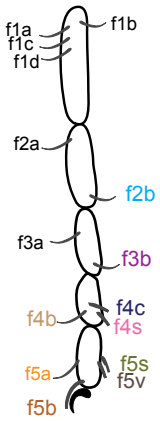
| Sensillum | L | S-a | S-b | S-c | I-a | I-b |
|---|---|---|---|---|---|---|
| Neurons | | | | | | |

| Neuron | Sensillum | Receptors |
|---|---|---|
| | L | Gr5a, Gr61a, Gr64a, Gr64e, Gr64f, Ir47a, Ir56b *Gr64b, Gr64c, Gr64d* |
| | S-a, S-b, S-c | Gr5a, Gr64a, Gr64e, Gr64f, Ir56b* *Gr64b, Gr64c, Gr64d* |
| | I-a, I-b | Gr5a, Gr64a, Gr64e, Gr64f *Gr61a, Gr64b, Gr64c, Gr64d* |
| | S-a | **Gr32a, Gr33a, Gr39a.a, Gr66a, Gr89a**, Gr8a, Gr22e, Gr22f, Gr28a, Gr28b.a, Gr28b.d, Gr28b.e, Gr36a, Gr36c, Gr39b, Gr59a |
| | S-b | **Gr32a,Gr33a,Gr39a.a, Gr66a, Gr89a,** Gr8a,Gr22b, Gr22d, Gr22e, Gr28a, Gr28b.a, Gr28b.e, Gr36b, Gr36c, Gr39a.b, Gr39a.d, Gr39b, Gr57a Gr58b, Gr59c, Gr59d, Gr92a, Gr93a, Gr93b, Gr98b, Gr98c, Gr98d, Ir56a* |
| | I-a | **Gr32a, Gr33a, Gr39a.a, Gr66a, Gr89a,**Gr59c, Ir56a* |
| | I-b | **Gr32a, Gr33a, Gr39a.a, Gr66a, Gr89a,** Gr22b, Gr28a, Gr28b.a, Gr28b.e,Gr47a |
| | L | Ir76b |
| | L, S-a, S-b, S-c | ppk28 |
| | S-a, S-b, S-c | ppk23, ppk29 |
| | L | unknown |
| | S-c | unknown |

| Sensillum | Receptors |
|---|---|
| I (Sweet, ?) | Ir56d, Ir94e* (Only in I0) |
| L (Sweet, ?) | Ir56d, Ir94e* (Only in a subset) |
| S (Sweet, ?) | Ir47a, Ir56d* (Only in S2 and S6) |
| S (Deterrent) | TrpA1 |
| S (Deterrent) | TrpL |

**B**

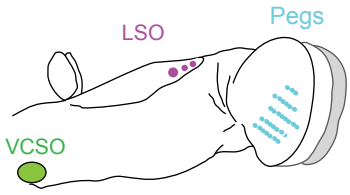
Female Tarsi




| Neuron | Sensillum | Receptors |
|---|---|---|
| | f2b, f3b | Gr5a, Gr61a, Gr64c, Gr64e, Gr64f |
| | f5v | Gr43a, Gr61a, Gr64a, Gr64c, Gr64e, Gr64f |
| | f4b, f5s | Gr5a, Gr61a, Gr64a, Gr64b, Gr64c, Gr64e, Gr64f |
| | f5a | Gr61a, Gr64b, Gr64f |
| | f4s, f5b | Gr5a, Gr61a, Gr64a, Gr64b, Gr64c, Gr64f |
| | f4s, f4c, f5b | **Gr32a, Gr33a, Gr39a,a, Gr89a,** Gr58c |
| | f5s | **Gr33a, Gr39a,a, Gr66a, Gr89a,** Gr8a, Gr22b, Gr22a, Gr22d, Gr28a, Gr28b.c, Gr28b.d, Gr28b.e, Gr39b, Gr59a, Gr59d, Gr93b, Gr98d |

| Sensillum | Receptors |
|---|---|
| f1a, f1c, f1d, f2a | Ir52a, Ir52c, Ir52d, Ir56d |
| f1b, f3a, f4b, f5a | Ir52a, Ir52c, Ir52d |
| f2b | Ir52a, Ir56b, Ir56d |
| f3b | Ir52a, Ir56b |
| f4s, f5b | Ir47a, Ir56b |
| f5s, f5v | Ir20a, Ir47a, Ir56b, Ir62a, Ir94h |
| Not mapped (Low salt) | Ir76b |
| Not mapped (Water) | ppk28 |

**C**




Pharynx

| Organs | Receptors |
|---|---|
| LSO | |
| Sweet | Gr43a, Gr61a, Gr64a, Gr64c, Gr64d, Gr64e, Gr64f, *Gr64b* |
| Bitter | Gr66a, TrpA1 |
| Not mapped | Ir20a, Ir56a, Ir60b, Ir67c, Ir94f, Ir94h |
| VCSO | |
| Sweet | Gr43a, Gr61a, Gr64a, Gr64c, Gr64e, Gr64f |
| Not mapped | Ir20a, Ir94a, Ir94c, Ir94h |

Pegs

| Taste | Receptors |
|---|---|
| Bitter | Gr66a |
| Sweet | Gr5a, Gr64a, Gr64b, Gr64c, Gr64e, Gr64f |
| Carbonation | unknown |
| Not mapped | Ir56d, Ir76b |

**Figure 1.1 Receptor to neuron map of known taste receptors in adults**

**(A)** Schematic of sensillar classes in the labellum, defined by receptor expression patterns and functional analysis (left). Tables indicating identified neurons (center) or sensilla (right) with their receptor expression patterns. Maps created from expression studies along with, in some instances, functional studies; with the exception of Gr64a, which is mapped to labellar sweet neurons by functional studies [31, 99]. Receptors marked with an asterisk are not expressed in every sensillum of the indicated class. Sweet receptors in italics have been mapped by knock-in reporter analysis but not by transgenic reporter experiments. Expression of *ppk23* and *ppk29* has been assigned to S sensilla based on observed pairing of *ppk23*$^{+}$ cells with *Gr66a*$^{+}$ cells. Receptors in bold are broadly expressed in bitter neurons. **(B)** Schematic of sensilla in the female fore tarsi (left) and tables indicating identified neurons (center) or sensilla (right) with their receptor expression patterns. **(C)** Schematic indicating the location of oral taste pegs and the labral sense organ (LSO; chemosensory sensilla in lilac, mechanosensory sensilla in black) and ventral cibarial sense organ (VCSO) in the pharynx. Tables indicating identified neurons (left) with their receptor expression patterns in the LSO and VCSO (center) and the taste pegs (right)

# Figure 1.2

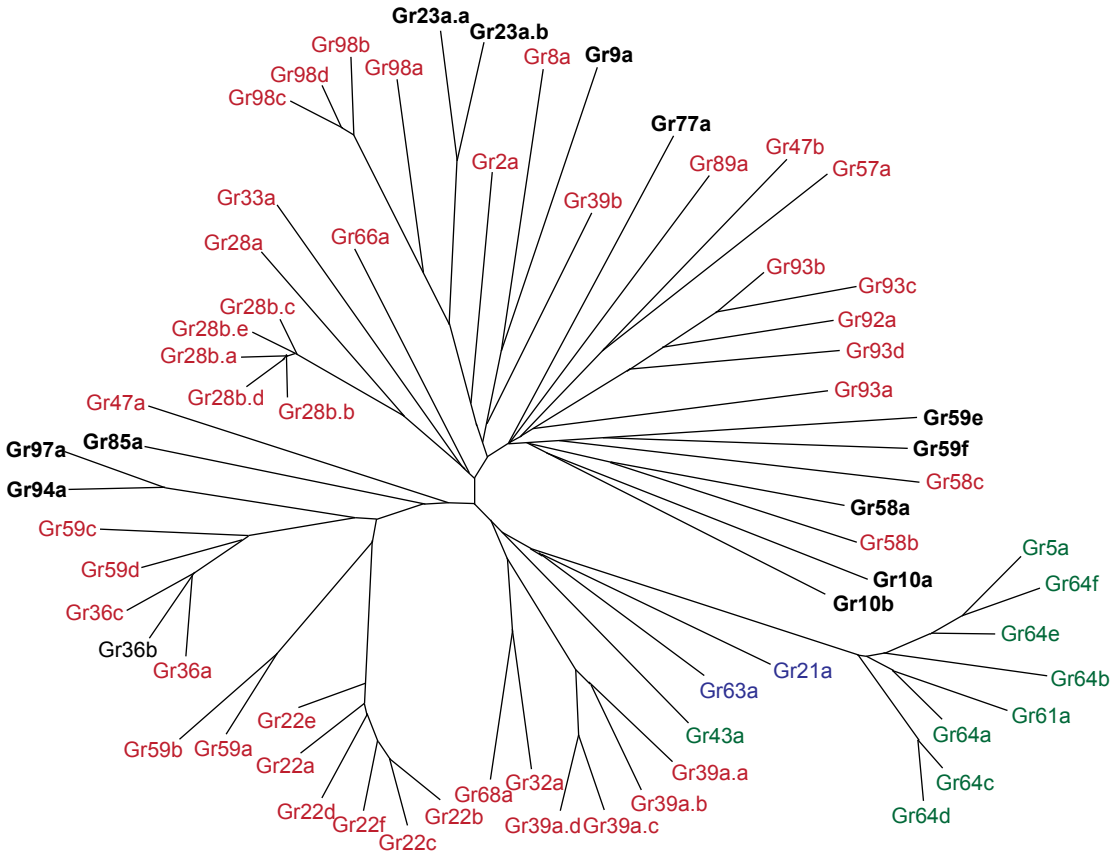

Gr23a.a
Gr23a.b
Gr98b
Gr98a
Gr8a
Gr9a
Gr98d
Gr98c
Gr77a
Gr47b
Gr89a
Gr57a
Gr2a
Gr33a
Gr39b
Gr66a
Gr28a
Gr93b
Gr93c
Gr28b.c
Gr92a
Gr28b.e
Gr93d
Gr28b.a
Gr28b.d
Gr28b.b
Gr93a
Gr47a
Gr59e
Gr97a
Gr85a
Gr59f
Gr94a
Gr58c
Gr58a
Gr59c
Gr58b
Gr59d
Gr10a
Gr10b
Gr36c
Gr36b
Gr36a
Gr63a
Gr21a
Gr22e
Gr59b
Gr59a
Gr22a
Gr43a
Gr39a.a
Gr68a
Gr32a
Gr22d
Gr22f
Gr22b
Gr39a.d
Gr39a.c
Gr39a.b
Gr22c
Gr5a
Gr64f
Gr64e
Gr64b
Gr61a
Gr64a
Gr64c
Gr64d

**Figure 1.2 A phylogenetic Gr tree** adapted from [1]. Red receptors are expressed in bitter neurons, black have not been mapped to any taste neurons, blue receptors are the carbon dioxide receptors and green have been mapped to the sweet neurons

Figure 1.3

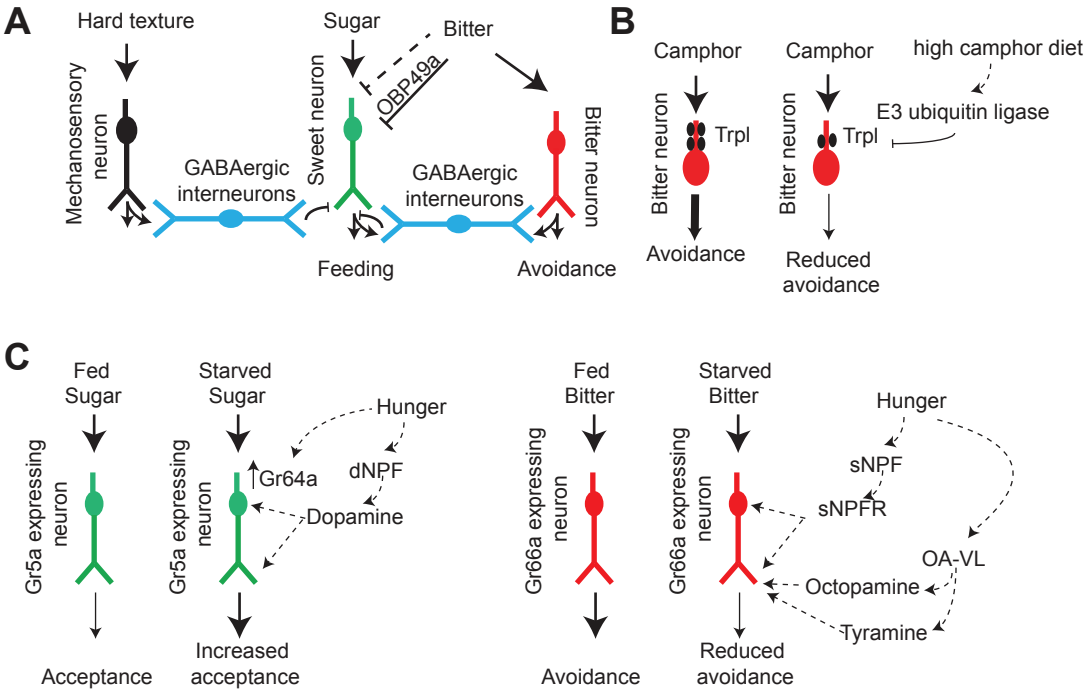

A
Hard texture
Mechanosensory neuron
GABAergic interneurons
Sweet neuron
Sugar
Bitter
OBP49a
GABAergic interneurons
Bitter neuron
Feeding
Avoidance
B
Camphor
Bitter neuron
Trpl
Avoidance
Camphor
high camphor diet
E3 ubiquitin ligase
Bitter neuron
Trpl
Reduced avoidance
C
Fed Sugar
Gr5a expressing neuron
Acceptance
Starved Sugar
Hunger
Gr64a
dNPF
Dopamine
Gr5a expressing neuron
Increased acceptance
Fed Bitter
Gr66a expressing neuron
Avoidance
Starved Bitter
Hunger
sNPF
sNPFR
OA-VL
Octopamine
Tyramine
Gr66a expressing neuron
Reduced avoidance

**Figure 1.3 Modulation of sweet and bitter tastant detection on the periphery.** (**A**) Inhibition of taste neurons (Left) (Green) sweet taste neurons and (Right) (Red) bitter taste neurons either directly or by GABAergic interneurons. (**B**) Modulation of bitter neuron sensitivity to camphor by high camphor diet ( **C**) Modulation of sweet neurons (left) or bitter neurons (right) by starvation. Upregulation of Gr64a upon starvation has only been found in wild derived strain TW1 of *D. melanogaster* [108].

## 2.0 Design and characterization of a heterologous system for functional expression of gustatory receptors *in D. melanogaster*

**Abstract:** Insects use the taste system to determine if a potential food source is suitable for consumption. *Drosophila melanogaster* uses a family of 68 gustatory receptors (Gr) to detect appetitive (sweet) and aversive (bitter) compounds. Grs are expressed in multiple combinations in either sweet or bitter neurons. As of now, it is unknown how these receptors detect tastants or which Grs are involved in ligand recognition of sweet or bitter compounds. A system that can functionally express single Grs from either bitter or sweet neurons to study ligand recognition is necessary to fill a critical gap in the field. We designed a system using a unique olfactory neuron that responds to $CO_2$. After expressing both sweet and bitter receptors individually in the $CO_2$ sensing neuron, we applied multiple tastants and discovered which receptors could confer ligand sensitivity. Both Gr5a and Gr64e are capable of detecting multiple sweet compounds. Expression of either Gr93a or Gr59c conferred sensitivity to multiple bitter compounds. Surprisingly, we were able to successfully express an internal fructose receptor, Gr43a, and its mosquito ortholog, AgGr25, both of which conferred sensitivity to fructose and other sugars. This suggests that we can use the $CO_2$ neuron to potentially deorphanize Gr families from other insects.

## 2.1 Introduction

The taste system is essential for animals to assess the palatability of potential food sources and to avoid toxic compounds. *Drosophila melanogaster* use a highly divergent family of gustatory receptors (Gr) genes to taste the chemical world around them [1, 2]. This family is necessary for the detection of multiple aversive and appetitive compounds. Using multiple Gal4 lines for members of the Gr family, many of these receptors have been shown to be expressed in either bitter or sweet neurons [3-7]. Forty-four Grs are expressed in bitter neurons and nine Grs are expressed in sweet neurons (**Fig.1.2**). None of the Grs are found in both bitter and sweet neurons, indicating that no single Gr functions as an obligate co-receptor required for both sweet and bitter receptor complexes. Despite the lack of overlap between sweet and bitter Grs, these receptors are expressed in a multitude of unique combinations, making the role of each Gr protein more difficult to ascertain (**Fig. 1.1**).

Despite multiple attempts, there are only a few examples of successful deorphanizing of Grs. Two Grs are able to function when expressed singly in cell culture. When Gr5a is expressed in S2 cells, it confers trehalose sensitivity [8]. Expression of Gr43a confers fructose detection to cos7 cells [9]. As of now, no other Grs have worked in cell culture when singly expressed. Other examples of functional expression include expressing receptors in pairs. Using *ΔGr5a; ΔGr61, ΔGr64* flies, individual sweet Grs were expressed in a sweet tarsal neuron where only Gr43a is endogenously expressed [10]. Changes in neural activity were

recorded by calcium imaging. Five out of the eight receptors in the sweet clade conferred new sensitivity for one to three different sweet compounds. Despites its success, there are some caveats when using this system to deorphanize Grs. One issue is that Gr43a can detect sucrose and fructose when expressed alone in this neuron, making it difficult to determine the role of each Gr. Both Grs could be involved in ligand detection of sweet tastants or one may merely be necessary for signaling. Another issue is that Gr43a expression in taste neurons is exclusive to one tarsal sensillum and the pharynx [6, 10, 11]. While Gr43a could be a co-receptor for some responses, other Grs must be able to form functional sweet receptor complexes without Gr43a. Furthermore, there is evidence of overlap in detection of taste compounds between Grs. When Gr64a and Gr64e are expressed together in a sweet neuron devoid of all other Grs, the neuron is able to respond to sucrose and maltose [10]. Gr43a expressed alone in a sweet neuron can detect sucrose as well. Expression of Gr64a and Gr64f in labellar taste neuron in *ΔGr64* flies rescues sucrose detection. As of now, it is unknown how much overlap in detection exists in the Gr family or which sweet receptors are involved in ligand recognition.

Bitter Grs have similar issues as sweet Grs. Bitter Grs may be expressed either singly or expressed in combination with as many as 28 bitter Grs which increases the complexity in finding which receptors are responsible of bitter tastant recogniton [4]. As of now, only one receptor combination has been discovered that confers detection of a bitter compound. Expression of Gr8a,

Gr93b, and Gr66a bestows L-canavanine detection to both S2 cells and sweet taste neurons [12]. There are examples of single mutants of bitter Grs causing reductions in neural responses to some bitter compounds. Neurons that lack Gr93a, Gr66a, or Gr33a have a deficit in caffeine detection and Gr47a is necessary for strychnine detection [13-15].

On the labellum, each bitter neuron was shown by GAL4 analysis to express five putative co-receptors, known as the Commonly Expressed Receptors (CERs): Gr33a, Gr32a, Gr66a, Gr89a, and Gr39a.a. All bitter Grs are coexpressed with at least one member of the CERs [3, 4]. Previous experiments have shown that loss of Gr32a, Gr33a, or Gr66a, causes a dramatic reduction in avoidance behaviors and neuronal response to multiple bitter stimuli [15-17]. It was originally thought that the CERS are involved in the receptor complex formation and the other thirty-nine bitter Grs are involved in ligand recognition but this may not be the CERS only function. In one class of labellar bitter neurons called I-a type bitter neurons, Gr59c is solely expressed with the five CERS [4]. Misexpression of Gr59c in another bitter neuron confers new ligand sensitivity but deletion of Gr59c from its endogenous I-a bitter neurons also confers new neural responses to multiple bitter compounds [18]. This suggests that at least one of the CERs is involved in ligand recognition. Gr59c appears to be involved in both ligand recognition of some bitter compound and inhibition of other bitter compounds. Misexpression of other bitter receptors in I-a type bitter neurons with and without Gr59c shows that Gr59c inhibits responses of other bitter Grs as

well. From these results, it appears bitter Grs have both excitatory and inhibitory functions. Because of interactions between bitter Grs and the complexity of bitter Grs expression patterns, it is difficult to study the involvement of each Gr in ligand recognition. A single system to study the individual properties of each Gr would fill a critical gap in the field. As of now, no one system has been designed that could be used to deorphanize both bitter and sweet Grs.

Using the ab1C neuron in the olfactory system, we designed a heterologous expression system to deorphanize individual taste Grs. The ab1C neuron is unique in that it expresses no olfactory receptors, but instead expresses two Grs, Gr21a and Gr63a. Together, these receptors form the $CO_2$ receptor and there is no evidence suggesting these receptors are involved in detecting taste compounds [19, 20]. After expressing a sweet Gr, Gr5a, in the ab1C neuron; we discovered that Gr5a can confer novel responses to trehalose and other sugars. Furthermore, the response to trehalose is both ligand and receptor gene dose dependent. Analysis in a Gr63a mutant background shows that functional expression of Gr5a is not dependent on a functional $CO_2$ receptor. We also discovered that a second sweet Gr, Gr64e, could be successfully expressed in the ab1C neuron and conferred glycerol sensitivity. Moreover, we were able to functionally express two bitter Grs. Expression of either Gr59c or Gr93a conferred detection of multiple bitter compounds. Surprisingly, we are able to express a functional mosquito sweet receptor in the ab1C neuron. Ab1C neuron expressing AgGr25 responded to fructose as well as other sugars,

suggesting this system could be used to deorphanize taste receptors from other insects.

## 2.2 Methods

### 2.2.1 Fly maintenance

Flies were maintained on standard cornmeal–dextrose medium at 25 °C and 40% humidity and were tested between 3-7 days after pupation. Full genotypes are in Table 2.1.

### 2.2.2 Olfactory recordings

Extracellular single unit recordings from the ab1C neuron were performed as described [21]. Tastant stimuli were prepared in sensillum lymph ringer (SLR) and stored at −20 °C. Two recording electrodes, one with electrolyte alone (SLR) and a second with stimulus solution dissolved in SLR (stimulus) were held on the same manipulator. Recordings were first obtained with SLR from three ab1 sensilla for ∼6 s to measure baseline activity of the ab1 neurons. Subsequently, ∼6-s recordings were obtained from the same three sensilla stimulus with the stimulus. 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 from 9 sensilla —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. Unless otherwise indicated, baseline SLR activity of the ab1C neuron was subtracted from the stimulus-evoked response recorded from the same sensillum.

### 2.2.3 Tastants

All compounds were obtained at the highest available purity from Sigma-Aldrich and were as follows: Sweet compounds used were: trehalose (T9531), maltose (M9171), sucrose (S7903), fructose (47740), maltotriose (M8378), melezitose (M5375), glycerol (G7893), glucose (G7528), methyl α glucopyranoside (M9376). Bitter compounds used were: caffeine (C0750), denatonium (D5765), escin quinine (Q1125), lobeline (141789) and theophylline (T1633)

### 2.2.4 Statistics

Using SSPS software, all statistics are two-way ANOVA with *post hoc* Dunnett's *t*-tests unless otherwise indicated.

## 2.3 Design of a heterologous expression system

### 2.3.1 Development of heterologous expression systems

Taste receptors can be expressed in the ab1C neuron by utilizing the *GAL4*/Upstream Activating Sequence (*UAS*) system [22]. We used *Gr21a-GAL4*

and/or *Gr63a-GAL4* drivers in combination with *UAS-gDNA* or *UAS-cDNA* transgenes for the gene of interest. In flies expressing a selected taste receptor in the ab1C neuron (ab1C:GrX), the sensillum was pierced twice, first with sensillar lymph ringer solution (SLR) for baseline activity, then a second time with SLR plus a stimulus. The ab1C neuron is housed with three other neurons in the ab1 large basiconic sensilla which are found on the third segment of the antenna. Activity from all four neurons in the ab1 sensillum was recorded and the C neuron was differentiated by spike amplitude as the one with the third largest spike (**Fig 2.1**). To determine the neural activity due to stimulus-Gr interactions, the baseline activity spike count was subtracted from the second recording with the stimulus.

### 2.3.1 Development of delivery stimuli

The ab1C neuron detects volatile compounds, mainly $CO_2$. Since we were using non- or low volatility compounds, a new delivery system was required. Our solution was to add taste compounds to the SLR of the recording microelectrode. The compound would then be delivered via diffusion through sensillar lymph. To determine if adequate amounts of the stimulus would reach the neuron using this approach, we compared delivery via microelectrode to standard odor delivery methods using a volatile activator that is water soluble.

Ethyl acetate is an activator of the ab1A neuron and is water-soluble at $10^{-4}$ dilution. We delivered ethyl acetate through the humidified airstream in a 0.5

sec impulse over the antenna. Then, we dissolved the same concentration of ethyl acetate in the SLR of the recording electrode and inserted the electrode into the same sensillum. By comparing the neural activity between odor delivery and diffusion from the electrode, we found that ethyl acetate can diffuse from the electrode to the sensillar lymph and activate the ab1A neuron (**Fig. 2.2**) The neural activity from diffusion is not as high as from odor delivery but it demonstrates that this method can be used as a delivery method for taste compounds. Other studies have shown success using this method for non-volatile compound delivery as well [23, 24].

### 2.3.2 Gr5a can function in the ab1C neuron

Gr5a has been shown to be both necessary for trehalose detection [25] as well as sufficient to confer trehalose sensitivity [8]. We expressed Gr5a in the ab1C neuron (ab1C:Gr5a) to determine if a single sweet receptor could function in the $CO_2$ sensing neuron. Using the two-electrode system, we recorded using SLR alone, 100 mM trehalose, and 100 mM sucrose. Ab1C:Gr5a neurons were able to detect trehalose but not sucrose (**Fig. 2.3 A and B**). Gr5a is not necessary for sucrose detection [25].This indicates that Gr5a can function in the ab1C neuron and that ab1C:Gr5a neurons have specificity for trehalose. Furthermore when we increased gene dosage of Gr5a by using 2 GAL4 transgenes and two UAS-Gr5a transgenes, there was an increase in response to trehalose but not to sucrose (**Fig. 2.3 B**). Trehalose detection appears to be

dependent on the level Gr5a protein expressed. For all further experiments, ab1C:GrX indicates two GAL4 lines and two UAS lines were used, unless otherwise specified.

Previously, flies lacking Gr5a were shown to have defects for sensing other sweet compounds besides trehalose including melezitose, methyl-α-glucopyranoside (m-glucoside), and glucose [25]. It is unknown if Gr5a is involved in ligand recognition of these sweet compounds or if Gr5a is part of the complex responsible for detecting these compound but is not directly involved in ligand detection. To distinguish between these two possibilities, we applied these four compounds to ab1C:Gr5a neurons. We saw an increase in neural response to all four sugars, indicating Gr5a is capable of detecting multiple sweet compounds (**Fig. 2.3 C**). Moreover, these responses were not dependent on a functional $CO_2$ receptor. ab1C neurons need both Gr63a and Gr21a to detect $CO_2$ [19, 20]. After recording from ab1C:Gr5a neurons in flies with and without Gr63a using trehalose, melezitose, glucose, and m-glucoside, we found no significant difference in sugar detection between flies expressing Gr63a and those that do not (**Fig. 2.3 D**).

Since both *Gr63a-GAL4* and *Gr21a-GAL4* lead to expression in the ab1C neuron, we wanted to determine if both drivers led to comparable activity or if one GAL4 line has higher functional expression levels. We compared neural responses to all five Gr5a dependant sugars from ab1C:Gr5a neurons using one copy of either *Gr21a-GAL4* or *Gr63a-Gal4* and one copy of *UAS-Gr5a*. We found

no statistical difference in responses to all five compounds between the two drivers (**Fig. 2.3 E**). For future experiments, we used *Gr21a*- and *Gr63a-GAL4* drivers interchangeably.

Each sensillum was being punctured twice, instead of once as is normally done in olfactory recordings [21]. It was imperative to see if multiple insertions would disrupt the baseline activity of the ab1C neuron. We first recorded the baseline activity using SLR alone in ab1C:Gr5a neurons, then recorded with 100mM trehalose, and then a third recording with SLR alone. This meant the same ab1 sensillum was punctured three times. Fortunately, we discovered there was no significant difference in baseline activity before and after testing with trehalose suggesting that multiple insertions do not significantly alter the neural activity of the c neuron (**Fig. 2.3 F**).

Since expression of a sweet Gr in the ab1C neuron conferred sugar sensitivity, we wondered if other characteristic traits of taste neurons are present in these neurons. A characteristic trait of extracellular recordings from taste neurons is its fast adapting phase where there is a rapid burst of activity that adapts quickly usually within hundredths of a second after contact. Olfactory neurons possess a longer phasic phase which can last several minutes especially when continuously exposed to a strong activator [21]. To see if ab1C neurons expressing a sweet Gr possess an initial fast adapting phase, we counted the activity of six ab1C:Gr5a neurons in response to trehalose in 100ms bins for 2 seconds without subtracting the baseline activity (**Fig. 2.3 G**). The

neural response for all recordings is steady and there is no significant drop in activity over two seconds, suggesting that ab1C:Gr5a neurons lack the fast adapting phase.

Since Gr5a functions in the ab1C neuron, our next step was to see if other Grs could function as well.

### 2.3.3 Gr64e, a glycerol receptor

Previously, our lab discovered that *Drosophila* attraction to beer is partially due to beer's glycerol content which is detected by sweet labellar taste neurons [26]. Furthermore, a different *Drosophila* species, *D. pseudoobscura,* does not have the same preference for glycerol as *D. melanogaster*. One key difference between these two species is *D. pseudoobscura* does not have a functional copy of Gr5a or Gr64e [27]. When recording from labellar taste hairs, *D. melanogaster* flies lacking Gr5a showed no defects in glycerol sensitivity. Flies lacking Gr64e lost sensitivity to glycerol and 1,2 propanediol. Sucrose detection was unaffected in *ΔGr64e* flies. Since Gr64e is necessary for glycerol and 1,2 propanediol detection, we wondered if Gr64e could confer sensitivity for these compounds to the ab1C neuron. After expressing Gr64e in ab1C neuron (ab1C:Gr64e), we applied glycerol, 1,2 propanediol, and sucrose to ab1C neuron. Both glycerol and 1,2 propanediol caused an increase in neural response but sucrose did not (**Fig. 2.4 A and B**). As observed for Gr5a and

trehalose, we found that glycerol detection was dose dependent, and increased with increasing concentrations of glycerol (**Fig. 2.4 C**).

In sweet taste neurons, glycerol detection is inhibited by both 3-amino-1,2-propanediol and 2-amino-1,3-propanediol [28]. It was unknown if these inhibitors could affect glycerol detection by Gr64e or if inhibition was dependent on the expression of other taste Grs or via another nonspecific mechanism. To solve this question, we mixed 3-amino-1,2-propanediol or 2-amino-1,3-propanediol with 10% glycerol and applied both mixtures to ab1C:Gr64e neuron. Both mixtures had a reduced response compared to glycerol alone, indicating both compounds could inhibit the glycerol mediated neural response (**Fig 2.4D**). Furthermore, none of these compounds affected the endogenous $CO_2$ response suggesting that inhibition of glycerol response is due to inhibition of Gr64e and not the ab1C neuron itself (**Fig 2.4E**).

### 2.3.5 Expression of bitter taste receptors

After the successful expression of two sweet Grs, our next step was to test bitter Grs. A system that could express bitter Grs individually would be an incredible asset due to multiple factors. Bitter Grs are expressed in multiple combinations with as many as twenty eight Grs in a single neuron [4]. As previously stated, bitter Grs can confer ligand sensitivity to some compounds but also repress detection of other compounds [18]. Furthermore, the role of the

CERS is unknown and there is evidence suggesting they might be involved ligand recognition.

Gr59c was the first receptor we picked because it is expressed alone with the five CERS in one class of labellar bitter neurons, I-a bitter neurons (**Fig. 1.1**). It can also confer higher sensitivity to bitter compounds when misexpressed in another class of labellar bitter neurons. This suggests that Gr59c can confer new ligand sensitivity and makes it an ideal proof of principle receptor to test in a new heterologous system. Previously, Gr59c was shown to enhance sensitivity to lobeline and denatonium when misexpressed [4]. After recording from ab1C neurons expressing Gr59c (ab1C:Gr59c), we discovered that Gr59c confers sensitivity to lobeline, denatonium, and escin but not to caffeine, or quinine (**Fig. 2.5 A**). This response profile is similar to that of I-a bitter neurons. Escin mediated response in ab1C:Gr59c neurons is concentration dependent as well (**Fig. 2.5 B**). Moreover like Gr5a, functional expression of Gr59c was not dependent on expression of Gr63a and there is no significant difference in neural responses between ab1C:Gr59c neurons with and without Gr63a (**Fig. 2.5 C**).

We next tested two other bitter Grs, Gr93a and Gr33a. Both of these receptors plus Gr66a are necessary for caffeine detection [15]. Since three receptors are necessary for caffeine sensitivity, it was unknown if one or all of these receptors were involved in ligand detection of caffeine. ab1C neurons expressing Gr93a (ab1C:Gr93a) had an increase in neural responses to caffeine and lobeline (**Fig. 2.5 D**). Surprisingly, single mutants for Gr93a do not show a

defect in lobeline sensitivity [15]. Considering Gr93a is normally coexpressed with twenty-seven other Grs and some bitter Grs can inhibit other bitter Grs, there may be overlap in lobeline detection [4]. Gr33a, one of the CERS, is also necessary for caffeine detection and for sensitivity to other bitter compounds [16, 29]. When we expressed Gr33a in ab1C neurons ( ab1C:Gr33a), the neurons did not respond to any of the bitter compound tested which suggests that Gr33a alone cannot confer ligand detection for these compounds (**Fig. 2.5 D**).

Since Gr33a does not confer sensitivity to any tested bitter compounds, perhaps Gr33a performed a different role such as increasing sensitivity of Gr93a to bitter compounds. We coexpressed Gr33a and Gr93a in ab1C neurons. Coexpression involved using one copy of UAS-Gr93a, one copy of *UAS-Gr33a* and two GAL4 drivers. Responses to caffeine and lobeline were similar whether ab1C neurons were expressing two copies of *UAS-Gr93a* or one copy of both *UAS-Gr93a* and *UAS-Gr33a* (**Fig. 2.5 D**). Surprisingly, there was a novel response to theophylline that was not seen when either Gr93a or Gr33a were expressed alone in the ab1C neuron. This indicates that coexpression of two bitter Grs can lead to unique responses that are not seen when bitter receptors are expressed singly.

### 2.3.6 Expression of an internal fructose sensor and its mosquito ortholog

Previously, it's been shown that Gr43a is expressed in the central nervous system of adult flies and larva and is as an internal fructose detector [11, 30].

Gr43a has also been shown to function as a sugar gated ion channel in cell culture [9]. Moreover, Gr43a is highly conserved with orthologs found in multiple insects including the malaria vector, *Anopheles gambiae (A. gambiae)* [27]. Because of how highly conserved Gr43a is, we decided to test Gr43a in ab1C neurons to determine if Gr43a can confer sensitivity to fructose and other sugars. When we expressed Gr43a in the ab1C neuron (ab1C:DmGr43a), we discovered that ab1C:Gr43a neurons respond to fructose in a dose dependent manner and also respond to other sugars (**Fig. 2.6 A and B**).In cell culture experiments, Gr43a expressing cells only responded to fructose and was hypothesized to be narrowly tuned for fructose [9]. Surprisingly, we saw responses to glucose, maltose and maltotriose which suggest that Gr43a may be more broadly tuned than previously expected.

Because of the broad compatibility between Grs in *Drosophila* and the ab1C neuron, we decided to test a sweet Gr from *A. gambiae* to see if Grs from other species can function in *Drosophila. D. melanogaster* and *A. gambiae* Gr families are highly divergent with few one to one orthologs found in the Gr family between both species [27]. *AgGr25* and *DmGr43a* are one of the few ortholog pairs found and they share ~30% amino acid identity [31]. After expression of AgGr25 in ab1C neurons (ab1C:AgGr25), we discovered that these neurons respond to fructose and similar sugars as DmGr43a (**Fig. 2.6 B**). This suggests that mosquito Grs can function in a *Drosophila* ab1C neuron in the absence of

mosquito specific cofactors and indicates that the *Drosophila* ab1C neuron may be a suitable system to study ligand recognition of other insect taste receptors.

## 2.4 Discussion

In summary, we used the Gr21a/Gr63a $CO_2$-sensing olfactory neuron as a host for *in vivo* expression of sweet and bitter receptors. We demonstrated that this system is suitable to study the interactions between taste receptors and multiple types of stimuli. Both bitter and sweet Grs are able to function in the ab1C neuron in the absence of a functional $CO_2$ receptor. Surprisingly, we were able to successfully express a sweet receptor from the malaria vector, *A. gambiae.* This work sets the platform to deorphanize all the sweet receptors in *Drosophila* as well as in mosquitoes.

It is quite remarkable that these receptors are able to function in an olfactory neuron not meant for detecting taste compounds. This suggests that taste receptors can function in the absence of taste neuron specific co-factors or co-receptors. Although a functional $CO_2$ receptor does not seem to be necessary, we only tested *ΔGr63a* flies so expression of Gr21a may still be required. Recordings from double mutants of Gr21a and Gr63a are necessary to see if either Gr is necessary for responses to taste compounds in the ab1C neuron. With the exception of Gr5a and Gr43a, Grs expressed alone have not been successful in conferring ligand detection. There are some pairs of sweet receptors have been shown to respond to some sweet compounds in the

absence of other receptors [10]. This may explain why Grs can function in the ab1C neuron but questions still remain about what is actually going on in taste neurons because receptor interactions can create new responses and also inhibit responses.

Though taste receptors function in the ab1C neuron, there are also differences in temporal responses between ab1C:GrX neurons and taste neurons. Tastants normally evoke an initial strong and fast-adapting response in taste neurons. Ab1C:GrX neurons lack this characteristic. Another difference is that ab1C:GrX neurons have lower neural responses to taste compounds as compared to the endogenous taste neuron. This may be due to the nature of the expression system. Since we are using an artificial expression system and taste Grs are not normally found in the ab1C neuron, the neural responses may be weaker due to the ab1C neuron lacking important cofactors. It could also be due to the delivery system. The stimulus is delivered from a microelectrode with a pore that is only a few micrometers compared to taste tip recordings where the sensillar pore is enveloped by the compound [32].

Both sweet and bitter receptors functioning in the ab1C neuron is surprising. There is no overlap of expression of sweet or bitter receptors in taste neurons. Only one combination of bitter Grs has worked in sweet neurons. When expressed in labellar sweet taste neurons, Gr8a, Gr98b, and Gr66a bestows L-canavanine detection [12]. As of now, multiple combinations of sweet receptors have not worked in the bitter neuron (unpublished). This suggests that there is

something unique about the ab1C neuron that allows both bitter and sweet Grs to function. It may simply be that either Gr21a or Gr63a are somewhat promiscuous and can function with both sweet and bitter Grs while sweet and bitter Grs are unable to function with each other.

Bitter Grs may be more complex than we originally thought. Gr93a can detect caffeine and lobeline in the ab1C neuron, though mutants show no deficiency to lobeline. One reason could be that this receptor is thought to be expressed with 27 other receptors in a labellar bitter neuron class, S-b type bitter neurons (**Fig 1.1 A**). S-b bitter neurons respond to a broad selection of bitter compounds and there may be significant overlap for lobeline detection by different Grs. Another possibility is that another bitter Gr inhibits lobeline detection by Gr93a, a type of interaction for which there is emerging evidence [18]. It may require multiple bitter Grs being added one at time to determine how each bitter Gr affects that response of other bitter Grs. Coexpression of both Gr33 and Gr93a in the ab1C neuron was required to confer theophylline sensitivity. However, the ab1C neuron is not very tractable for the expression of multiple bitter Grs. This approach is more useful in showing what compounds a single bitter Gr can detect without interference from other Grs. Understanding the breadth of ligand recognition by each bitter Gr could help us understand how bitter Grs have evolved to detect multiple bitter compounds that are incredibly structurally diverse.

The most exciting discovery is that a mosquito Gr can confer novel responses to the *Drosophila* $CO_2$ neuron. Previous studies have shown that mosquito Ors can function in a *Drosophila* olfactory neuron but this is the first time a mosquito taste receptor has functioned in a *Drosophila* background [33]. These results demonstrate that mosquito Grs can function in the absence of mosquito specific factors. Both ab1C:AgGr25 and ab1C:DmGr43a neurons had similar response profiles, which suggest that detection of fructose and other sugars is evolutionarily conserved between these two species and potentially other insects. This is in spite of millions of years of evolution between them [27]. Furthermore, functional expression of a mosquito Gr leads to the possibility of other insect taste receptors being deorphanized.

In summary, we have designed a system to study ligand detection of both bitter and sweet Grs in two insect species. This creates the platform to study the properties of individual taste Grs in multiple insects.

**2.5 Abbreviations**

Gustatory receptors (Gr) Pg. 48

Commonly expressed receptors (CERs) Pg.51

Sensillum lymph ringer (SLR) Pg. 53

Upstream Activating Sequence (*UAS*) Pg. 54

ab1C neuron expressing GrX protein( ab1C:GrX) Pg. 54

Gr5a expressed in the ab1C neuron (ab1C:Gr5a) Pg.56

methlyl-α-glucopyranoside (m-glucoside) Pg. 56

## 2.6 References


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Figure 2.1

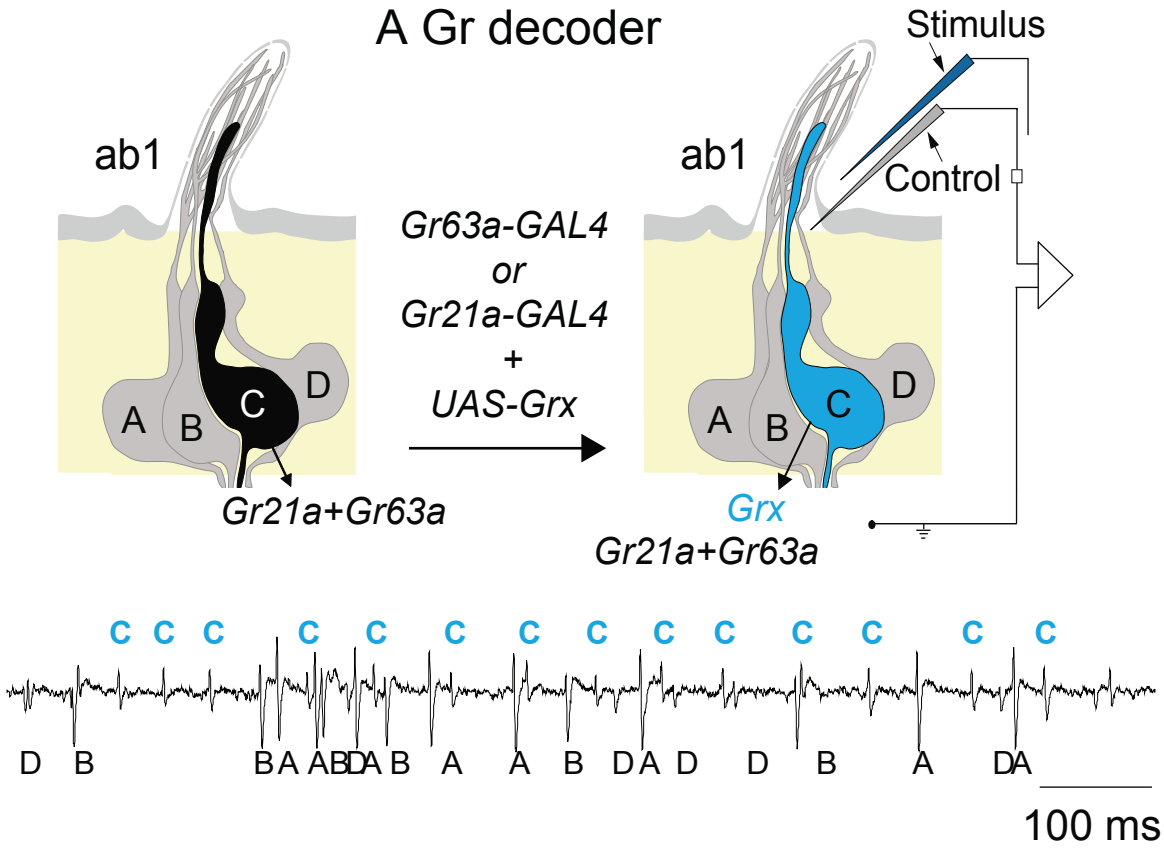

A Gr decoder
ab1
A
B
C
D
Gr21a+Gr63a
Gr63a-GAL4
or
Gr21a-GAL4
+
UAS-Grx
ab1
Stimulus
Control
A
B
C
D
Grx
Gr21a+Gr63a
C C C C C C C C C C C C C C C
D B B A A B D A B A A B D A D D B A D A
100 ms

**Figure 2.1Compounds in recording electrode can activate neurons** (**A**) Representative odor trace from Ab1 sensillum to $10^{-4}$ ethyl acetate. Line indicate 0.5 sec odor. Red dots indicate ab1A neuron spikes. (**B**) Representative trace from Ab1 sensillum to $10^{-4}$ ethyl acetate in SLR of recording electrode. Red dots indicate ab1A neuron spikes.

Figure 2.2

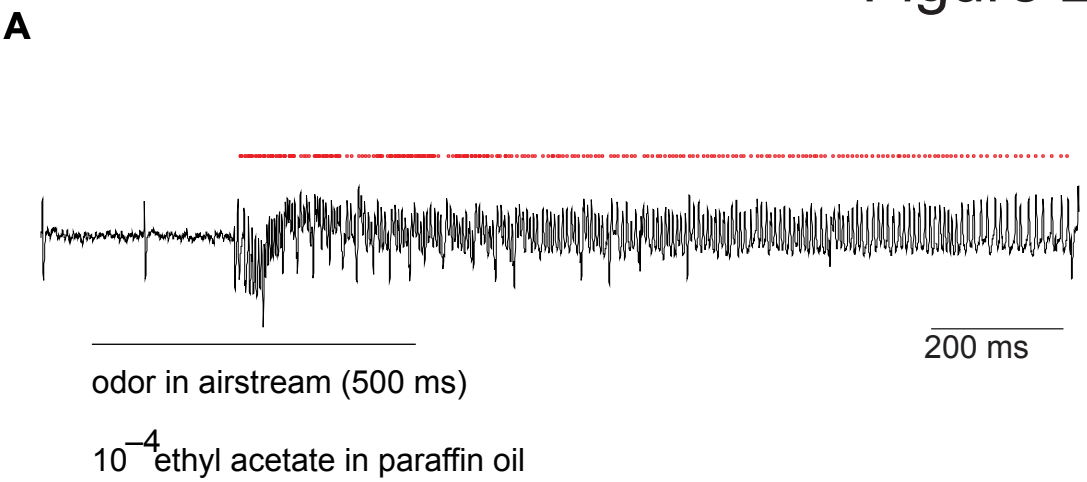

A
200 ms
odor in airstream (500 ms)
10–4 ethyl acetate in paraffin oil
B
200 ms
odor in recording micropipette
10 –4 ethyl acetate in SLR

**Figure 2.2 An in vivo ectopic expression system for analysis of individual Grs.** Schematic of the ectopic expression in the ab1C neuron with trace from a *wild type* ab1 sensillum depicting activities of the four ab1 neurons. Glass micropipettes for tastant recordings contain sensillum lymph ringer (SLR) control (gray) or stimulus in SLR (blue).

# Figure 2.3

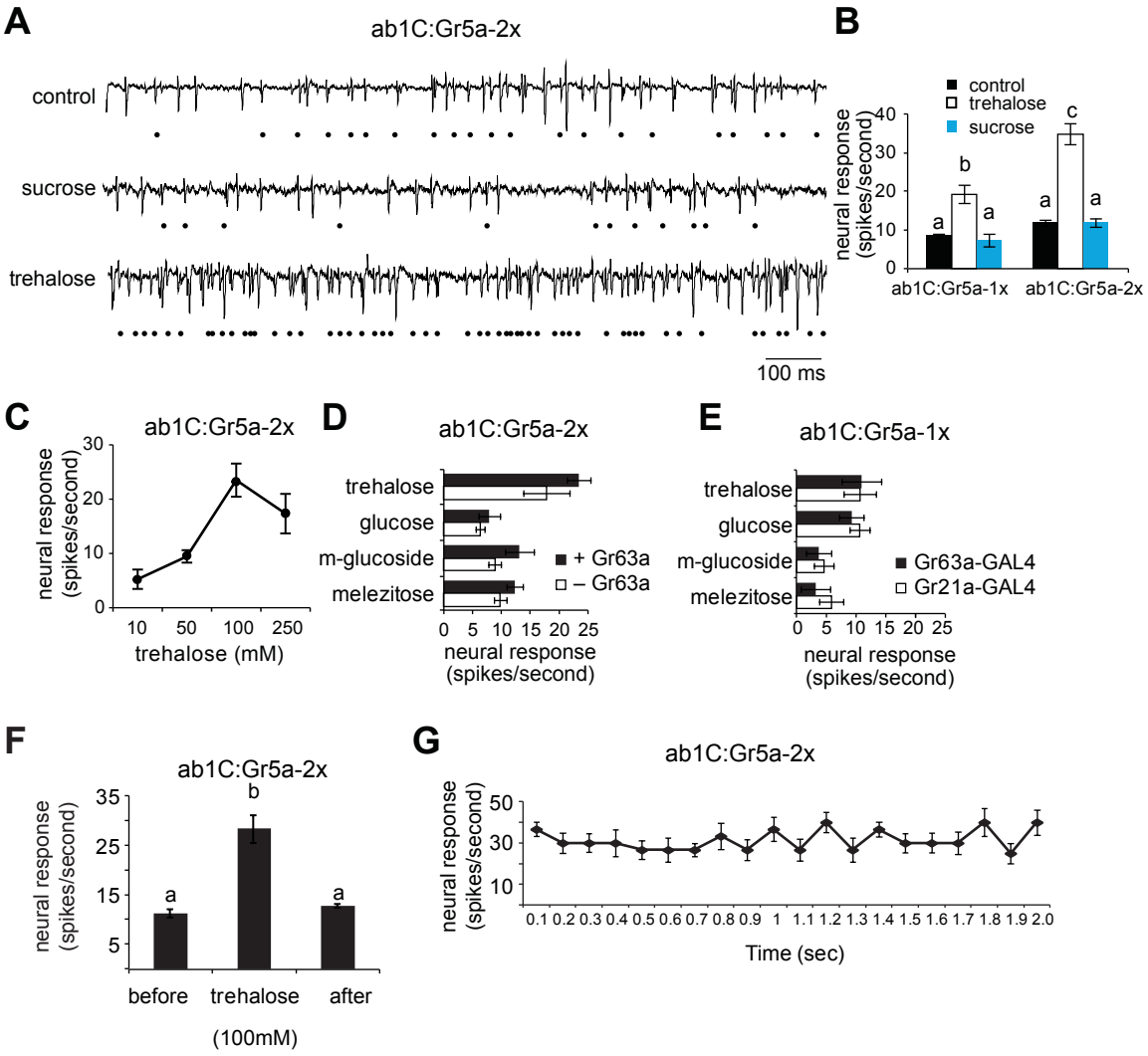
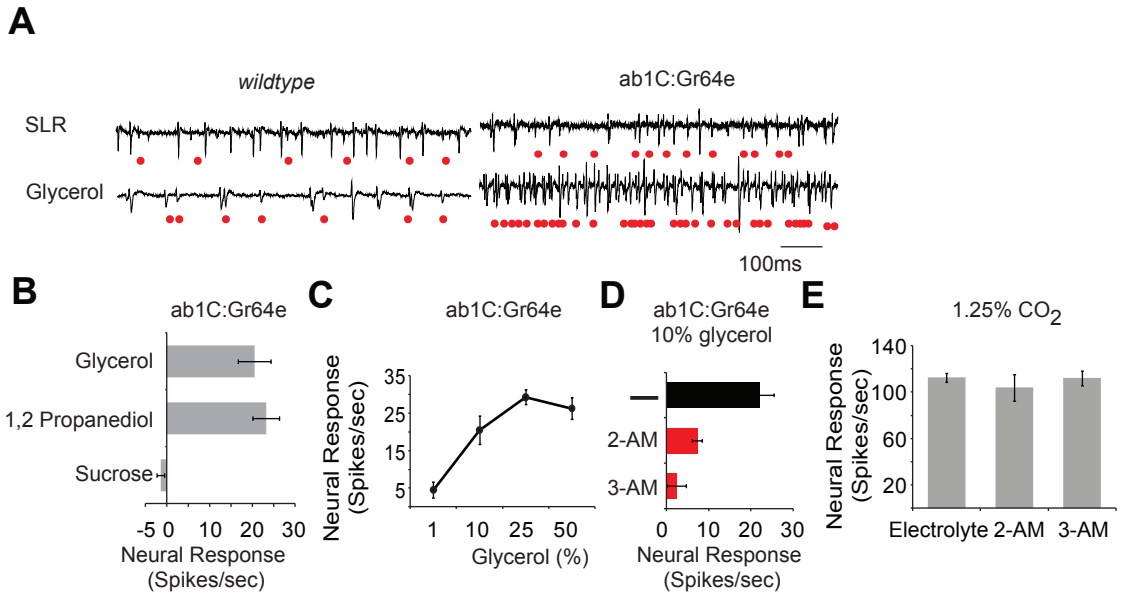

A
ab1C:Gr5a-2x
control
sucrose
trehalose
100 ms
B
control
trehalose
sucrose
neural response (spikes/second)
ab1C:Gr5a-1x
ab1C:Gr5a-2x
C
ab1C:Gr5a-2x
neural response (spikes/second)
trehalose (mM)
D
ab1C:Gr5a-2x
trehalose
glucose
m-glucoside
melezitose
+ Gr63a
– Gr63a
neural response (spikes/second)
E
ab1C:Gr5a-1x
trehalose
glucose
m-glucoside
melezitose
Gr63a-GAL4
Gr21a-GAL4
neural response (spikes/second)
F
ab1C:Gr5a-2x
neural response (spikes/second)
before
trehalose
after
(100mM)
G
ab1C:Gr5a-2x
neural response (spikes/second)
Time (sec)

**Figure 2.3 Ab1C:Gr5a neurons responds to trehalose and other sugars** (**A**) Sample ab1 recordings in flies expressing Gr5a in ab1C neurons (ab1C:Gr5a-2x). Black dots indicate ab1C spikes. (**B**) Mean responses of ab1C:Gr5a-1x and ab1C:Gr5a-2x neurons. Baseline activity to SLR is not subtracted from stimulus-evoked activity. Sugars were tested at a concentration of 100 mM. Letters indicate statistical significance ($P < 0.001$; one-way ANOVA with Tukey's post hoc test; n= 6–12). (**C**) Dose-dependent response of ab1C:Gr5a neurons to trehalose (n= 6–12). (**D**) Mean responses of ab1C:Gr5a-2x neurons in *wild type* (+Gr63a) or *ΔGr63a* (–Gr63a) flies to 100 mM sugars (n= 10–14). (**E**) Mean responses of ab1C:Gr5a neurons generated with Gr21a– or Gr63a–GAL4 as indicated to 100 mM sugars (n = 6). (**F**) Mean responses of ab1C:Gr5a-2x neurons using three recording from the same sensillum to the indicated stimuli. (n=6) All genotypes in D and E were compared with each other by using two-way ANOVA with Tukey's post hoc test. Only genotypes with one copy of UAS–Gr5a in (**E**) are significantly different from genotypes with two copies of UAS–Gr5a (**F**) (*$P < 0.05$). (**G**) Mean responses counted in 100ms bins for ab1C-Gr5a-2x neurons over 2 sec.

# Figure 2.4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wildtype
ab1C:Gr64e
SLR
Glycerol
100ms
B
ab1C:Gr64e
Glycerol
1,2 Propanediol
Sucrose
-5 0 10 20 30
Neural Response
(Spikes/sec)
C
ab1C:Gr64e
Neural Response
(Spikes/sec)
35
25
15
5
1 10 25 50
Glycerol (%)
D
ab1C:Gr64e
10% glycerol
2-AM
3-AM
0 10 20 30
Neural Response
(Spikes/sec)
E
1.25% CO2
Neural Response
(Spikes/sec)
140
100
60
20
Electrolyte 2-AM 3-AM

**Figure 2.4 Gr64e is a glycerol detector** (A) Representative traces from ab1C neuron in *wild type* (left) and ab1C:Gr64e (right) flies responding to indicated stimuli. Red dots indicate ab1C neuron firing (B) Mean responses of ab1C neuron to indicated stimuli in ab1C:Gr64e flies. (n=6-12)(C) Mean responses of ab1C neuron to glycerol in ab1C:Gr64e flies. (n=6-12) (D) Mean responses of ab1C neuron to mixtures of 10%glycerol and 2-amino 1,3 propanediol(2-AM) and 3-amino 1,2 propanediol (3-AM) in ab1C:Gr64e flies. (n=6-12). Red bars indicate statistically significant using Dunnett's t-test ($P<0.05$). (E) Mean response of ab1c neuron in *wild type* flies to 1.25% $CO_2$ and indicated stimuli. (n=6).

# Figure 2.5

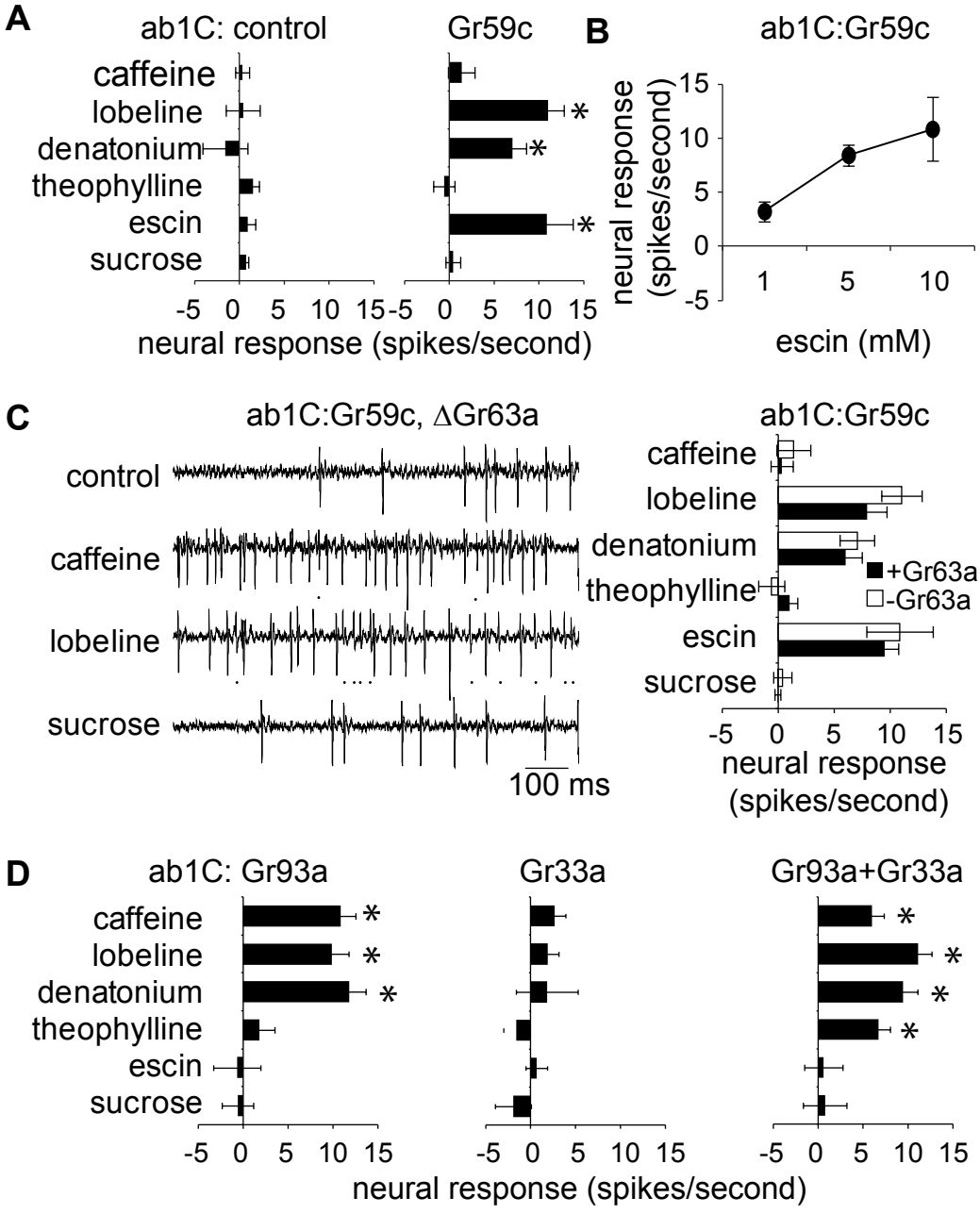
A
ab1C: control
Gr59c
caffeine
lobeline
denatonium
theophylline
escin
sucrose
-5 0 5 10 15
neural response (spikes/second)
B
ab1C:Gr59c
neural response (spikes/second)
15
10
5
0
-5
1 5 10
escin (mM)
C
ab1C:Gr59c, ΔGr63a
control
caffeine
lobeline
sucrose
100 ms
ab1C:Gr59c
+Gr63a
-Gr63a
neural response (spikes/second)
D
ab1C: Gr93a
Gr33a
Gr93a+Gr33a
neural response (spikes/second)

**Figure 2.5 Multiple bitter Grs detect aversive compounds.** (**A**) Mean responses of ab1C neurons in control *w1118* flies (ab1C) and flies expressing Gr59c (ab1C:Gr59c). *P < 0.05 (vs. control; n=6–12). Sucrose was tested at a concentration of 100 mM and bitter compounds at 10 mM. (**B**) Dose-dependent response of ab1C:Gr59c (n=6–12). (**C**) Sample recordings and mean responses of ab1C:Gr59c in wild-type (+Gr63a) and ΔGr63a (–Gr63a) flies to indicated stimuli (10 mM). Genotypes are not significantly different (P > 0.05; n = 6). Concentrations were as in A. (**D**) Mean responses of ab1C neurons in flies expressing Gr93a (ab1C:Gr93a) (left), flies expressing Gr33a (ab1C:Gr33a) (middle), and flies expressing Gr33a and Gr93a (ab1C:Gr93a+Gr33a) (right). *P<0.05 (n=6-12).

# Figure 2.6

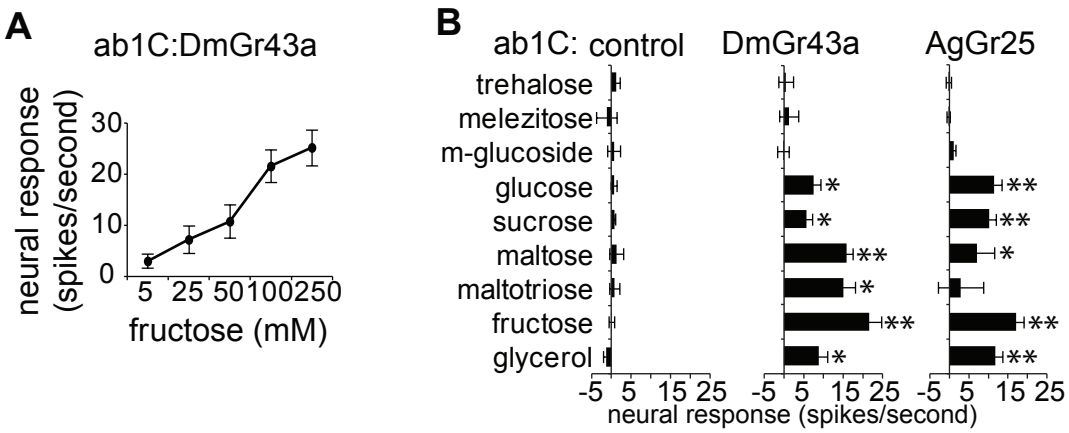
A
ab1C:DmGr43a
neural response (spikes/second)
30
20
10
0
5 25 50 100 250
fructose (mM)
B
ab1C: control
DmGr43a
AgGr25
trehalose
melezitose
m-glucoside
glucose
sucrose
maltose
maltotriose
fructose
glycerol
-5 5 15 25
neural response (spikes/second)

**Figure 2.6 Mosquito receptor functions in fly**. (**A**) Dose dependent responses in flies expressing *Drosophila* Gr43a in the ab1C neuron (ab1C:DmGr43a). (n=6-12) (**B**) Mean responses of ab1C neurons in control $w^{1118}$ flies (ab1C) and flies expressing Drosophila Gr43a in the ab1C neuron (ab1C:DmGr43a) or its mosquito ortholog (ab1C:AgGr25) to indicated stimuli tested at a concentration of 100 mM, except maltotriose (250 mM) and glycerol (10%). *P < 0.05; **P < 0.001 (vs. ab1C; n = 6–12).

## Table 2.1 List of Genotypes used in chapter 2

| Line name | Genotype/ source | Figures |
|---|---|---|
| Gr21a-GAL4 | Kristin Scott | |
| Gr63a-GAL4 on III | BDSC (# 9942) | |
| Gr63a-GAL4 on II | BDSC (# 9943) | |
| Δ*Gr63a* | BDSC (# 9941) | |
| UAS-Gr5a | Dahanukar laboratory | |
| UAS-Gr64e | Dahanukar laboratory | |
| UAS-Gr59c | Carlson laboratory | |
| UAS-Gr33a | Montell laboratory | |
| UAS-Gr93a | Montell laboratory | |
| UAS-Gr43a | Dahanukar laboratory | |
| UAS-Gr43a | Dahanukar laboratory | |
| *wild type* | *w1118* | 2.1, 2.2, 2.3, 2.4, 2.5 |
| ab1C:Gr5a-1x | *UAS-Gr5a-8/+; Gr63a-GAL 4/+* | 2.3 |
| ab1C:Gr5a-2x (same as ab1C:Gr5a) | *UAS-Gr5a-8/UAS-Gr5a-8; Gr63a-GAL4/Gr63a-GAL4* | 2.3 |
| ab1C:Gr5a-1x | *UAS-Gr5a-8/+; Gr63a-GAL4/+* | 2.3 |
| Gr63a-GAL4 | *UAS-Gr5a-8/+; Gr63a-GAL4/+* | 2.3 |
| Gr21a-GAL4 | *Gr21a-GAL4/+; UAS-Gr5a-3/+* | 2.3 |
| + Gr63a | *UAS-Gr5a-8/UAS-Gr5a-8; Gr63a-GAL4/Gr63a-GAL4* | 2.3 |
| − Gr63a | *UAS-Gr5a-8/UAS-Gr5a-8; ΔGr63a,Gr63a-GAL4/ΔGr63a, Gr63a-GAL4* | 2.3 |
| ab1C:Gr64e | *UAS-Gr64e-3/UAS-Gr64e-3; Gr63a-GAL4/Gr63a-GAL4* | 2.4 |
| ab1C:Gr59c | *Gr21a−GAL4/Gr63a−GAL4;UAS−59c-9d,UAS−Gr9c-9d* | 2.5 |
| +Gr63a | *Gr21a−GAL4/Gr63a−GAL4;UAS−59c-9d,UAS−Gr9c-9d* | 2.5 |
| -Gr63a | *UAS−Gr59c-14d/UAS−Gr59c-14d; ΔGr63a,Gr63a−GAL4/ΔGr63a, Gr63a−GAL4* | 2.5 |
| ab1C:Gr93a | *UAS-Gr93a-2d/Gr21-GAL4;UAS-Gr93a-3/Gr63a-GAL4* | 2.5 |
| ab1C:Gr33a | *UAS-Gr33a/UAS-Gr33a;Gr63a-GAL4/Gr63a-GAL4* | 2.5 |
| ab1C: Gr93a+Gr33a | *UAS-Gr33a/UAS-Gr93a-2D;Gr63a-GAL4/Gr63a-GAL4* | 2.5 |
| ab1C:DmGr43a | *UAS-Gr43a-8d/Gr21a-GAL4; Gr63a-GAL4/UAS-Gr43a-5d* | 2.6 |
| ab1C:AgGr25 | *Gr21a-GAL4/Gr63a-GAL4; UAS-AgGr25-3D/UAS-AgGr25-3L* | 2.6 |

## 3.0 All sweet Grs are capable of detecting sweet compounds

**Abstract** Sweet taste plays an essential role in food selection and feeding behaviors. In *D. melanogaster,* sweet neurons express eight gustatory receptors (Grs) belonging to a highly conserved clade in insects. Despite multiple attempts, little is known about how these receptors detect sweet tastants. Using the ab1C $CO_2$ sensing olfactory neuron as a unique in vivo decoder, we expressed each receptor of the sweet clade individually and recorded the neural activity to a panel of stimuli. Each of the eight receptors of this group conferred sensitivity to two or more sweet tastants, indicating direct roles in ligand recognition for all sweet receptors. We validated receptor response profiles by analysis of taste responses in available corresponding Gr mutants. The response matrix shows extensive overlap in Gr-ligand interactions and loosely separates sweet receptors into two groups: one which detects Gr5a dependent sugars and one which detects Gr64a dependent sugars. Furthermore, coexpression of two receptors, Gr64a and Gr64e, in the ab1C neuron enhanced sensitivity to sweet compounds as compared to when each receptor was expressed alone.

## 3.1 Introduction

The taste system is essential for the detection of high caloric appetitive compounds. Sugars are readily available high calorie food sources found in fruit, nectar, and honeydew. As such, many animals and insects have receptors that are used to detect sugars and other sweet compounds leading them to seek out fruits and other produce for consumption. Because of this behavior, crop destruction by insects is a major problem causing billions of dollars of waste every year. By studying how insects detect sweet compounds and understanding the mechanisms involved, we could create new deterrents to prevent crop loss.

Sucrose, fructose, and glucose are the most abundant sugars found in fruits and nectars and are considered highly appetitive for many types of insects [1, 2]. Insects are able to detect other sweet compounds including multiple monosaccharides, polysaccharides, glucosides, and sugar alcohols [3, 4]. Sweet compounds are detected by a highly conserved clade of gustatory receptors (Grs) with members found in all insects and arthropods including a crustacean, *Daphnia pulex* [5]. *Drosophila melanogaster* expresses members of this sweet clade and has multitudes of genetic tools available, making it an ideal model organism to study sweet receptor interactions.

In *Drosophila*, there are eight Grs that have been mapped to sweet taste neurons and are part of a distinct clade (**Fig. 3.1A, 1.2**) [4]. These receptors are expressed in multiple combinations and are found in all taste organs, some neuroendocrine cells and in the central nervous system (**Fig. 1.1**) [6-10]. Two

sweet receptors, Gr5a and Gr64a, are broadly required for responses to two large complementary subsets of sugars [3]. The other receptors are co-expressed with Gr5a and Gr64a in varying combinations (**Fig. 3.1B-D**) [6, 11]. *ΔGr61a* flies have reduced responses to glucose [12] and Gr64e is necessary for glycerol detection [13]. Less is known about the other sweet receptors but there is evidence suggesting they are necessary for feeding behaviors involving specific sweet tastants [14].

While multiple members of the sweet clade are necessary for sugar responses, little is known about how each of these receptors contributes to sugar detection. It is unclear from genetic analyses, for example, whether one or more of these receptors serve a general function as the obligate co-receptor, similar to how OrCo is necessary for responses to odors in olfactory neurons [15], or which ones are directly involved in ligand detection. This embodies a critical gap in the field. Few receptors have been successfully expressed in cell culture, Gr5a and Gr43a, and are involved in ligand recognition [16, 17]. However, in both of these cases, a single receptor responded to a single sweet compound while there is strong evidence that Grs can function in either pairs or as heteromeres [10, 18, 19]. Furthermore, there is evidence that suggests multiple combinations of sweet Grs are capable of detecting the same sugar [10, 18]. Gr43a, a sweet receptor found outside of the sweet clade, responds to sucrose and fructose when it is endogenously expressed alone in a tarsal sweet taste neuron in*ΔGr5a; ΔGr61a, ΔGr64* flies. In a sweet taste neuron devoid of all sweet Grs, artificially

expressing both Gr64a and Gr64e confers sucrose detection[18]. Expression of Gr64a and Gr64f rescues sucrose sensitivity in labellar sweet taste neurons in *ΔGr64* flies where only Gr5a and Gr61a are endogenously expressed [10]. By studying each sweet receptor individually, we can understand if each receptor is capable of detecting multiple sweet compounds and determine how much overlap exist for detecting a single sugar by the sweet clade.

Previously in Chapter 2, we discovered that expression of Gr5a in the ab1C neuron conferred sensitivity to multiple compounds including trehalose (**Fig. 2.3**). Continuing with the same setup, we expressed each receptor of the sweet clade individually in the ab1C neuron and applied panel of monosaccharides, polysaccharides, a glucoside, and glycerol to evoke a neuronal response. We discovered that every receptor was activated by at least two sweet tastants, and that each tastant induced a response from at least one receptor. Gr5a and Gr64a were activated by separate and complementary subsets of compounds, as suggested by mutant analyses [3]. Typically, ectopic responses of the other six receptors appeared to overlap with either Gr5a dependent sugars or Gr64a dependent sugars, but not with both. Responses of the ab1C:GrX neurons were validated by reductions in neural responses from the endogenous taste neuron in available corresponding *Gr* mutants. By comparing mutant analyses to ectopic expression, we found incidents where a Gr was necessary for a response in the endogenous taste neuron but not sufficient to confer sensitivity when expressed in the ab1C neuron, but supporting a model

where members of sweet clade function together in complexes. We did not find any cases where a gain of response in ectopic expression was not validated by a loss of response in a correlating mutant. Although coexpression of receptors with Gr5a did not lead to an increase in sugar response or a broadening of the sugar response profile, experiments with inhibitory compounds suggest that coexpressed receptors in the ab1C neuron interact with each other.

## 3.2 Methods and materials

### 3.2.1 Fly maintenance

Flies were maintained on standard cornmeal–dextrose medium at 25 °C and were tested between 3-10 days after pupation. Full genotypes are in Table 3.1.

### 3.2.2 Single sensillum taste recordings

Female flies that were between 3-7 days old were used and kept on regular diet. Solutions were made by dissolving compounds in miliQ water. Stock solutions of sugars and tricholine citrate TCC were stored at -20°C for up to 6 months. 100% Glycerol was stored at room temperatures. After thawing stock solutions, the testing solutions were made from mixing sugar, TCC, and milliQ water until sugar and TTC were at appropriate concentrations. Prepared testing solutions were kept at 4°C unless being used and were disposed of after 5 days. Tip recordings from taste sensilla were as described in [3] using 30 mM TTC. All compounds

were tested at 100 mM concentration except maltotriose (250mM) and glycerol (10%) unless otherwise stated. We recorded from at most three labellar L-type hairs on at least two flies. All the spikes in each trace were counted for 1 sec. from contact

### 3.2.3 Olfactory recordings

Extracellular single unit recordings from the ab1C neuron were performed as described[20]. Protocol for olfactory recordings is the same as chapter 2

### 3.2.3 Tastants

Tastants used are same as in chapter 2.

### 3.2.4 Statistics

Using SSPS software, all statistics are one-way ANOVA with post hoc Dunnett's *t*-tests, unless otherwise indicated.

## 3.3 Results

### 3.3.1 Each receptor of the sweet clade responds to sugars

In the previous chapter, we established that the ab1C neuron is suitable for the expression of Gr proteins (**Fig. 2.1**). We expressed two receptors of the sweet clade, Gr5a and Gr64e, individually in the ab1C neuron. Gr5a conferred

sensitivity to trehalose, melezitose, methyl α glucopyranoside (m-glucoside), and glucose (**Fig. 2.3**). Ab1C neurons expressing Gr64e responded to glycerol and 1,2 propanediol (**Fig. 2.4**). Based on successful expression of two sweet receptors, we decided to express each receptor of the sweet clade individually and test each receptor using a panel of sweet compounds. This panel included four sugars that are dependent on Gr5a for responses in endogenous taste neurons (trehalose, melezitose, m-glucoside, and glucose), four others that are dependent on Gr64a (sucrose, maltose, maltotriose, and fructose), and glycerol, which is a known ligand for Gr64e. We used concentrations of 100 mM for all sugars except maltotriose (250mM) and glycerol (10%), which are comparable to those that evoke robust responses in taste sensilla.

We found that Gr64a and Gr5a were broadly responsive to two completely non overlapping subsets of sugars (**Fig. 3.2 A**). Gr5a responded to all of the Gr5a dependent sugars but not to the Gr64a dependent sugars or glycerol (**Fig. 3.2 A**). Gr64a responded to all the Gr64a dependent sugars and glycerol but not to any of the Gr5a dependent sugars. In chapter 2, we demonstrated the dose dependent relationships between Gr5a and trehalose, and Gr64e and glycerol (**Fig 2.3 & 2.4**). Here, we applied increasing concentrations of maltotriose, sucrose, and glycerol to ab1C neurons expressed Gr64a (ab1C:Gr64a). We found that with increasing concentrations of each stimulus, there is a correlating increase in neural activity of ab1C:Gr64a neuron supporting these being bonafide responses (**Fig. 3.2 B**).

Each of the eight receptors of the sweet clade responded to at least two sweet compounds of our panel (**Fig. 3.2 A**). Barring the exception of sucrose, each sweet compound was detected by multiple receptors. This indicated that all receptors participate, to some extent, in ligand recognition of various compounds, and that no single receptor is solely responsible for responses to any of the tested compounds.

Using the responses from ab1C neuron expressing GrX (ab1C:GrX) responses, we generated an activity heat map for receptors arranged in the order of sequence similarity (**Fig. 3.2 C**). Based on both sequence similarities and response profiles, we found two loosely separated sub-groups of receptors: one that overlapped with Gr5a and a second with Gr64a.

It has been previously shown that Gr5a and Gr64f are required for trehalose sensitivity [3, 10]. Also, labellar sweet taste neurons lacking both Gr5a and Gr64a do not respond to any sweet compounds. Furthermore, there are examples of pairs of sweet receptors functioning in a neuron devoid of other sweet receptors [18]. Previous evidence suggests that sweet Grs function together in a receptor complex to confer sugar sensitivity. We coexpressed each receptor of the sweet clade with Gr5a to determine if coexpression increases neural responses to sweet compounds. We expressed each of the receptors individually with Gr5a in the ab1C neuron, using 2 Gal4 drivers, 1 copy of *UAS-Gr5a*, and 1 copy of *UAS-GrX*. After applying four Gr5a dependent sugars to the ab1C neuron, we found no significant increase in neural response in any of the

combinations (**Fig. 3.2 D**). In fact, we observed a reduction in Gr5a mediated responses when Gr5a and Gr64a were expressed together.

### 3.3.2 Ectopic responses in the ab1C neuron are validated by mutant analysis

In an effort to validate the ectopic responses discovered in the ab1C neuron, we compared the response profiles of ab1C:GrX neurons to those of sweet taste neurons in the corresponding *Gr* mutants. As of this point, there were four previously reported single *Gr* mutants for Gr5a, Gr61a, Gr64a, and Gr64e [3, 13]. In addition, we found a *Minos* element insertion allele for Gr64f. We chose to record from labellar L-type sensilla since these sensilla house sweet taste neurons that have robust responses to a broad range of sweet taste compounds [11]. We recorded the neural activity from sweet neurons in each mutant using our panel of nine sweet tastants. As previously reported [3], $\Delta$*Gr5a* and $\Delta$*Gr64a* mutant flies showed reduced responses to two non overlapping, complementary subsets of sugars (**Fig. 3.3 A and B**). In addition, there was a complete correspondence of Gr5a and Gr64a receptor-ligand interactions determined via ectopic expression in the ab1C:GrX neurons and reduced sensitivity in the appropriate *Gr* mutant. For Gr61a, Gr64e, and Gr64f, every occurrence of a ligand evoked ectopic response in the ab1C:GrX neuron corresponded to a significant reduction in ligand evoked sweet taste neuron activity in the *Gr* mutant (**Fig. 3.3 A and B**). Using a scatter plot, we examined

the relationship between the gain of function ectopic responses and percent loss of endogenous response in each Gr mutant and compared the trend using a Spearman's correlation (**Fig. 3.3 C**). We found a strong positive correlation, which further authenticates the analysis of singly expressed Grs in the ab1C neuron ($r_s = 0.759$, $n = 45$, $P < 0.001$). Furthermore, the lack of interactions in the ab1C neuron that don't corroborate with mutant analysis makes it unlikely that sweet receptors are conferring any off target responses in this system.

Our mutant analysis also revealed eight instances in which a Gr was necessary for a response to a particular tastant in sweet taste neurons, but was not sufficient to confer sensitivity to that tastant in the ab1C:GrX neuron (**Fig. 3.3 B**). These instances were not restricted to either Gr5a or Gr64a dependent tastants. *Gr61a*, *Gr64e*, and *Gr64f* mutants all showed significant reductions in response to one or more tastants of both subsets. *ΔGr64e* flies have reduced responses to maltose and maltotriose but expression of Gr64e is unable to confer sensitivity to these compounds in ab1C neuron. Gr61a is necessary for trehalose, glucose, and m-glucoside sensitivity but ab1C:Gr61a neurons do not respond to trehalose. Lastly, ab1C:Gr64f neurons respond to trehalose, melezitose and m-glucoside, yet Gr64f is also necessary for glucose, sucrose, and maltose sensitivity in sweet taste neurons. This supports previous research where expression of Gr64a with Gr64f in *ΔGr64* flies is required to rescue responses to sucrose, glucose, and maltose in labellar L-type sweet taste neurons [10]. These results suggests that these receptors are necessary for

forming a functional receptor complex in the endogenous taste neuron but may not be involved in ligand recognition of those compounds.

Based on the mutant analysis, there were a few instances in which the loss of a single receptor led to a complete loss in neuronal response to a selected tastant at 100 mM. Examples include: Gr5a necessary for response to trehalose, and both Gr64a and Gr64e are necessary for responses to maltotriose and glycerol. On the other hand, the substantial amount of overlap in sweet compounds detection by the sweet receptor clade indicates that other receptors can detect these compounds. For example, trehalose is detected by Gr5a, Gr64b, Gr64e, and Gr64f. Gr64a, G64e, Gr64c, and Gr64d can confer glycerol detection to the ab1C neuron. Also, maltotriose is detected by Gr64a, and Gr64c. We wondered if single mutants of Gr5a, Gr64a, and Gr64e were insensitive at these concentrations but would respond at higher concentrations. We recorded from L-type sweet taste neurons on *ΔGr5a, ΔGr64a, ΔGr64e, ΔGr61a,* and *ΔGr64f* flies using our panel at 1 M concentration except glycerol (25%). We discovered that some of the mutant flies could respond to the sugars at a higher concentration but not all (**Fig. 3.4 A and B**). Labellar L-type sweet taste neurons on *ΔGr5a* flies responded to 1 M trehalose and *ΔGr64a* responded to glycerol. Interestingly, L-type sweet neurons on both *ΔGr64a* and *ΔGr64e* still did not respond to maltotriose even at higher concentrations. One possibility is that both Gr64a and Gr64e receptors may function together and are required in L-type sweet neurons to detect maltotriose.

### 3.3.3 Coexpression of Gr64a and Gr64e alters response and inhibition

In chapter 2, we described that ab1C:Gr64e neurons confer sensitivity to glycerol, which can be inhibited by 3-amino-1,2 propanediol and 2-amino-1,3 propanediol (**Fig. 2.4**). ab1C:Gr64a neurons can also confer sensitivity to glycerol. We therefore wondered if the compounds that inhibited Gr64e dependent glycerol response could also inhibit Gr64a dependent glycerol response. To test this possibility, we recorded from ab1C:Gr64a flies using 10% glycerol and mixtures of 10% glycerol and 3-amino-1,2 propanediol or 2-amino-1,3 propanediol . Surprisingly, Gr64a dependent glycerol response was inhibited by 3-amino-1,2 propanediol but was immune to inhibition by 2-amino-1,3 propanediol (**Fig. 3.5 A**). Since Gr64a dependent glycerol response was not inhibited by 2-amino- 1,3 propanediol and Gr64e dependent glycerol response was inhibited by both of the glycerol inhibitors, we wondered if coexpression of Gr64a and Gr64e would alter responses to glycerol or its inhibitors. We used two GAL4 drivers, two copies of *UAS-Gr64a* and two copies of *UAS-Gr64e* (ab1C:Gr64a +Gr64e) in order to compare with the results of experiments in which either receptor was expressed alone via two copies of UAS-Gr transgenes. As compared to previous coexpression experiments in which we tested only one copy of each UAS-GrX (**Fig. 3.2 D**), we found an increase in the response to glycerol that may be attributed to the presence of both receptors (**Fig. 3.5 B**). Moreover, ligand sensitivities conferred when each receptor is singly expressed

are still present when Gr64a and Gr64e are coexpressed. Surprisingly, the neural response to glycerol was inhibited by both 3-amino-1,2 propanediol and 2-amino-1,3 propanediol when both Gr64a and Gr64e are expressed together ( **Fig. 3.5 A and B**). Furthermore, ab1C:Gr64a +Gr64e neurons' neural response to glycerol was inhibited by 2-amino 1,3 propanediol (67%) to a similar degree as in ab1C:Gr64e neurons (72%). Since the glycerol mediated response is similar in both ab1C:Gr64a neurons (16.6 spikes/sec) and ab1C:Gr64e neurons (20.1 spikes/sec), these results suggests that coexpression of Gr64a and Gr64e led to an overall enhanced sensitivity to inhibitors, supporting the possibility of interactions between coexpressed Grs in the ab1C neuron.

### 3.4 Discussion

We have shown that each Gr belonging to the sweet clade can function in the ab1C neuron and is capable of detecting multiple ligands. Each receptor can detect more than one tastant and can be placed loosely into one of two groups: one that overlaps with Gr5a dependent sugar and one that overlaps with Gr64a dependent sugars. Moreover, there is broad overlap in detection of sweet compounds where each taste compound is typically detected by more than one receptor of the sweet clade. There is a strong correlation between sweet tastant induced responses that are affected by loss of a Gr, and the responses that are conferred by expression of that Gr in the ab1C neuron. While pairwise coexpression of Gr5a with other receptors did not cause a significant increase in

sugar responses, coexpressed Gr64a and Gr64e response to glycerol had enhanced sensitivity to inhibition by 2-amino 1,3 propanediol that Gr64a lacked when artificially expressed alone in the ab1C neuron. The percent inhibition of the glycerol mediated response by 2-amino 1,3 propanediol in ab1C:Gr64a+Gr64e neurons is similar to the degree of inhibition of the Gr64e dependent glycerol response by the same inhibitor. The level of inhibition by 2-amino 1,3 propanediol of the coexpressed receptors argues for functional interactions between these two receptors.

Our analysis shows that all sweet Grs can be involved directly in ligand recognition. Gr proteins are most closely related to Or proteins, which function in heteromeric complexes with an obligate coreceptor, Orco, that does not respond to any natural compounds [20, 21]. Compared to Ors, there does not seem to be an OrCo-like counterpart in the sweet clade, or in the Gr family. As previously stated there multiple receptor combinations can detect the same sweet compound which leads one to wonder how much overlap exists for sweet compounds in the taste system[18]. Based on our analysis, it appears that as many as five sweet receptors can detect trehalose and as few as two sweet Grs, Gr64a and Gr43a, can detect sucrose. One caveat is that responses that none of the sweet Grs are normally expressed with Gr21a or Gr63a. As such, some novel responses could be missed which is an issue that previous heterologous expression experiments seemed to have as well. For example, expression of Gr5a in the ab1C neuron conferred glucose, trehalose, and melezitose

sensitivity. In a different system, artificial expression Gr5a with endogenously expressed Gr43a in a tarsal sweet neuron in *ΔGr5a; ΔGr61a, ΔGr64* flies conferred sensitivity to trehalose and melezitose but not glucose [18]. It may explain why Gr64a is the only Gr of the sweet clade that conferred sensitivity to sucrose, yet *ΔGr64a* labellar sweet taste neuron had reduced responses to sucrose but not a complete loss. The other receptor known to respond to sucrose, Gr43a, is not expressed in labellar taste hairs. This suggests that either another receptor or combination of receptors is capable of detecting sucrose.

Since Gr5a and Gr64a respond to and are necessary for complementary subsets of sugars and the loss of Gr5a and Gr64a causes labellar L-type taste hairs to not respond to any sweet compounds, it argues for these two receptors forming complexes with other Grs but not each other. In fact, one hypothesis suggests that the eight receptors originate from a single ancestral gene which led to two lineages for Gr5a and Gr64a [4]. By gene duplication and mutations, two groups, one from Gr5a and the other Gr64a, could have further specialized into the sweet clade. This could explain the division between the sweet receptors and which ligands they can detect. Furthermore, each sugar only evoked a strong response from one or two receptors in the ab1C neuron (>12.7 spikes/sec) which suggests further specializing by the receptors to detect specific compounds. As such, the sensitivity of a sweet taste neuron to a specific sweet compound would increase with the addition of each sweet Gr.

Based on our mutant analysis, there were few examples where loss of a Gr led to no neural responses to a sweet compound at 1M or 25% glycerol (**Fig. 2.4**). These include: Gr64e is necessary for glycerol responses, detection of maltotriose or fructose is dependent on Gr64a and Gr64e, and Gr5a is necessary for melezitose mediated responses. Since both Gr64a and Gr64e are necessary for neural responses to maltotriose and fructose in labellar sweet taste neurons, Gr64a and Gr64e may function together in a complex potentially with other receptors to detect these compounds. Single mutants for Gr64c, and Gr64d, both of which respond to at least one of these compounds, were not available so it is not possible to make predictions if these receptors are just as vital for detecting maltotriose or fructose. Our analysis from chapter 2 suggests that Gr43a can detect maltotriose as well as fructose. As with sucrose, it appears that there may more than one combination of receptors that detect fructose and maltotriose: one with Gr43a and another combination including Gr64a and Gr64e and potentially Gr64c and Gr64d as well.

We wondered about what the potential evolutionary advantage of having multiple sweet Grs to detect a single compound is. For example, trehalose is detected by potentially four Grs: Gr5a, Gr64b, Gr64e, and Gr64f. One possibility could be that redundancy of potential binding sites prevents significant loss of detection of a sugar. Another possibility is that each Gr may have different levels of sensitivity which are activated with higher concentrations of sugar. At 100mM trehalose, single mutants of Gr5a, Gr61a, Gr64e, and Gr64f had similar reduction

in neural responses. When we increased concentration to 1M trehalose, we saw different levels in responses from mutants. Both *ΔGr64f* and *ΔGr61a* flies had near *wild type* responses to trehalose. *ΔGr64e* responses were still dramatically reduced while *ΔGr5a* flies responded very weakly to 1M trehalose. This suggests that Gr64f and Gr64e are necessary for trehalose responses at low concentrations but not at high concentrations, indicating that overlap of ligand detection may be to increase the sensitivity of sweet taste neurons to sweet compound.

In our study, coexpression of sweet Grs with Gr5a in the ab1C neuron did not increase sensitivity to Gr5a dependent sugars. This may be due to several factors. The presence of Gr21a and Gr63a could be interrupting formation of a receptor complex that allows heightened sensitivity. The ab1C neuron may lack potential taste cofactors to support higher responses. It could also be that we lack the appropriate receptor combination or expression levels to confer responses. Interestingly, expression of Gr64e with Gr64a in the ab1C neuron had higher responses to glycerol and was inhibited to the same degree as ab1C:Gr64e neurons (**Fig. 2.5**). ab1C:Gr64a neurons were immune to inhibition by 2-amino 1, 3 propanediol. This suggests that coexpressed sweet Grs are capable of interactions in the ab1C neuron. It would be interesting to see if Gr21a or Gr63a interferes with coexpressed sweet receptor functioning together by comparing responses from pairwise receptor combination in the absence of Gr21a and Gr63a.

There are discrepancies between previously published work and our analysis here. It has been previously shown that labellar L-type hairs in *ΔGr64* flies do not respond to any taste compounds where only Gr5a and Gr61a are endogenously expressed. Expression of Gr64f in the labellar taste neuron of *ΔGr64* flies, restores the full trehalose response. When we recorded from single mutants, these same neurons in *ΔGr64e* flies have reduced responses to trehalose which suggests that Gr64e is necessary trehalose response as well. One possibility for the disparity may be due to Gr proteins being endogenously expressed at incredibly low concentrations [22]. When using GAL4/UAS system, we are artificially expressing high levels of Gr protein that normally do not exist and may account for the higher response to trehalose even when Gr64e is not expressed. There is precedence for overexpression of Grs leading to increased sensitivity [3, 23].

Overall, we have shown that all sweet Grs can participate in ligand recognition and that there is overlap in detection of sweet compounds. Recordings from single mutants supports a model where the addition of each receptor increases sensitivity to specific sweet compounds but rarely does a loss of a single receptor create a complete deficit in sweet detection of a sweet compound. The ability to increase sensitivity to specific high caloric compounds as well as adapt to the loss of a receptor would be a highly useful evolutionary advantage.

### 3.5 Abbreviations

Gustatory receptors (Gr) Pg. 85

Methlyl-α-glucopyranoside (m-glucoside) Pg. 90

Ab1C neurons expressed Gr64a (ab1C:Gr64a) Pg. 91

Ab1C neuron expressing indicated sweet Gr( ab1C:GrX) Pg. 92

Ab1C neuron expressing two copies of *UAS-Gr64a* and two copies of *UAS-Gr64e* (ab1C:Gr64a +Gr64e) Pg. 96

## 3.6 References


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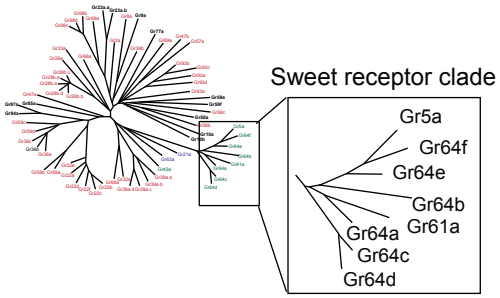
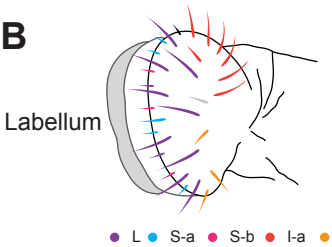
# Figure 3.1

**A**



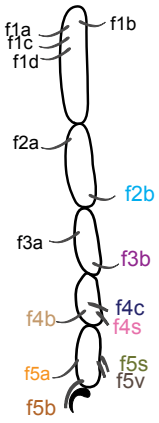

**B**




| Sensillum | Receptors |
|---|---|
| L | Gr5a, Gr61a, Gr64a, Gr64e, Gr64f, *Gr64b, Gr64c, Gr64d* |
| S-a, S-b, S-c | Gr5a, Gr64a, Gr64e, Gr64f, *Gr64b, Gr64c, Gr64d* |
| I-a, I-b | Gr5a, Gr64a, Gr64e, Gr64f *Gr61a, Gr64b, Gr64c, Gr64d* |

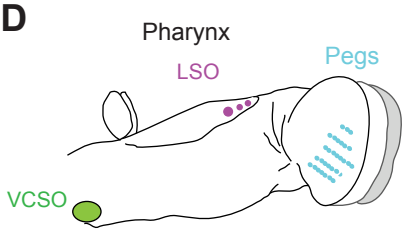
**C**

Female Tarsi




| Sensillum | Receptors |
|---|---|
| f2b, f3b | Gr5a, Gr61a, Gr64c, Gr64e, Gr64f |
| f5v | Gr43a, Gr61a, Gr64a, Gr64c, Gr64e, Gr64f |
| f4b, f5s | Gr5a, Gr61a, Gr64a, Gr64b, Gr64c, Gr64e, Gr64f |
| f5a | Gr61a, Gr64b, Gr64f |
| f4s, f5b | Gr5a, Gr61a, Gr64a, Gr64b, Gr64c, Gr64f |

**D**




| Organs | Receptors |
|---|---|
| LSO | Gr43a, Gr61a, Gr64a, Gr64c, Gr64d, Gr64e, Gr64f, *Gr64b* |
| VCSO | Gr43a, Gr61a, Gr64a, Gr64c, Gr64e, Gr64f |

**Figure 3.1 Sweet Grs are expressed in multiple combinations.** ( **A**) A phylogenetic tree adapted from [2]. Red receptors are expressed in bitter neurons, black have not been mapped to any taste neurons, blue receptors are the carbon dioxide receptors and green have been mapped to the sweet neurons. The box highlights the sweet receptor clade. **(B)** Schematic of sensillar classes in the labellum (top). Tables indicating identified neurons (bottom) with their receptor expression patterns of sweet Grs. Maps created from expression studies along with, in some instances, functional studies; with the exception of Gr64a, which is mapped to labellar sweet neurons by functional studies [3, 11]. Sweet receptors in italics have been mapped by knock-in reporter analysis but not by transgenic reporter experiments. **(C)** Schematic of sensilla in the female fore tarsi (left) and tables indicating identified sweet neurons (right) with their receptor expression patterns. **(D)** Schematic indicating the location of oral taste pegs and the labral sense organ (LSO; chemosensory sensilla in lilac, mechanosensory sensilla in black) and ventral cibarial sense organ (VCSO) in the pharynx. Tables indicating identified neurons (right) with their receptor expression patterns in the LSO and VCSO.

# Figure 3.2

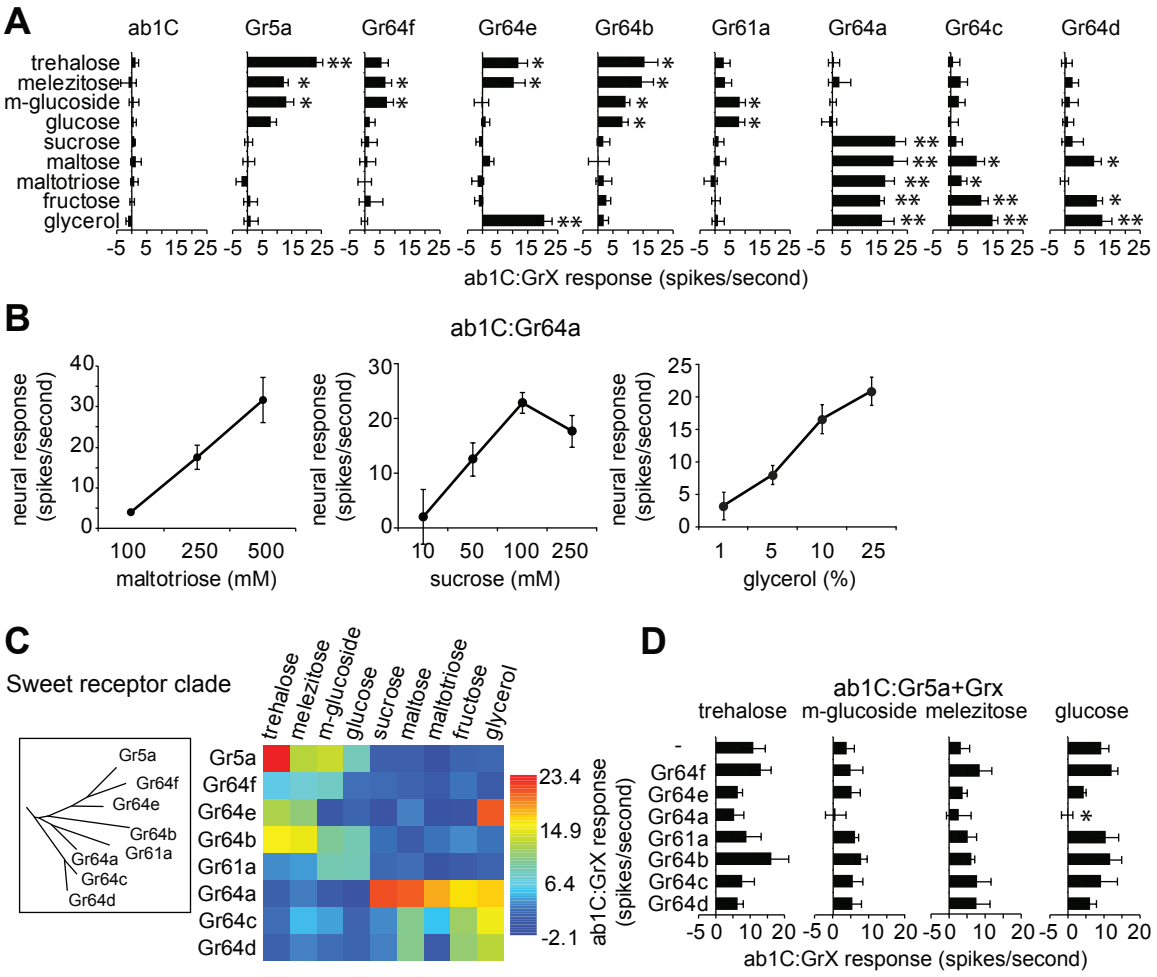

A
ab1C
Gr5a
Gr64f
Gr64e
Gr64b
Gr61a
Gr64a
Gr64c
Gr64d
trehalose
melezitose
m-glucoside
glucose
sucrose
maltose
maltotriose
fructose
glycerol
ab1C:GrX response (spikes/second)
B
ab1C:Gr64a
neural response (spikes/second)
maltotriose (mM)
sucrose (mM)
glycerol (%)
C
Sweet receptor clade
Gr5a
Gr64f
Gr64e
Gr64b
Gr61a
Gr64a
Gr64c
Gr64d
23.4
14.9
6.4
-2.1
ab1C:GrX response (spikes/second)
D
ab1C:Gr5a+Grx
trehalose
m-glucoside
melezitose
glucose
ab1C:GrX response (spikes/second)

**Figure 3.2 Tastant response profiles of sweet taste receptors.** (**A**) Mean electrophysiological responses of ab1C:GrX neurons. All sugars were tested at a concentration of 100 mM, except maltotriose at 250 mM and glycerol at 10% (vol/vol). For each data point, n = 6-14. *P < 0.05; **P < 0.001 [vs. control ab1C flies (*w1118*)]. (**B**) Dose dependent curves for ab1C:Gr64a neurons to indicated stimuli. (n=6-12) (**C**) Phylogenetic tree of sweet Grs adapted from [2] (Left) and heat map of mean neuronal responses of ab1C:GrX neurons to indicated sweet tastants. Data are the same as in 3.2A. Heat map was made with PAST (**D**) Mean electrophysiological responses of Gr5a expressed alone (-) or with the indicated receptor in ab1C neurons to indicated stimuli. For each data point, n = 6-7. *P < 0.05; **P < 0.001 [vs. Gr5a alone (-)]

# Figure 3.3

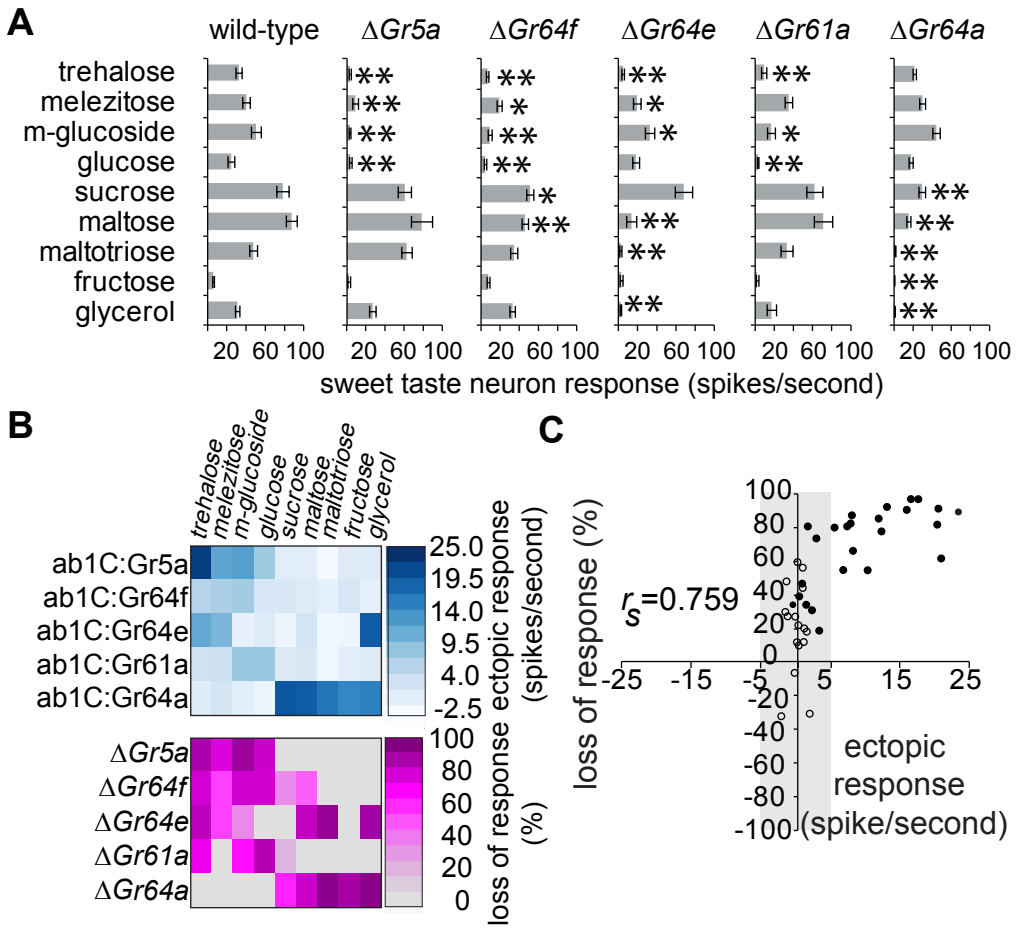

A
wild-type
ΔGr5a
ΔGr64f
ΔGr64e
ΔGr61a
ΔGr64a
trehalose
melezitose
m-glucoside
glucose
sucrose
maltose
maltotriose
fructose
glycerol
20 60 100
sweet taste neuron response (spikes/second)
B
ab1C:Gr5a
ab1C:Gr64f
ab1C:Gr64e
ab1C:Gr61a
ab1C:Gr64a
ΔGr5a
ΔGr64f
ΔGr64e
ΔGr61a
ΔGr64a
25.0
19.5
14.0
9.5
4.0
-2.5
100
80
60
40
20
0
loss of response ectopic response (spikes/second)
(%)
C
loss of response (%)
rs=0.759
ectopic response (spike/second)

**Figure 3.3 Sweet taste responses in Gr mutants.** (**A**) Mean responses of sweet

taste neurons in L-type sensilla to indicated tastants. Indicated genotypes were: *$w^{1118}$ (wild-type), ΔEP(X)-5 (ΔGr5a), $Gr64f^{MB12243}$ (ΔGr64f), $Gr64e^{MB03533}$ (ΔGr64e), $Gr61a^{1}$ (ΔGr61a), and $Gr64a^{1}$ (ΔGr64a).* All stimuli were tested at a concentration of 100 mM, except glycerol (10%). *P < 0.05; **P < 0.001 (one-way ANOVA with one-tailed Dunnett's t test vs. wild -type; n = 6–22). (**B**) Heat maps of ab1C:GrX responses (Upper) and percent reduction in taste neuron responses in corresponding GrX mutants (Lower); the latter only includes data points significantly different from wild-type in C. Percent loss of response was calculated by using [(wild type – mutant)/wild type] x 100. Heat maps were made by using JMP 10 (www.jmp.com). (**C**) Scatter plot of percent loss of response in Gr mutant and ab1C:GrX response (gain) for each GrX–ligand combination. Filled circles indicate taste neuron responses that are significantly reduced in mutant flies (ΔGrX); open circles indicate those that are not. Shaded area indicates ab1C:GrX responses that are not statistically significant.

# Figure 3.4

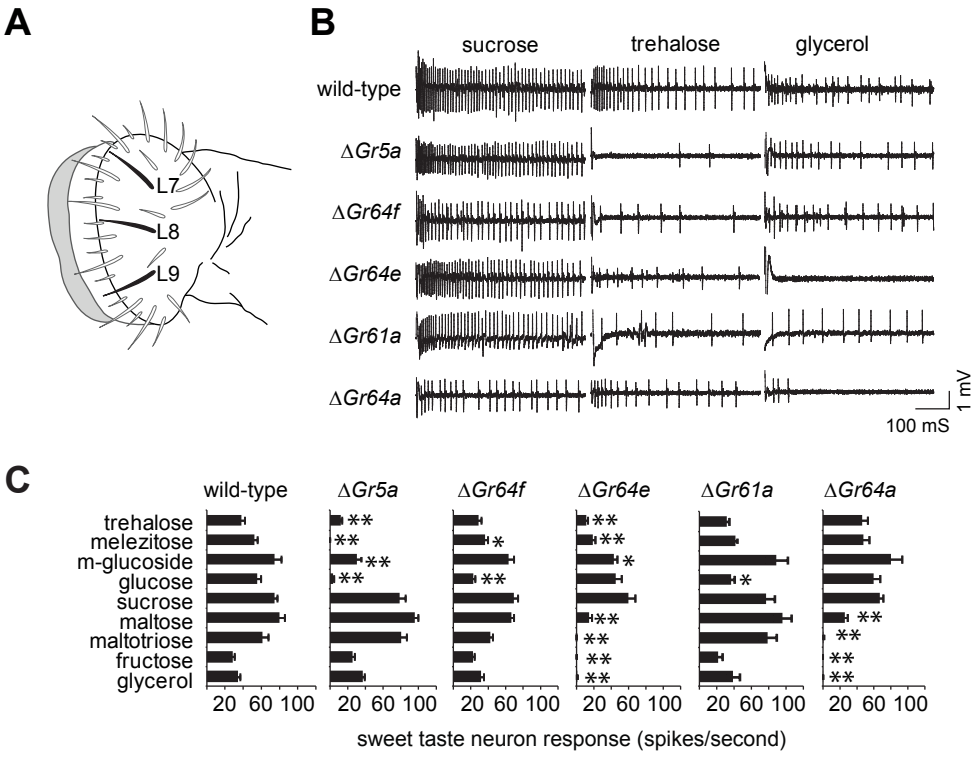

A
L7
L8
L9
B
sucrose
trehalose
glycerol
wild-type
ΔGr5a
ΔGr64f
ΔGr64e
ΔGr61a
ΔGr64a
1 mV
100 mS
C
wild-type
ΔGr5a
ΔGr64f
ΔGr64e
ΔGr61a
ΔGr64a
trehalose
melezitose
m-glucoside
glucose
sucrose
maltose
maltotriose
fructose
glycerol
20 60 100
sweet taste neuron response (spikes/second)

**Figure 3.4 Single Gr mutants have reduced sensitivity not loss of response.** (**A**) Schematic of the fly labellum highlighting L-type sensilla that were used for electrophysiological recordings in black. (**B**) Sample traces of recordings with 100 mM sweet stimuli obtained from L-type sensilla. Genotypes are as follows: *$w^{1118}$ (wild type), ΔEP(X)-5 (DGr5a), $Gr64f^{MB12243}$ (ΔGr64f), $Gr64e^{MB03533}$ (ΔGr64e), $Gr61a^{1}$ (ΔGr61a), and $Gr64a^{1}$ (ΔGr64a). (***B**) Mean responses of sweet taste neurons in L-type sensilla to indicated sugars tested at a concentration of 1 M and glycerol at 50%. *P < 0.05; **P < 0.001 (vs. wild-type; one-way ANOVA with Tukey's post hoc test; n = 6 –12).

# Figure 3.5

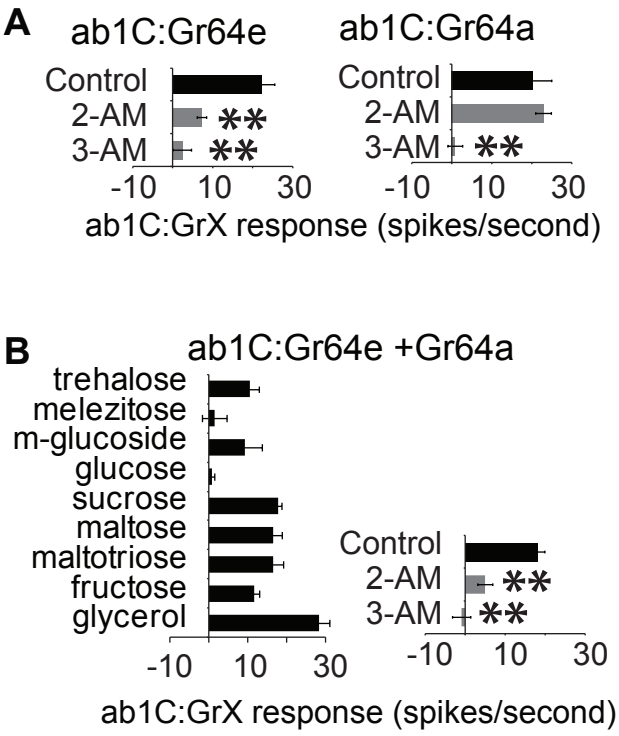

A
ab1C:Gr64e
ab1C:Gr64a
Control
2-AM
3-AM
**
**
**
-10
10
30
-10
10
30
ab1C:GrX response (spikes/second)
B
ab1C:Gr64e +Gr64a
trehalose
melezitose
m-glucoside
glucose
sucrose
maltose
maltotriose
fructose
glycerol
-10
10
30
Control
2-AM
3-AM
**
**
-10
10
30
ab1C:GrX response (spikes/second)

**Figure 3.5 Gr64a and Gr64e coexpression alters inhibition by glycerol inhibitors in the ab1C neuron.** (**A**) Mean responses of ab1C:Gr64e (left) and ab1C:Gr64a (right) neurons to mixtures of 10%glycerol and 2-amino 1,3 propanediol(2-AM) or 3-amino 1,2 propanediol (3-AM) (n=6-12). *P<0.05 and **P<0.001 indicate statistically significant using Dunnett's t -test. (**B**) Mean responses of the ab1C neuron in neurons expressing both Gr64a and Gr64e (ab1C:Gr64e+Gr64a) to indicated stimuli(left) and to mixtures of 10%glycerol and 2-amino 1,3 propanediol(2-AM) or 3-amino 1,2 propanediol (3-AM) (right) (n=6). *P<0.05 and **P<0.001 indicate statistically significant using Dunnett's t -test.

## Table 3.1 List of genotypes used in chapter 3

| Line name | Genotype/ source | Figures |
|---|---|---|
| wild type | *w[1118]* | 3.2 |
| Gr21a-GAL4 | Kristin Scott | |
| Gr63a-GAL4 on III | BDSC (# 9942) | |
| Gr63a-GAL4 on II | BDSC (# 9943) | |
| Δ*Gr63a* | BDSC (# 9941) | |
| UAS-Gr5a | Dahanukar laboratory | |
| UAS-Gr61a | Dahanukar laboratory | |
| UAS-Gr64a | Dahanukar laboratory | |
| UAS-Gr64b | BDSC (# 27324) | |
| UAS-Gr64c | Dahanukar laboratory | |
| UAS-Gr64d | Dahanukar laboratory | |
| UAS-Gr64e | Dahanukar laboratory | |
| UAS-Gr64f | Dahanukar laboratory | |
| Δ*Gr5a* | Δ*EP(X) -5* | 3.3, 3.4 |
| Δ*Gr64f* | *Gr64f[MB12243],* BDSC (# 27883) | 3.3,3.4 |
| Δ*Gr64e* | *Gr64e[MB03533],* BDSC (#23628) | 3.3, 3.4 |
| Δ*Gr61a* | *Gr61a[1]* | 3.3, 3.4 |
| Δ*Gr64a* | *Gr64a[1]* | 3.3,3.4 |
| ab1C | *w[1118]* | 3.2 |
| ab1C:Gr5a | *UAS-Gr5a-8/UAS-Gr5a-8; Gr63a-GAL4/Gr63a-GAL4* | 3.2 |
| ab1C:Gr64f | *UAS-Gr64f-2/UAS-Gr64f-2; Gr63a-GAL4/Gr63a-GAL4* | 3.2 |
| ab1C:Gr64e | *UAS-Gr64e-3/UAS-Gr64e-3; Gr63a-GAL4/Gr63a-GAL4* | 3.2, 3.5 |
| ab1C:Gr64b | *UAS-Gr64b/Gr21a-GAL4; UAS-Gr64b-2/Gr63a-GAL4* | 3.2 |
| ab1C:Gr61a | *UAS-Gr61a-2/Gr21a-GAL4; Gr63a-GAL4/UAS-Gr61a-4* | 3.2 |
| ab1C:Gr64a | *UAS-Gr64a-4/UAS-Gr64a-4; Gr63a-GAL4/Gr63a-GAL4* | 3.2, 3.5 |
| ab1C:Gr64c | *Gr21a-GAL4/UAS-Gr64c-B; Gr63a-GAL4/UAS-Gr64c-2* | 3.2 |
| ab1C:Gr64d | *Gr21a-GAL4/UAS-Gr64d-4; Gr63a-GAL4/UAS-Gr64d-3* | 3.2 |
| ab1C:Gr5a-1x | *UAS-Gr5a-8/+; Gr63a-GAL4/+* | 3.2 |
| ab1C:Gr5a+Gr64f | Gr21a−GAL4/UAS−Gr5a-8; Gr63a−GAL4/UAS−Gr64f-3 | 3.2 |
| ab1C:Gr5a+Gr64e | Gr21a−GAL4/UAS−Gr5a-8; Gr63a−GAL4/UAS−Gr64e-2L | 3.2 |
| ab1C:Gr5a+Gr64a | UAS−Gr5a-8/UAS−Gr64a-4; Gr63a−GAL4/Gr63a−GAL4 | 3.2 |
| ab1C:Gr5a+Gr61a | UAS−Gr5a-8/UAS−Gr61a-2; Gr63a−GAL4/Gr63a−GAL4 | 3.2 |
| ab1C:Gr5a+Gr64b | Gr21a−GAL4/UAS−Gr5a-8; Gr63a−GAL4/UAS−Gr64b-2 | 3.2 |
| ab1C:Gr5a+Gr64c | Gr21a−GAL4/UAS−Gr5a-8; Gr63a−GAL4/UAS−Gr64c-2 | 3.2 |
| ab1C:Gr5a+Gr64d | UAS−Gr5a-8/UAS−Gr64d-4; Gr63a−GAL4/Gr63a−GAL4 | 3.2 |
| ab1C:Gr64e +Gr64a | Gr21a-GAL4,UAS-Gr64a-4/UAS-Gr64e-3; Gr63a-GAL4, UAS-Gr64e-2L/UAS-Gr64a-10 | 3.5 |

## 4.0 Bitter tastant mediated Inhibition of individual sweet taste receptors

**Abstract:** Many potential food sources are mixtures of energy rich nutrients and potentially toxic compounds. Animals discriminate nutritious food from toxic substances using their sense of taste. In *D. melanogaster*, bitter tastants can directly suppress sugar induced action potentials in sweet tastes neurons in the absences of bitter neurons. Here, we investigate whether receptors of an evolutionarily conserved sweet clade are involved in inhibition by bitter compounds using heterologous expression system. After misexpressing single sweet receptors in the $CO_2$ sensing neuron, we recorded the neural activity from mixtures of sweet and bitter tastants using a panel 20 aversive compounds. We found that receptors of the sweet clade are directly inhibited by bitter tastants and each receptor is inhibited by a unique subset of compounds. This property is a distinguishing feature of sweet clade since neither Gr43a nor its mosquito ortholog AgGr25 are inhibited by bitter compounds. Furthermore, coexpression of two sweet Grs alters inhibition profiles where inhibition is enhanced or reduced depending on both the bitter tastant and the receptor combination suggesting that inhibition in sweet neurons is dependent on the receptor combination that is expressed in each sweet neuron.

## 4.1 Introduction

There are over 4000 known alkaloids many of which are toxic or aversive [1]. Between 10-15% of all plants have some type of alkaloid that is usually found in leaves, seeds, and fruit. It is believed that plants use alkaloids and other aversive compounds as deterrents for foraging insects and animals. Many of these plant defense compounds are undesirable and toxic to insects. Because of the risk involved in consuming a potentially toxic compound, detection and avoidance of bitter compounds is essential to avoid deleterious effects. Recent studies have uncovered multiple mechanisms that operate to prevent ingestion of potentially harmful compounds. First, activation of bitter neurons results in taste rejection [2-5]. Activation of bitter neurons can also lead to presynaptic inhibition of sweet neurons via GABAergic interneurons [6]. Recently, many aversive compounds such as alkaloids, DEET and acids were shown to inhibit the activity of sweet taste neurons in both the labellum and the tarsi [7-10]. This occurs via a direct mechanism that is independent of a canonical bitter neuron. An odorant binding protein, OBP49a, is involved in bitter tastant-mediated inhibition of sweet neurons [8]. Using a YFP-based protein complementation assay, OBP49a was shown to be closely associated with Gr64a suggesting sweet Grs play a role in inhibition of the sweet neuron by bitter compounds, yet is still unknown if or how sweet Grs are involved. Here, we show that a broad panel of bitter compounds can inhibit both the sweet neuron and receptors of the sweet clade in *Drosophila*.

Using an expanded 20 compound panel, we demonstrate that the *Drosophila* sweet taste neuron can be inhibited by a wide array of aversive compounds than previously reported, including atropine, a compound that does not activate labellar bitter neurons. We validate OBP49a's role in bitter tastant mediated inhibition of the sweet neuron. Surprisingly, when using higher but still environmentally relevant concentrations of bitter and sweet compounds, we found no significant difference in neural responses from labellar L-type sweet neurons in both *ΔOBP49a* and *wild type* flies. This suggests that other proteins play a role in bitter tastant mediated inhibition of the sweet neuron. Since Gr64a was shown to be closely associated with OBP49a, we hypothesized that sweet receptors are directly inhibited by bitter tastants. We expressed each receptor in the ab1C neuron where OBP49a is not thought to be expressed in sensillar lymph and applied mixtures of sugar and bitter compounds [8, 11]. Surprisingly, we discovered that each receptor of the sweet clade is inhibited by bitter compounds and each receptor displayed a unique inhibition response profile. A comparison of the inhibition at two concentrations of bitter compounds showed that each Gr exhibits different levels of sensitivity to multiple bitter compounds. Bitter tastant mediated inhibition appears to be a property exclusive to the sweet clade, because neither Gr43a nor its mosquito ortholog, AgGr25, were inhibited by any of the bitter compounds tested.

## 4.2 Methods

### 4.2.1 Fly maintenance

Flies were maintained on standard cornmeal–dextrose medium at 25 °C and were tested between 3-10 days after pupation. Full genotypes are in Table 4.1.

### 4.2.2 Single sensillum taste recordings

Tip recordings from taste sensilla were as described [12] using 30 mM tricholine citrate. Refer to Chapter 3.2 Methods. All recordings from Labellar L-type sensilla and s6 recording were counted for 0.5 sec. from contact and multiplied by 2 for (spikes/sec)

### 4.2.3 Olfactory recordings

Extracellular single unit recordings from the ab1C neuron were performed as described. Refer to Chapter 2.2 Methods.

Tastant stimuli were prepared in sensillum lymph ringer (SLR) and stored at 4 °C and were used within seven days. Two recording electrodes, one with electrolyte alone (SLR) and a second with stimulus solution (stimulus) were held on the same manipulator. Recordings were first obtained with SLR from three ab1 sensilla for ~6 s to measure baseline activity of the ab1 neurons. Subsequently, ~6-s recordings were obtained from the same three sensilla stimulus with the stimulus. 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 stimuli—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. Baseline SLR activity of the ab1C neuron was subtracted from the stimulus-evoked response recorded from the same sensillum.

Sugars, bitter compounds, and tricholine citrate TCC were stored at -20°C for up to 6 months. 100% Glycerol was stored at room temperatures. After thawing stock solutions, the testing solutions were made from mixing bitter tastants, sugar, TCC, and milliQ water until all were at appropriate concentrations. Prepared testing solutions were kept at 4°C unless being used and were disposed of after 5 days.

### 4.2.4 Tastants

All compounds were obtained at the highest available purity from Sigma-Aldrich and were as follows: Sweet compounds used were: trehalose (T9531), maltose (M9171), sucrose (S7903), fructose (47740), melezitose (M5375), Bitter compounds used were: atropine (A0132 ), caffeine (C0750), coumarin (C4261), denatonium (D5765), escin (E1378), histamine (H7125), noscapine (N1300000), papaverine (P3510), pilocarpine (P6503), quercetin (337951), quinine (Q1125), scopolamine(S0929), sinigrin (85440),

strychnine(S8753), theobromine (T4500), theophylline (T1633), umbelliferone(H24003), and yohimbine(Y3125).

### 4.2.5 Statistics

Using SSPS software, all statistics are two-way ANOVA with *post hoc* Dunnett's *t*-tests comparing sugar alone vs. sugar/bitter mixtures unless otherwise indicated.

## 4.3 Results

### 4.3.1 OBP49a enhances sensitivity of the sweet neuron to bitter tastant-mediated inhibition.

OBP49a is present in the sensillar lymph of taste hairs on the labellum and is expressed by the thecogen cells near the chemosensory neurons [8]. Previously, it was shown that OBP49a is necessary for inhibition of labellar sweet taste neurons by a panel of nine bitter tastants. To validate these results, we tested mixtures of 10 μM bitter compounds and 10 mM sucrose on labellar L-type sensilla, which do not express any bitter receptors, in *wild type* and two mutants of OBP49a, $OBP49a^{1}$ *and* $OBP49a^{D}$. In agreement with previous work, *wild type* sucrose response was inhibited by bitter compounds, but the inhibition was lost in both $OBP49a^{1}$ *and* $OBP49a^{D}$ flies (**Fig. 4.1 A**).

To characterize the relationship between OBP49a and bitter/sugar concentrations, we repeated the experiment using mixtures of 100mM sucrose and increasing concentrations of bitter compounds (0.1mM, 1mM, and 10mM). *Wild type* flies showed dose dependent inhibition with increasing concentrations of bitter compound leading to further reductions in neural responses to 100 mM sucrose. Surprisingly, we found no difference in inhibition between either OBP49a mutants or *wild type* flies using these sucrose/bitter concentrations (**Fig. 4.1 B**). Furthermore, inhibition was dose dependent for all genotypes. Suggesting that although OBP is required for the strength of inhibition observed in *wild type* flies, its activity is not essential when higher concentrations of bitter and sweet tastants are used.

### 4.3.2 Bitter tastant-mediated inhibition of sweet taste neurons is dependent on both the sugar and bitter compound used in mixture.

Recent studies have compared inhibition levels for multiple bitter compounds but no one has tested if inhibition of the sweet neurons varies by the agonist used for activation [7-9]. A previous study discovered that OBP49a is found in close proximity to Gr64a at the membrane of sweet neurons, suggesting that Gr64a and potentially other sweet Grs may play a role in the inhibition of the sweet neuron directly by bitter compounds. In Chapter 3 (**Fig. 3.2**), we found that different sweet receptors are able to detect different sugars where Gr5a and Gr64a detect two broad and nonoverlapping groups of sweet compounds. We

hypothesized that if multiple sweet Grs are involved in bitter tastant mediated inhibition, then the sugar used in mixtures with bitter compounds could affect the degree of inhibition of the sweet neuron. To determine the role agonists play in sweet neuron inhibition, we picked two Gr64a dependent sugars (sucrose and maltose) and two Gr5a dependent sugars (trehalose and melezitose). For each sugar, we selected a concentration that would yield a response that was comparable to that evoked by 50 mM sucrose (~42 spikes per second) representing the $EC_{50}$ for sucrose based on the neural response of labellar L-type sweet neurons. Sugars were tested in mixtures with 5 mM and 1mM of bitter tastant. The neural response of L-type labellar sweet neurons to the mixtures was compared to sugar alone. We found that responses to all of our agonists were inhibited by bitter compounds (**Fig. 4.2 A & B**). Interestingly, when we compared the degree of inhibition observed for each sugar-bitter mixture, we found that inhibition was influenced by sugar used in the mixtures. For example, 1mM denatonium inhibited the neural response to sucrose by 35% while the other sugars responses were inhibited by 59 %. Though we chose two Gr64a dependent sugars and two Gr5a dependent sugars, we only found one example where the neural response to these two sugar groups responded differently. At 1mM, strychnine significantly inhibited melezitose and trehalose while the sucrose and maltose mediated responses were not inhibited by strychnine.

### 4.3.3 Sweet taste neurons are inhibited by multiple classes of bitter compounds

The number of compounds that are aversive to flies is orders of magnitude larger than appetitive compounds. Recent papers have shown that many structurally diverse compounds including all classes of alkaloids, some amino acids and glycolisides are aversive to flies [3, 7, 9, 13-17]. As of now, only 10 bitter compounds have been shown to be capable of inhibiting the sweet neuron (caffeine, lobeline, quinine, strychnine, berberine, papaverine, denatonium, escin, DEET and nicotine) but there may be more bitter compounds capable of inhibiting the sweet neuron [7, 8]. To study the relationship between bitter compounds and sweet taste neuron inhibition, we decided to expand our bitter panel to 20 structurally diverse bitter compounds including nine compounds that have never been tested for avoidance behavior (**Table 4.2**)[18-33] . We selected bitter compounds to increase structurally diversity and picked alkaloids from different classes, plus other compounds that are found in plants . Some compounds were selected for their structural similarity to see if similar compounds could be distinguished from each other. These include caffeine, theophylline, and theobromine where the only difference is the addition or removal of a methyl group.

Since some of these compounds have never been tested before and have not been shown to be aversive to *Drosophila*, another lab member recorded the neural activity of the bitter neuron in labellar S-6 hairs to these bitter compounds.

He showed that all the bitter compounds could be detected by labellar bitter neurons except atropine which has been previously found not to activate any labellar bitter neurons but was aversive in behavioral studies (**Fig. 4.3 A**) [16]. Next, we used our panel of bitter compounds on labellar L-type sensilla. Using mixtures of 100mM sucrose and 5mM bitter tastant, we discovered that many of these compounds can inhibit the sweet taste neuron in *wild type* flies (**Fig. 4.3 B**). Moreover, we found that different bitter compounds elicited different levels of inhibition. Some compounds such as histamine and escin were weak inhibitors, whereas others such as lobeline and strychnine were strong inhibitors. Furthermore, atropine could inhibit labellar sweet neurons despite labellar bitter neurons not responding to it. The sweet neuron was also able to distinguish between structurally similar bitter compounds. For examples, theobromine did not inhibit sucrose mediated responses of the sweet neuron while both caffeine and theophylline did to similar degrees.

As we have previously shown, Obp49a was not necessary for inhibition of the sweet neuron when using mixtures with higher concentrations of sugar and bitter compound. Since we used a small panel of five bitter compounds previously, we cannot eliminate the possibility that OBP49a might be necessary for inhibition of the sweet neuron by the other bitter compounds in our panel when using 100mM sucrose and 5 or 1 mM bitter compounds. To see if OBP49a enhanced sensitivity of the sweet neuron to any of the bitter compounds in our panel at these concentrations, we tested responses of L-type sensilla in

*ΔOBP49a* flies to mixtures of bitter compounds at two different concentrations (5mM and 1mM) with 100mM sucrose (**Fig. 4.3 C**). For most of the compounds tested, we did not see any change in inhibition between *wild type* and *ΔOBP49a* flies. There were even a few instances where flies lacking OBP49a had higher degrees of inhibition. When we compared the responses from *wild type* and *ΔOBP49a* flies using 5mM bitter compound and 100mM sucrose, there was no statistical difference between the two genotypes, which suggests that OBP49a may not play a significant role at these concentrations for the bitter compounds in our panel (1-way Anova *wild type* vs. *ΔOBP49a*, $P>0.05$).

### 4.3.4 Gr64a can be directly inhibited by bitter tastants

Loss of OBP49a does not stop interactions between sweet neurons and bitter compounds. Furthermore, OBP49a was previously shown to be closely associated with Gr64a in the membrane [8]. This supports the possibility that Gr64a maybe involved in bitter tastant mediated inhibition of the sweet neuron. Since we have created a system that can successfully express individual sweet receptors, we can determine whether sweet Grs are directly inhibited by bitter compounds (Chapter 2&3). Another advantage of using an olfactory neuron is that it eliminates other inhibitory mechanisms that are specific to the sweet neuron. Secondly, we can express each sweet Gr individually in the ab1C neuron which allows us to see if all or only a few sweet Grs are directly involved in inhibition by bitter compounds.

We expressed Gr64a in the ab1C neuron (ab1C:Gr64a) and recorded using mixtures of 100 mM sucrose and varying concentrations of lobeline (**Fig. 4.4 A and B**). Lobeline inhibited the neural response of ab1C:Gr64a neurons to sucrose in a dose dependent manner, where increasing concentrations of lobeline further reduced the activity of ab1C:Gr64a neuron. Our next step was to determine if inhibition was dependent on the expressed sweet Gr or the ab1C neuron itself. We measured the baseline activity of the ab1C neuron in control flies *(+/+;Gr63a-GAL4/Gr63a-GAL4*) and its response to 1% $CO_2$, ,ab1C's endogenous ligand, when exposed to 5 bitter compounds at 10mM. We chose bitter compounds that were either moderate or strong inhibitors of labellar sweet neurons (**Fig. 4.3**): caffeine, lobeline, quinine, strychnine and denatonium. We found that neither the baseline activity nor response to 1% $CO_2$ were affected by bitter tastants (**Fig. 4.4 C and D**). Since bitter alkaloids did not alter the ab1C neuron response to $CO_2$ or its baseline activity, it indicates that the inhibitory action of bitter tastants is specific to the Gr64a sweet taste receptor.

We next tested mixtures of 100mM sucrose and 5 bitter compounds at 10mM on ab1C:Gr64a neurons. Both lobeline and quinine inhibited ab1C:Gr64a sucrose response (Fig**. 4.4 E**). We tested mixtures using another sugar that strongly activates ab1C:Gr64a, maltose, to determine if inhibition was dependent on the expressed receptor or the sugar. Quinine and lobeline also inhibited ab1C:Gr64a maltose response suggesting that inhibition is dependent on Gr64a (**Fig. 4.4 E**).

Caffeine, lobeline and strychnine inhibited labellar sweet taste neurons, but ab1C:Gr64a neurons were immune to inhibition by these compounds. We wondered if other sweet Grs could be inhibited by these bitter compounds. Since Gr64a and Gr5a are necessary for the detection of nonoverlapping subsets of sweet compounds, we wondered if Gr5a and Gr64a are involved in inhibition by different nonoverlapping subsets of bitter compounds. To test this theory, we expressed Gr5a in the ab1C neuron (ab1C:Gr5a) and recorded neural activity using mixtures of 100mM trehalose or melezitose and 10mM bitter compounds. Surprisingly, the neural response of ab1C:Gr5a neurons to both trehalose and melezitose were inhibited by strychnine and quinine (**Fig. 4.4 F**). This suggests that multiple sweet Grs maybe involved in bitter tastant mediated inhibition of the sweet neuron. Both ab1C:Gr64a and ab1C:Gr5a neurons are inhibited by quinine, but ab1C:Gr64a was also inhibited by lobeline and ab1C:Gr5a by strychnine indicating that different Grs are inhibited by different bitter compounds.

### 4.3.5 Receptors of the sweet clade have distinct inhibition profiles

Since both Gr64a and Gr5a can be inhibited by bitter compounds, we wondered if inhibition by bitter compounds is unique to some sweet Grs or if other Grs are inhibited as well. We decided to use our previous panel of 20 bitter compounds since there was a broad range of weak to strong inhibitors of the sweet neuron. Furthermore, many bitter compounds in our panel were previously shown to range from mildly to strongly aversive in feeding assays [16]. By using

a large panel, we can see if there is any correlation between inhibition of the sweet neuron and inhibition of each sweet Gr. Moreover since Gr5a and Gr64a were inhibited by different bitter compounds, a large panel allows us to see if each receptor is inhibited by a distinct class of bitter compounds. Also, we can determine if there are any structural features unique to a class of bitter compounds and its ability to inhibit a sweet Gr.

We tested all but Gr61a, which yielded only weak responses to sugars (**Fig. 3.2**). After expressing each receptor in the ab1C neuron (ab1C:GrX) , we mixed 5mM or 10mm bitter compounds with either 100mM maltose or 100mM trehalose. Two sugars were chosen because no sugar is detected by all of the sweet Grs. In chapter 3, we discovered that sweet receptors could be loosely grouped into two nonoverlapping groups based on which sugars they could detect when expressed in the ab1C neuron (**Fig. 3.2**). We chose trehalose and maltose because all of the sweet receptors respond to either trehalose or maltose, except Gr61a. Two concentrations of bitter compounds were chosen to compare differences in sweet receptor sensitivity.

We discovered that each bitter compound tested could inhibit at least one receptor at 10mM and that each receptor was inhibited by a unique subset of bitter compounds (**Fig. 4.5**). Moreover, some receptors were inhibited by bitter compounds at both 10 mM and 5mM while others were only inhibited when using 10mM. This indicates that some receptors were more sensitive to lower concentrations of specific bitter compounds than others. To visualize differences

in inhibition between receptors, we created heat maps of % inhibition for all the receptors at 2 concentrations (**Fig. 4.5 C**). By comparing the receptors at 5mM bitter compounds, it appears that Gr64f and Gr64a were inhibited by the most compounds. This is interesting because Gr64f is a trehalose receptor and Gr64a is a maltose receptor which suggests that each subgroup of receptors has at least one broadly inhibited receptor.

Based on ab1C:GrX bitter inhibition profiles, each receptor can be inhibited by a distinct panel of bitter compounds while the sweet taste neuron is broadly inhibited by our entire panel. We wondered if when sweet Grs are expressed together, could inhibition of one Gr inhibit the activity of other sweet Grs. Previously in chapter 3, the glycerol response from coexpressing Gr64e and Gr64a in the ab1C neuron was inhibited by two inhibitors when Gr64a was inhibited by only one of the inhibitors suggesting the coexpressed receptors had functional interactions. We wanted to determine if coexpression of two receptors would alter the inhibition profile. We tested two combinations that respond to trehalose: Gr5a+Gr64f and Gr5a+Gr64e. Our reasoning being is there is evidence that Gr5a functions with both Gr64f and Gr64e and all three are needed for trehalose detection [34, 35]. Plus, Gr64f is the only receptor inhibited by caffeine and would be a great indicator if coexpressing receptors can affect the inhibition profile. We used two copies of *Gr63a-GAL4*, and two copies *UAS-GrX* for each sweet Gr in the ab1C neuron. Next, we applied mixtures of 100mM trehalose and 10mM bitter compound and measured the neural responses which

were then compared to responses to 100mM trehalose alone. The inhibition profiles for both combinations were most similar to ab1C: Gr5a with few example of additive inhibition when compared to any of the singly expressed receptors (**Fig. 4.6**). We then calculated the % inhibition for each of the individual receptors and the co-expressed receptors (**Fig 4.6 C & D**). The results suggest that for some bitter compounds, inhibition of the coexpressed receptor was the combined inhibition of each receptor but not others. For example, ab1C:Gr5a+Gr64f response to trehalose was 53% inhibited by caffeine while ab1C:Gr64f was completely inhibited (100%) by caffeine and ab1C:Gr5a trehalose mediated response is unaffected. Similar behavior can be seen in ab1C:Gr5a+Gr64e neurons as well. ab1C:Gr5a +Gr64e trehalose mediated response is 54% inhibited by denatonium while ab1C:Gr5a neurons were immune to inhibition and ab1C:Gr64e response to trehalose is completely inhibited by denatonium. On the other hand, some cases where there isn't equal contribution include ab1C:Gr5a +Gr64f trehalose mediated response is 20% and 24% inhibited by denatonium and escin, respectively, yet ab1C:Gr64f neural response to trehalose is completely inhibited by both these compounds. As such it appears for some bitter-receptor interactions both receptors may contribute equally and in other interactions one of the receptor may take precedence.

### 4.3.6 Gr43a and AgGr25 are immune to inhibition by bitter compounds

All the receptors of the sweet clade, except Gr61a, have been shown to be inhibited by bitter compounds. There is one sweet receptor in *Drosophila* that is not part of the sweet clade, Gr43a. Previously, we showed that ab1C neurons expressing Gr43a (ab1C:Gr43a) respond to fructose and other sugars (**Fig 2.6**). We wondered if all sweet Grs could be inhibited by bitter compounds or if inhibition is specific to the sweet clade. To that end, we recorded from ab1C:Gr43a neurons using mixtures of 100mM fructose and 10mM bitter compounds. We used the same panel of bitter compounds as was previously used on the other sweet Grs. In contrast with the receptors in the sweet clade, none of the bitter compounds inhibited ab1C:Gr43a response to fructose (**Fig. 4.7**).

Gr43a is evolutionarily conserved as a fructose receptor and orthologs exist in insect species [36, 37]. In Chapter 2, we showed that ab1C neuron expressing Gr43a's mosquito ortholog, AgGr25, had a similar response profile to different sugars as ab1C:DmGr43a neurons and responded to fructose, sucrose, glucose, maltose, and glycerol (Fig**. 2.6**). Because of the sequence similarity (~27%) with DmGr43a, we wondered if AgGr25 is immune to bitter tastant mediated inhibition as well. After expressing AgGr25 in ab1C neuron (ab1C:AgGr25), we applied mixtures of 100mM sucrose and 10mM bitter compound. Similar to DmGr43a, ab1C:AgGr25 response to fructose was

resistant to inhibition by bitter compounds. This suggests that inhibition by bitter compounds is unique to the sweet clade.

### 4.4 Discussion

Multiple classes of bitter alkaloids and other aversive compounds can inhibit responses of the sweet taste neuron. Inhibition is dependent on the identity and concentration of the bitter tastant, and is via a direct effect on sweet taste neurons because it occurs in the absence of bitter taste neuron activity. Although inhibition is reduced in flies lacking OBP49a, it is not lost, invoking the presence of other targets of bitter tastant action in sweet neurons. Individually expressed receptors of the sweet clade can be directly inhibited and each receptor is inhibited by a unique subset of compounds suggesting that sweet receptors play a role in the inhibition of the sweet taste neuron by bitter compounds.

It is interesting that OBP49a supports inhibition at lower concentrations of bitter compounds but is not necessary at higher concentrations. When determining the relevance of using higher concentrations of bitter and sugar mixtures as compared to previous labs, we looked at the concentration in natural sources. Many sugars are found in nature at similar or higher concentrations [38-40]. Bitter compounds can be found at either lower or higher concentrations than what we chose, depending on the source (Table 4.2). Many aversive compounds are only slightly water soluble and some are found at low but still toxic

concentrations, so OPB49a may be necessary in these scenarios. As of now, we don't know if OBP49a enhances sweet neurons' sensitivity to bitter compounds at low concentrations no matter the agonist concentration or if OBP49a-dependent inhibition is only essential at low sugar concentration. Further experiments comparing neural responses from labellar sweet neurons in *OBP49a* mutants and *wild type* flies using mixtures of bitter compounds at low concentrations (10μM) and high sugar concentrations (100mM) would help determine whether OBP49a is important when evaluating food sources with high sugar concentrations.

Using a YFP based complementation assay, Jeong *et al*. (2013) discovered that OBP49a is either juxtaposed or directly interacts with Gr64a [8]. The fact that sweet neurons can be inhibited by bitter tastants in absence of OBP49a and individual sweet Grs can be directly inhibited in a heterologous system supports the possibility of OBP49a ferrying bitter compounds in the sensillar lymph and increasing the local concentration of bitter compounds around the sweet receptors to increase sensitivity. In fact, it was originally hypothesized that odorant binding proteins were involved in bringing low water soluble volatile compounds to olfactory receptors in olfactory sensilla [41]. Another possibility is that OBP49a has direct interactions with sweet receptors in the presence of bitter compounds therefore enhancing sensitivity of the sweet receptors to inhibition when exposed to potentially toxic food with low sugar concentrations. Future studies, possibly using coimmunoprecipitation, could test

if there are any direct interactions between Gr64a or other sweet Grs and OBP49a.

When we recorded from labellar sweet neurons using mixtures of 100mM sucrose and 5mM bitter compounds, we found that our panel gave us a continuum of strong, medium, or weak inhibitors. By comparing bitter tastant mediated inhibition of the sweet neuron to previous avoidance experiments, we found some correlation between sweet neuron inhibition and behavior. In Weiss *et al.* (2011), they compared the preference between 1mM sucrose and 5mM sucrose mixed with different concentrations of bitter compounds [16]. Then, they calculated the isoattractive point for each bitter tastant for these experiments. The isoattractive point is the bitter tastant concentration when mixed with 5mM sucrose would reduce preference for the mixtures so that flies had equal preference for mixture and 1mM sucrose. A lower isoattractive point suggests that a bitter compound is more aversive. In this experiment, denatonium, strychnine, and lobeline had the lowest isoattractive points [42]. These compounds were also the strongest inhibitors of the sweet neuron response to 100mM sucrose in our panel. Furthermore, caffeine, theophylline, and escin had higher isoattractive points and were weaker inhibitors of the sweet neurons than strychnine, lobeline, and denatonium. There were compound that did not match this trend and were aversive in behaviors studies but did not inhibit the sweet neuron at 5mM. Two examples include umbelliferone and coumarin where they did not inhibit sweet neurons' 100mM sucrose induced responses at 5mM but

have lower isoattractive points than caffeine in behave,or studies while 5mM caffeine did inhibit neural responses to 100mM sucrose. This suggests that while sweet neuron inhibition does play an important role in avoidance behaviors, other mechanisms such as activation of the bitter neuron and inhibition of the sweet neuron by GABAergic interneurons may have a more active role in prevention of consumption of these compounds [8, 43]. Furthermore, we only recorded from labellar L-type sensilla. Sweet neurons in other sensilla classes may have a different inhibition profile.

Surprisingly, each receptor of the sweet clade can detect a broad selection of bitter compounds, and each one detects a unique subset while the endogenous taste neuron is broadly inhibited by many compounds. Because of how structurally diverse bitter compounds are from sugars, it suggests that bitter compounds could be allosteric modulators of specific sweet taste subunits that form a sweet receptor complex in the taste neuron. Each receptor is inhibited by a broad and unique subset of bitter compounds but there does not appear to be any similar features between the compounds detected by each receptor based on structure or environmental source. Nevertheless, bitter tastant mediated inhibition of each sweet Gr is highly specific. For example, sweet Grs can distinguish between caffeine and theobromine, and caffeine is a stronger inhibitor of labellar sweet neurons than theobromine. Similar features have been found in the mammalian taste system. Some mammalian bitter taste receptors are able to detect a broad and diverse selection of bitter compounds with a high degree of

specificity. Moreover, these broadly tuned mammalian bitter receptors were shown to have one large orthosteric binding site that allows for broad detection for bitter compound [44]. Within the binding site, there exist multiple affinity points that interact with bitter compounds and allows for specificity. Receptors of the *Drosophila* sweet clade may have a similar feature for the detection of bitter compounds.

We have shown that labellar sweet neurons' response to multiple agonists can be inhibited by bitter tastants. The degree of inhibition was dependent on both the bitter compound and the sugar used in each mixture. While there are differences in inhibition for each agonist, they can't be separated as Gr64a dependent sugars and Gr5a dependent sugars. Instead, it appears each sugar response is uniquely inhibited by each bitter compound. Since different receptors can detect unique subsets of sweet and bitter compounds, inhibition may be dependent on the sweet receptor composition. When we coexpressed Gr5a with Gr64e or Gr64f in the ab1C neurons and applied mixtures of 100mM trehalose and 10mM bitter compound, we found that inhibition was not solely dependent on Gr5a, Gr64e, or Gr64f alone. Instead, each combination was inhibited by a combination of the bitter inhibition of each sweet Gr. Sensitivity to each bitter compound appeared to be upregulated or downregulated when compared to the bitter inhibition profile of each receptor alone. It may be that the presence of another receptor can reduce inhibition by a bitter compound or enhance it. This would mean that both activation and inhibition of a sweet receptor complex would

be dependent on all the receptor subunits found. Similar mechanisms has been found in bitter neurons where the presence of a new bitter Gr can confer new ligand sensitivity to bitter compounds or inhibit responses to other bitter tastants [45]. This would explain the selectivity of inhibition by bitter compounds for each sweet receptor subunit in the ab1C neuron and the broad inhibition of sugar responses in endogenous neuron. One advantage of each sweet receptor subunit being inhibited by a select panel could be that it allows the taste system to be highly adaptive. If sweet neurons are exposed to a specific toxin, receptors that are capable of detecting it could be unregulated or made more available to prevent ingestion. There is precedence for changes in receptor expression due to diet and starvation state [46, 47]. Considering how the LD50 for some aversive compounds could be incredibly low, being able to quickly adapt to a new environment and prevent ingestions would be highly desirable.

Heterologously expressed Gr43a and its mosquito ortholog, AgGr25, are immune to inhibition by bitter compounds. Since Gr43a is an important internal fructose receptor that is used to measure sugar levels in hemolyph, inhibition of Gr43a by bitter compounds could be disastrous for flies and might possibly cause some imbalance and as such it would be beneficial for Gr43a to not respond to bitter compounds [48]. Since members of the sweet clade are expressed in all taste organs, they play an important role as gatekeepers to encourage consumption of sweet compounds and in conjunction with bitter receptors to

prevent ingestions of bitter compounds. As such, the sweet clade appears to have evolved as dual sensors of both sweet and bitter compounds.

One feature lacking in our study is we did not test any aversive amino acids for sweet taste neuron inhibition. This is an important because L-canavanine, an amino acid, activates some bitter neurons but does not inhibit sweet neurons [49]. It may be that L-canavanine is a unique case or there may be other aversive amino acids that are detected only via activation of bitter neurons. Our panel was heavily reliant on different classes of bitter alkaloids. More studies need to be done to determine if the sweet neuron can be inhibited by other aversive amino acids and to test other bitter compounds found in plants besides alkaloids.

It is unknown if this feature is unique to insects or other types of sweet receptors can be inhibited by bitter compounds. Mammalian sweet receptors have not been shown to be inhibited by bitter compounds but it would be interesting to see if bitter inhibition is evolutionarily conserved outside of insects.

### 4.5 Abbreviations

Gr64a expressed in the ab1C neuron (ab1C:Gr64a) Pg. 128

Ab1C neuron expressing GrX (ab1C:GrX) Pg. 131

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Figure 4.1

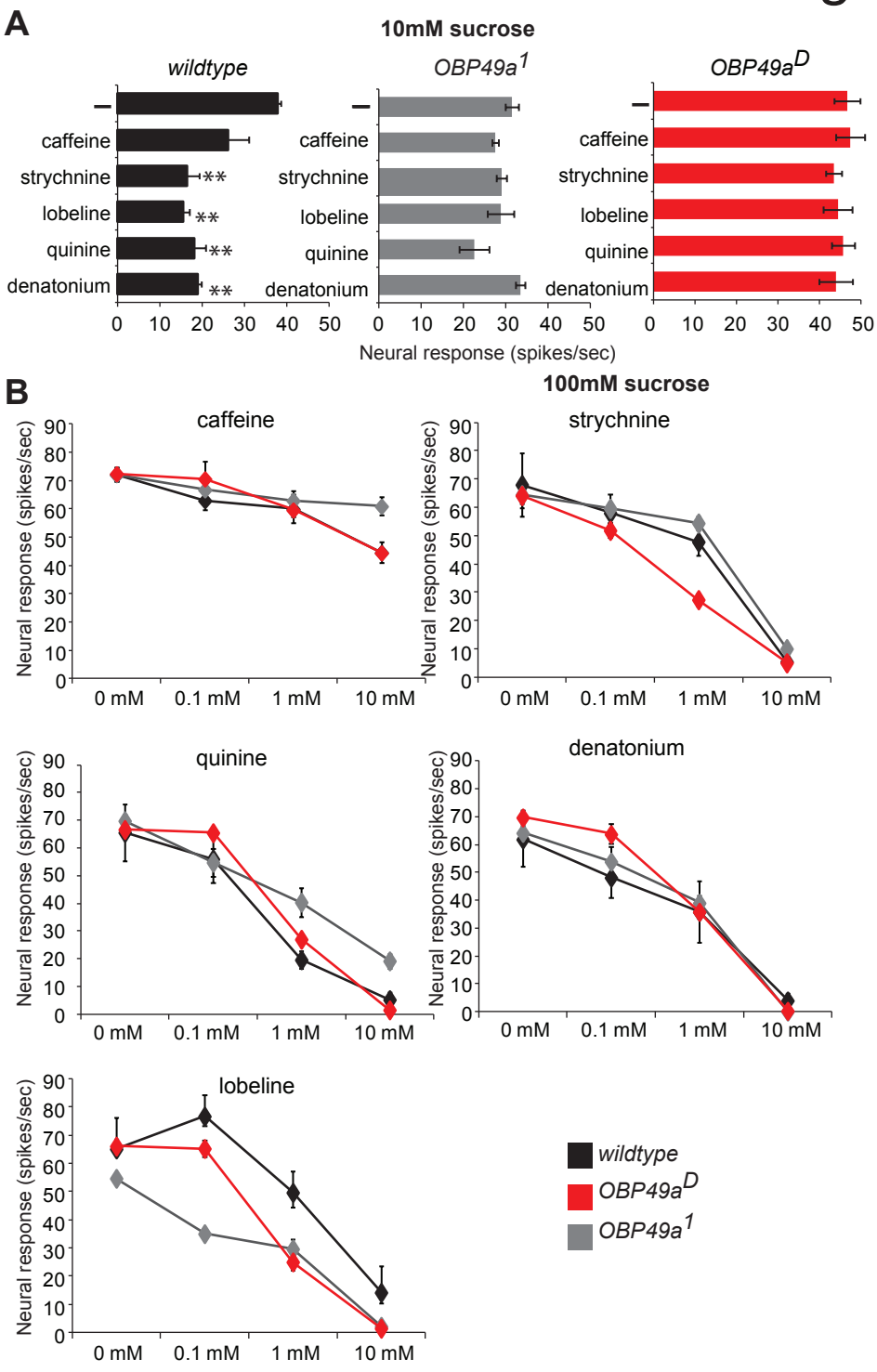
A
10mM sucrose
wildtype
OBP49a1
OBP49aD
–
caffeine
strychnine
lobeline
quinine
denatonium
**
0 10 20 30 40 50
Neural response (spikes/sec)
B
100mM sucrose
caffeine
strychnine
quinine
denatonium
lobeline
Neural response (spikes/sec)
0 10 20 30 40 50 60 70 80 90
0 mM
0.1 mM
1 mM
10 mM
wildtype
OBP49aD
OBP49a1

**Figure 4.1 OBP49a increases sensitivity to bitter tastants in low sugar concentrations.** (**A**) Mean responses of L-type sweet taste neurons in *wild type, OBP49a*$^{1}$*,* and *OBP49a*$^{D}$ flies to 10mM sucrose (-), and mixtures of 10mM sucrose and 10μM of indicated bitter compound. (n=6-10) **P<0.001 Dunnett's t-test vs. sugar alone. Traces were counted from contact to 1 sec. (**B**)Dose-dependent curves of L-type sweet taste neurons in *wild type, OBP49a*$^{1}$*,* and *OBP49a*$^{D}$ flies to mixtures of 100mM sucrose and indicated bitter compounds. (n=6-10).

# Figure 4.2

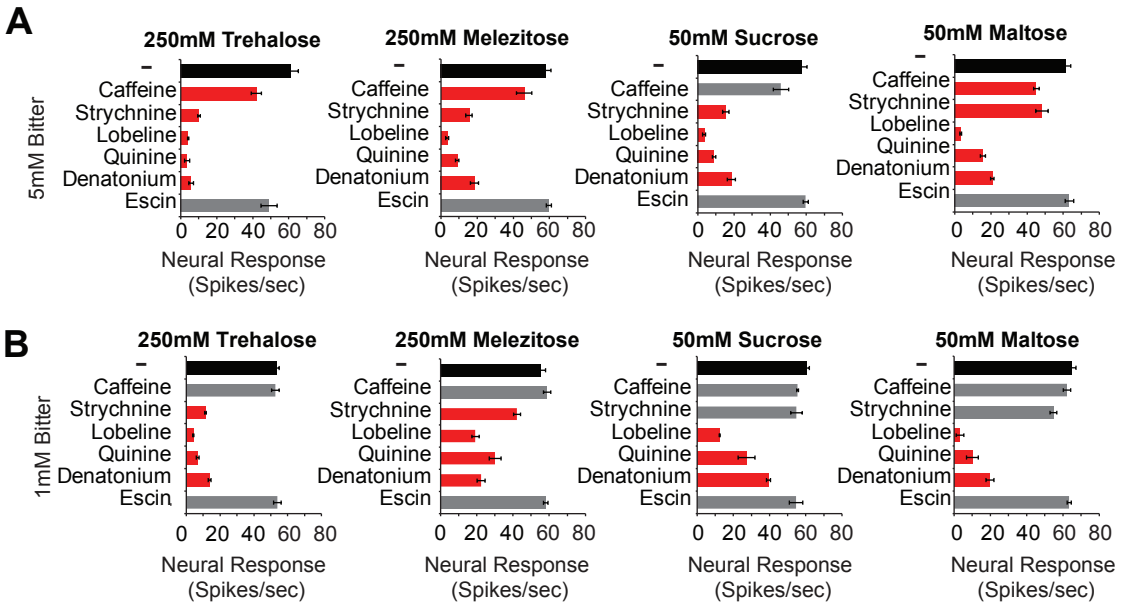
A
250mM Trehalose
250mM Melezitose
50mM Sucrose
50mM Maltose
5mM Bitter
–
Caffeine
Strychnine
Lobeline
Quinine
Denatonium
Escin
0 20 40 60 80
Neural Response
(Spikes/sec)
B
250mM Trehalose
250mM Melezitose
50mM Sucrose
50mM Maltose
1mM Bitter
–
Caffeine
Strychnine
Lobeline
Quinine
Denatonium
Escin
0 20 40 60 80
Neural Response
(Spikes/sec)

**Figure 4.2 Multiple sugars are inhibited by bitter compounds.** (**A**) Mean responses of L-type sensilla in $w^{1118}$ flies to mixtures of indicated sugar (top) and 5mM bitter compound or (**B**) 1mM bitter compound. Red bar indicated statistically significance. P<0.05 Dunnett's ttest vs. sugar alone. (n=6).

# Figure 4.3

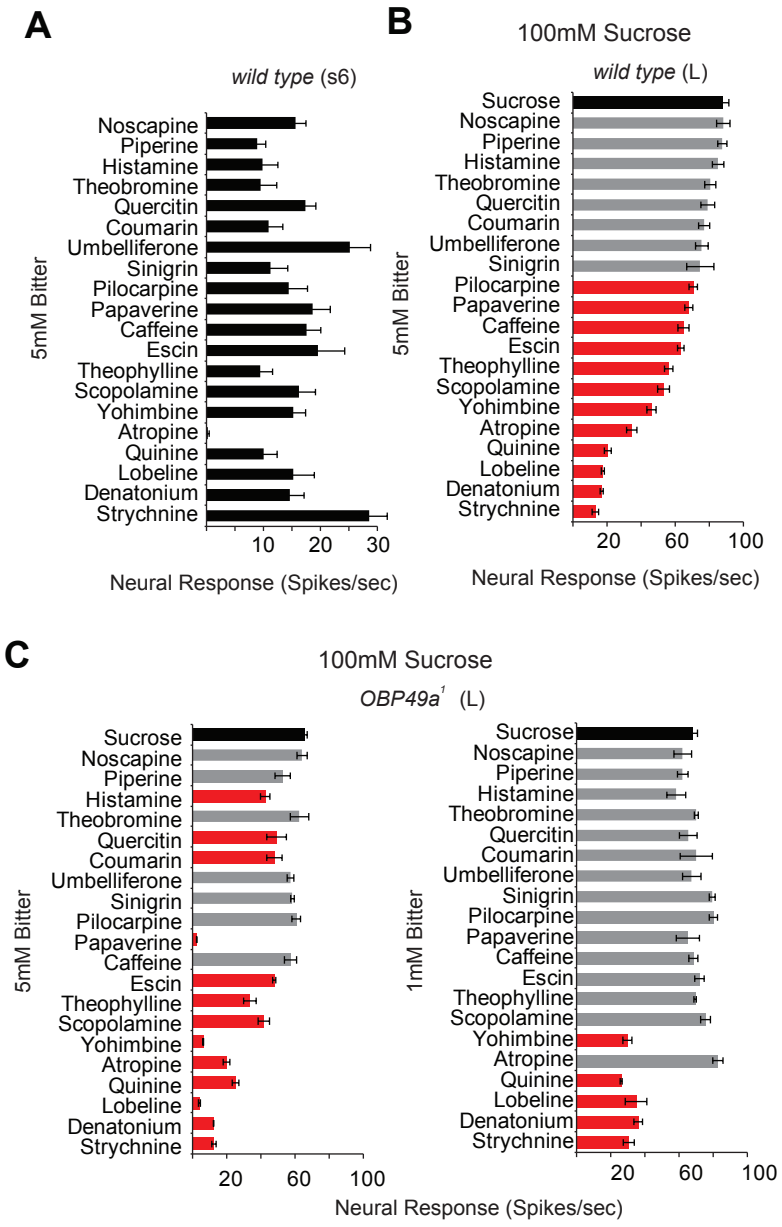
A
wild type (s6)
Noscapine
Piperine
Histamine
Theobromine
Quercitin
Coumarin
Umbelliferone
Sinigrin
Pilocarpine
Papaverine
Caffeine
Escin
Theophylline
Scopolamine
Yohimbine
Atropine
Quinine
Lobeline
Denatonium
Strychnine
5mM Bitter
10
20
30
Neural Response (Spikes/sec)
B
100mM Sucrose
wild type (L)
Sucrose
Noscapine
Piperine
Histamine
Theobromine
Quercitin
Coumarin
Umbelliferone
Sinigrin
Pilocarpine
Papaverine
Caffeine
Escin
Theophylline
Scopolamine
Yohimbine
Atropine
Quinine
Lobeline
Denatonium
Strychnine
5mM Bitter
20
60
100
Neural Response (Spikes/sec)
C
100mM Sucrose
OBP49a1 (L)
Sucrose
Noscapine
Piperine
Histamine
Theobromine
Quercitin
Coumarin
Umbelliferone
Sinigrin
Pilocarpine
Papaverine
Caffeine
Escin
Theophylline
Scopolamine
Yohimbine
Atropine
Quinine
Lobeline
Denatonium
Strychnine
5mM Bitter
1mM Bitter
20
60
100
Neural Response (Spikes/sec)

**Figure 4.3 Multiple bitter compounds inhibit sweet taste neurons** (**A**) Mean responses of L-type sweet taste neurons in *wild type* flies to 100mM sucrose (-), and mixtures of 100mM sucrose and 5mM of indicated bitter compound. (n=6-10) Red bar indicated statistically significance. P<0.01 Dunnett's ttest vs. sugar alone (**B**) Mean responses of L-type sweet taste neurons $OBP49a^{1}$ in flies to 100mM sucrose (-), and mixtures of 100mM sucrose and 5mM (left) or 1mM (right) of indicated bitter compound. (n=6-10) Red bar indicated statistically significance. P<0.01 Dunnett's ttest vs. sugar alone.

# Figure 4.4

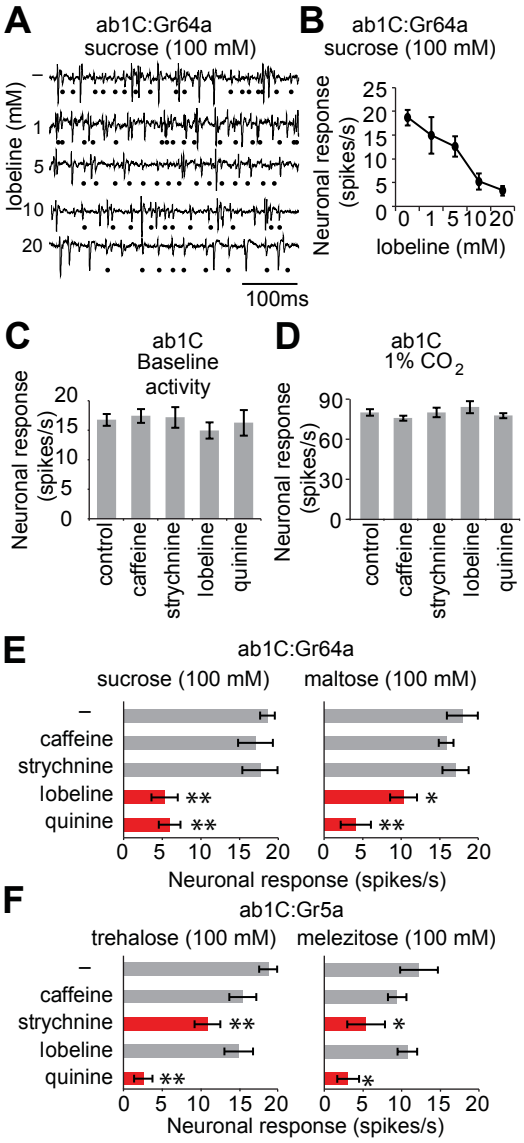
A
ab1C:Gr64a
sucrose (100 mM)
lobeline (mM)
–
1
5
10
20
100ms
B
ab1C:Gr64a
sucrose (100 mM)
Neuronal response (spikes/s)
25
20
15
10
5
0
0 1 5 10 20
lobeline (mM)
C
ab1C
Baseline activity
Neuronal response (spikes/s)
20
15
10
5
0
control
caffeine
strychnine
lobeline
quinine
D
ab1C
1% CO2
Neuronal response (spikes/s)
90
60
30
0
control
caffeine
strychnine
lobeline
quinine
E
ab1C:Gr64a
sucrose (100 mM)
maltose (100 mM)
–
caffeine
strychnine
lobeline
quinine
**
**
*
**
0 5 10 15 20
Neuronal response (spikes/s)
F
ab1C:Gr5a
trehalose (100 mM)
melezitose (100 mM)
–
caffeine
strychnine
lobeline
quinine
**
*
*
0 5 10 15 20
Neuronal response (spikes/s)

**Figure 4.4 A Gr receptor can be directly inhibited by bitter compounds** (**A**) Sample traces of recordings from ab1C:Gr64a neurons using 100mM sucrose and increasing concentrations of lobeline. (**B**) Dose dependent responses from ab1C:Gr64a neurons using 100mM sucrose and increasing concentrations of lobeline. n=6-14 (**C**) Mean baseline activity of ab1C neurons in control flies with SLR alone (control) or named bitter compounds at 10 mM, except lobeline at 20 mM. Flies were *+;Gr63a-GAL4* homozygous. Responses were not significantly different versus control . Dunnets ttest vs control. $P > 0.05$. n = 6. (**D**) $CO_2$ response of ab1C neurons in control flies with SLR alone (control) or named bitter compounds at 10 mM, except lobeline at 20 mM. Flies were *+;Gr63a-GAL4* homozygous. Dunnets ttest vs control. $P > 0.05$. $n = 6$ (**E and F**) Responses of ab1C:GrX neurons to indicated sugar alone (–) or in mixtures with 10 mM bitter compound. Red bars indicate responses that are significantly reduced. Dunnets ttest vs control. $P > 0.05$. n = 6-14.

# Figure 4.5

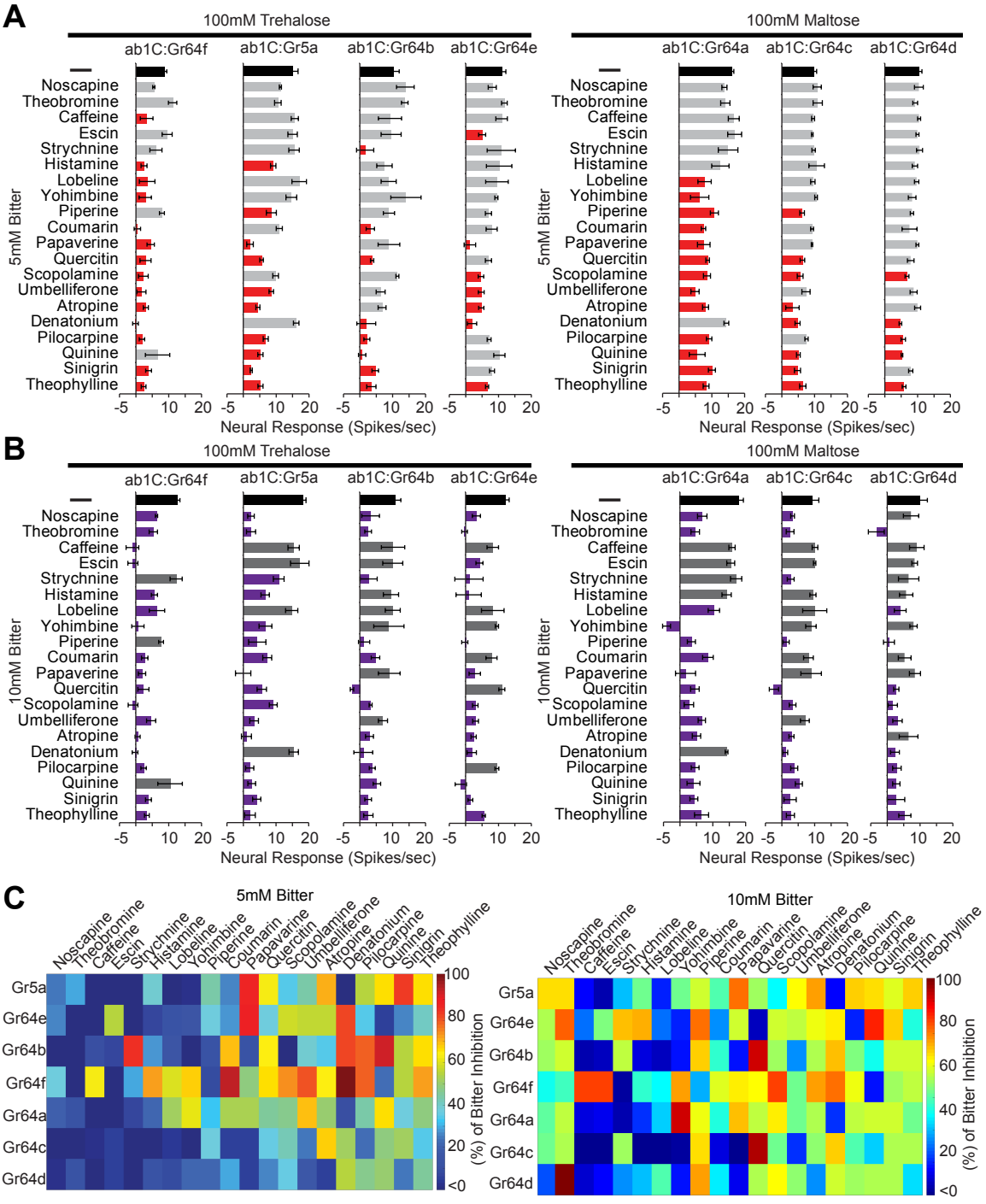
A
100mM Trehalose
ab1C:Gr64f
ab1C:Gr5a
ab1C:Gr64b
ab1C:Gr64e
100mM Maltose
ab1C:Gr64a
ab1C:Gr64c
ab1C:Gr64d
Noscapine
Theobromine
Caffeine
Escin
Strychnine
Histamine
Lobeline
Yohimbine
Piperine
Coumarin
Papaverine
Quercitin
Scopolamine
Umbelliferone
Atropine
Denatonium
Pilocarpine
Quinine
Sinigrin
Theophylline
5mM Bitter
-5 10 20
Neural Response (Spikes/sec)
B
10mM Bitter
C
5mM Bitter
10mM Bitter
Gr5a
Gr64e
Gr64b
Gr64f
Gr64a
Gr64c
Gr64d
100
80
60
40
20
<0
(%) of Bitter Inhibition

**Figure 4.5 All receptors of the sweet clade can be inhibited by bitter compounds** (**A**) Mean electrophysiological responses of ab1C:GrX neurons. To either 100mM trehalose (left) or 100mM maltose(right), either alone (-) or in mixtures of 5mM indicated bitter compound.For each data point, n = 6– 17. Red bars indicate significance*P < 0.05 [vs. sugar alone]. (**B**) Mean electrophysiological responses of ab1C:GrX neurons. To either 100mM trehalose (left) or 100mM maltose(100mM), either alone (-) or in mixtures of 5mM indicated bitter compound. For each data point, n = 6– 17. Purple bars indicate significance*P < 0.05 [vs. sugar alone]. (**C**) Heat maps of percent inhibition [(Sugar alone-sugar bitter mixture)/(sugar alone)*100] of each bitter/sugar combinations using 10mM bitter compound(left) and 5mM bitter compound(right) in ab1C:GrX neurons. Data used from 4.5 A and B. Heat maps were generated using PAST.

# Figure 4.6

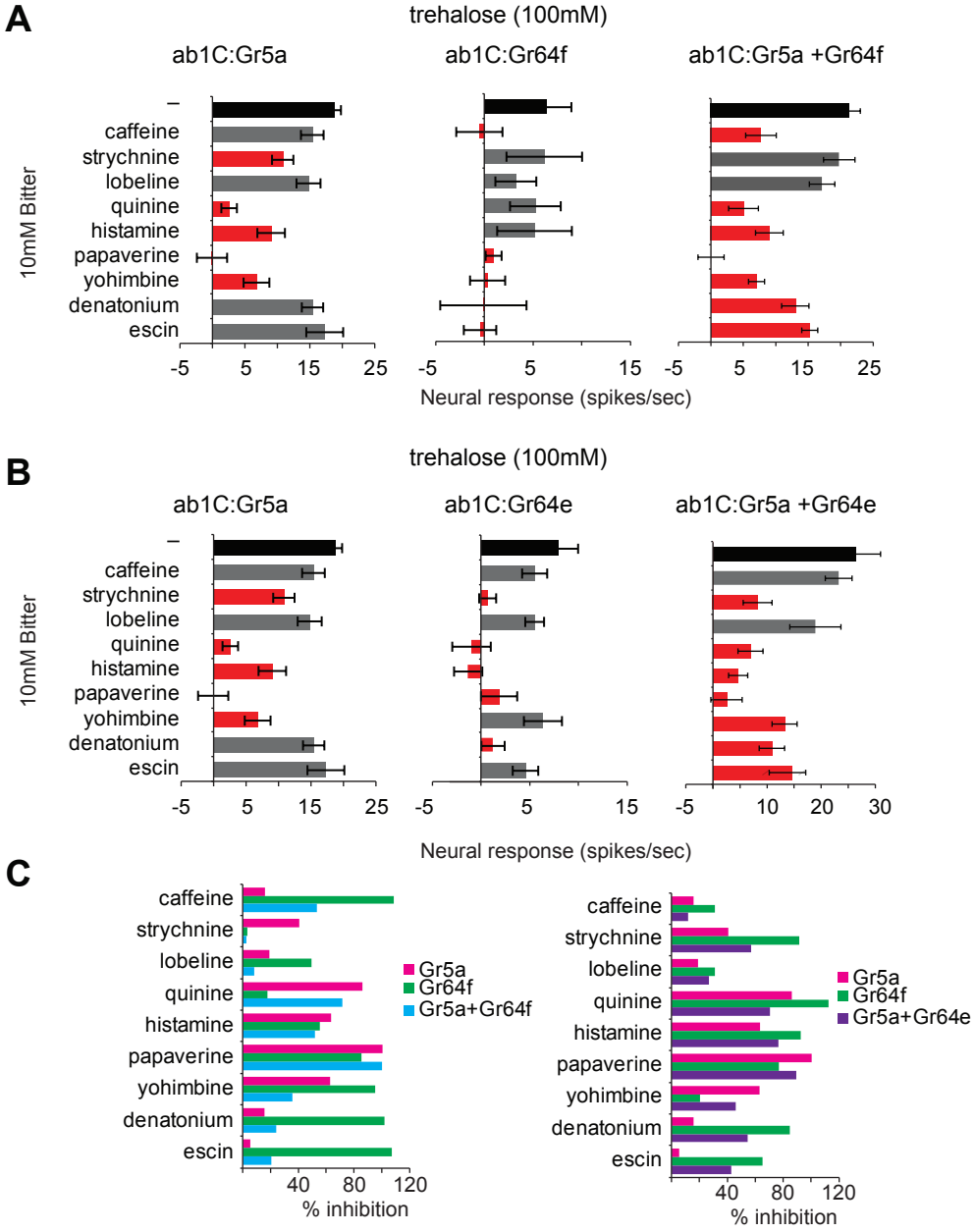
A
trehalose (100mM)
ab1C:Gr5a
ab1C:Gr64f
ab1C:Gr5a +Gr64f
10mM Bitter
–
caffeine
strychnine
lobeline
quinine
histamine
papaverine
yohimbine
denatonium
escin
-5
5
15
25
Neural response (spikes/sec)
B
trehalose (100mM)
ab1C:Gr5a
ab1C:Gr64e
ab1C:Gr5a +Gr64e
10mM Bitter
30
Neural response (spikes/sec)
C
Gr5a
Gr64f
Gr5a+Gr64f
Gr5a+Gr64e
40
80
120
% inhibition

**Figure 4.6 Coexpression of sweet Grs alters inhibition profiles**. (**A**) Mean responses from ab1C:Gr5a, ab1C:Gr64f and ab1C neurons expressing both Gr5a and Gr64f (ab1C:Gr5a+Gr64f) to 100mM trehalose alone (-) or in mixtures with 10mM of indicated bitter compounds. Data same as used in Figure 4.5. Red bars indicate significance *P<0.05. (n=6 for ab1C:Gr5a+Gr64f) (**B**) Mean responses from ab1C:Gr5a, ab1C:Gr64e and ab1C neurons expressing both Gr5a and Gr64e (ab1C:Gr5a+Gr64e) to 100mM trehalose alone (-) or in mixtures with 10mM of indicated bitter compounds. Data same as used in Figure 4.5. Red bars indicate significance *P<0.05. (n=6 for ab1C:Gr5a+Gr64e) (**C**) Percent inhibition [(Sugar alone-sugar bitter mixture)/(sugar alone)*100] of each compound in ab1C:GrX neurons. Data used from 4.6 A and B.

Figure 4.7

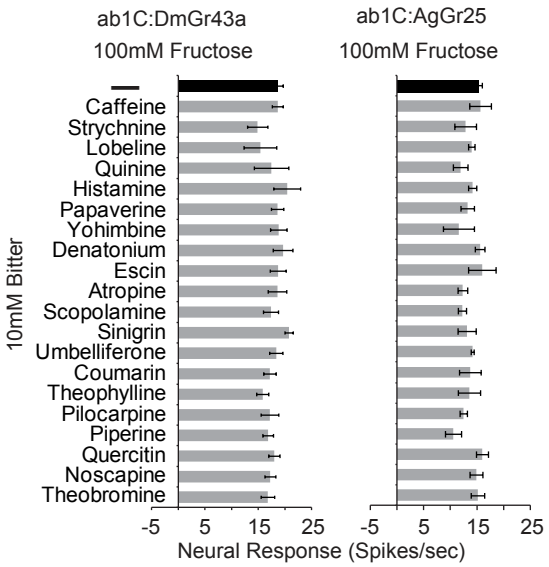
ab1C:DmGr43a
100mM Fructose
ab1C:AgGr25
100mM Fructose
10mM Bitter
—
Caffeine
Strychnine
Lobeline
Quinine
Histamine
Papaverine
Yohimbine
Denatonium
Escin
Atropine
Scopolamine
Sinigrin
Umbelliferone
Coumarin
Theophylline
Pilocarpine
Piperine
Quercitin
Noscapine
Theobromine
-5
5
15
25
Neural Response (Spikes/sec)

**Figure 4.7 The fructose receptor and its mosquito ortholog are immune to inhibition.** Mean responses in ab1C:DmGr43a and flies expressing AgGr25 (ab1C:AgGr25) to fructose alone(-) or in mixtures of 10mM bitter compound. No responses were statistically significant P>0.05 vs. fructose alone. (n=6-12)

**Table 4.1 List of Genotypes used in chapter 4**

| Line name | Genotype/ source | Figures |
|---|---|---|
| wild type | *w[1118]* | 4.1, 4.2, 4.3, 4.5 |
| OBP49a$^{1}$ | Montell laboratory | 4.1, 4.3 |
| OBP49a$^{D}$ | Montell laboratory | 4.1 |
| Gr21a-GAL4 | Kristin Scott | |
| Gr63a-GAL4 on III | BDSC (# 9942) | |
| Gr63a-GAL4 on II | BDSC (# 9943) | |
| UAS-Gr5a | Dahanukar laboratory | |
| UAS-Gr64a | Dahanukar laboratory | |
| UAS-Gr64b | BDSC (# 27324) | |
| UAS-Gr64c | Dahanukar laboratory | |
| UAS-Gr64d | Dahanukar laboratory | |
| UAS-Gr64e | Dahanukar laboratory | |
| UAS-Gr64f | Dahanukar laboratory | |
| ab1C | *+/+;Gr63a-GAL4/Gr63a-GAL4* | 4.4 |
| ab1C:Gr5a | *UAS-Gr5a-8/UAS-Gr5a-8; Gr63a-GAL4/Gr63a-GAL4* | 4.4, 4.5 |
| ab1C:Gr64f | *UAS-Gr64f-2/UAS-Gr64f-2; Gr63a-GAL4/Gr63a-GAL4* | 4.5, 4.6 |
| ab1C:Gr64e | *UAS-Gr64e-3/UAS-Gr64e-3; Gr63a-GAL4/Gr63a-GAL4* | 4.5, 4.6 |
| ab1C:Gr64b | *UAS-Gr64b/Gr21a-GAL4; UAS-Gr64b-2/Gr63a-GAL4* | 4.4 |
| ab1C:Gr64a | *UAS-Gr64a-4/UAS-Gr64a-4; Gr63a-GAL4/Gr63a-GAL4* | 4.4 |
| ab1C:Gr64c | *Gr21a-GAL4/UAS-Gr64c-B; Gr63a-GAL4/UAS-Gr64c-2* | 4.4 |
| ab1C:Gr64d | *Gr21a-GAL4/UAS-Gr64d-4; Gr63a-GAL4/UAS-Gr64d-3* | 4.4 |
| ab1C:Gr5a+Gr64f | *UAS-Gr64f-2/UAS-Gr64f-2; UAS-Gr5a-3, Gr63a-GAL4/UAS-Gr5a-3,Gr63a-GAL4* | 4.6 |
| ab1C:Gr5a+Gr64e | *UAS-Gr64e-3/UAS-Gr64e-3; UAS-Gr5a-3, Gr63a-GAL4/UAS-Gr5a-3,Gr63a-GAL4* | 4.6 |
| ab1C:DmGr43a | *UAS-Gr43a-8d/Gr21a-GAL4; Gr63a-GAL4/UAS-Gr43a-5d* | 4.7 |
| ab1C:AgGr25 | *Gr21a-GAL4/Gr63a-GAL4; UAS-AgGr25-3D/UAS-AgGr25-3L* | 4.7 |

## Table 4.2 Panel of bitter compounds and sources in nature

| Compound | Common Source | In Nature | 5mM | 10mM | From Reference | Reference |
|---|---|---|---|---|---|---|
| | | % | % | % | | |
| Noscapine | poppy seed | 2.37E-02 | 0.21 | 0.41 | 237 µg/g | [18] |
| Piperine | peppercorn | 5-9 | 0.14 | 0.29 | 5-9% | [23] |
| Histamine | stinging nettle flowers | 0.20 | 0.06 | 0.11 | 1:500 | [22] |
| Theobromine | cocoa seeds | 1.89 | 0.09 | 0.18 | 0.189 | [24] |
| Quercitin | plums | 0.12 | 0.15 | 0.30 | 12 mg/100g | [19] |
| Coumarin | cinnamon bark | 1.68 | 0.07 | 0.15 | 16.8 g/kg | [29] |
| Umbelliferone | chamomile flower | 0.08 | 0.08 | 0.16 | 0.089-0.838g/kg | [25] |
| Sinigrin | mustard seeds | 1.38E-05 | 0.20 | 0.40 | 55umol/g | [26] |
| Pilocarpine | jaborandi flowers | 6.00E-04 | 0.10 | 0.21 | 6ug/g | [27] |
| Papaverine | poppy seed | 6.70E-03 | 0.19 | 0.38 | 67 µg/g | [18] |
| Caffeine | coffee bean (green) | 8.60 | 0.10 | 0.19 | 86.42mg/g | [28] |
| Escin | horse chestnut | 3.70 | 0.57 | 1.13 | 3.70% | [31] |
| Theophylline | tea leaves | 0.25 | 0.09 | 0.18 | 0.25% | [28] |
| Scopolamine | jimson weed seeds | 0.04 | 0.15 | 0.30 | 0.387ug/mg | [30] |
| Yohimbine | yohimbi bark | 2.00 | 0.18 | 0.35 | 19.95mg/g | [33] |
| Atropine | jimson weed seeds | 0.27 | 0.14 | 0.29 | 2.71ug/mg | [30] |
| Quinine | remijia bark | 0.5-2 | 0.16 | 0.32 | 0.5-2% | [32] |
| Lobeline | lobelia whole | 1.05 | 0.17 | 0.34 | 1.05% | [20] |
| Denatonium | synthetic | NA | 0.16 | 0.33 | | |
| Strychnine | strychnine seeds | 3.90 | 0.17 | 0.33 | 3.9-5.6% | [21] |

## 5.0 Sugar receptors and sweet neurons in mosquitoes possess evolutionarily conserved mechanisms for detecting sweet and bitter compounds.

**Abstract:** Mosquitoes are the deadliest animal family in the world and spread diseases to millions of people worldwide. While mature female *Anopheles gambiae (A. gambiae)* require blood feeding for egg production, newly emerged and male mosquitoes are solely nectar feeders. Sugar feeding is necessary for mosquito survival, making sugar-baited lethal traps an essential tool for controlling mosquito populations. Despite the success of sugar-baited traps, little is known about sugar detection in mosquitoes. By recording from hairs on the labellum, we discovered that labellar sweet neurons from both *A. gambiae* and *Aedes aegypti (A. aegypti)* can detect sweet tastants and be directly inhibited by bitter tastants. This suggests that inhibition by bitter tastants is evolutionarily conserved. Using the $CO_2$ sensing neuron in *D. melanogaster* to ectopically express sweet receptors, we discovered every receptor of the *A. gambiae* sweet clade is involved in ligand recognition of at least one sweet tastant. Furthermore, we measured the neural response from one of the *A. gambiae* sweet receptors, AgGr15, expressed in the *D. melanogaster* $CO_2$ sensing neuron to mixtures of sweet and bitter tastants. Like the *D. melanogaster* sweet clade, AgGr15 is inhibited by bitter tastants, suggesting bitter tastant mediated inhibition of sweet receptors may be evolutionarily conserved.

## 5.1 Introduction

According to the CDC, each year *A. gambiae* transmits diseases such as malaria to over 300 million people worldwide, resulting in ~1 million deaths per a year. *A. aegypti* spread diseases such as zika, dengue, yellow fever, and chikungunya, while treatment and prevention costs billions of dollars every year. Female *A. gambiae* and *A. aegypti* require a blood meal to produce mature eggs after mating [1]. It is during blood feeding that female mosquitoes transfer and spread disease from host to host. Male mosquitoes only sugar feed, and, female mosquitoes also prefer sugar over blood feeding outside of the reproductive cycle [2]. This makes the use of sugar-baited lethal traps a critical tool for controlling mosquito populations and preventing the spread of diseases. Although sugar-baited lethal traps are an effective way to control the spread of insects [3-5], our understanding of how mosquitoes detect sweet compounds is limited. All insects, including disease vectors such as mosquitoes, use the gustatory system to detect potential food sources and determine the presence of toxins. Mosquitoes mostly feed on floral and extrafloral nectars as well as honeydew [4]. Males and newly emerged mosquitoes of both sexes feed exclusively on these sources [2, 4, 6], which are mainly comprised of sugars, with the most abundant ones being sucrose, fructose, and glucose. Other sweet compounds that are found in nectar, extrafloral nectar, and honeydew include methyl-alpha glucopyranoside (m-glucoside), trehalose, turanose, galactose, maltose, raffinose, melezitose, stachyose and glycerol [2, 6-9]. Three taste

sensilla on the mosquito labellum have been shown to respond to sucrose and other sugars while the other hairs have not been studied comprehensively [10, 11]. Moreover, one labellar taste sensillum that responds to sucrose can be inhibited by both quinine and denatonium [12], a phenomenon conserved between flies and mosquitoes [13, 14].

Like the *D. melanogaster* genome, the *A. gambiae* genome encodes a divergent family of gustatory receptor (Gr) genes that are hypothesized to be involved in detecting bitter and sweet compounds, yet no one has mapped expression of these receptors nor deorphanized them except for AgGr25 (**Fig 2. 6**) [15, 16]. Based on phylogeny and sequence alignment, eight Grs in the *A. gambiae* genome were found to cluster with the sweet clade from *D. melanogaster* [15, 16] (**Fig. 5.1**). Sequence similarities between these receptors and the *D. melanogaster* sweet clade suggests sweet taste function for seven of the eight AgGrs and along with AgGr25 are thought to encompass the majority of sweet taste in *A. gambiae* (AgGr19 is a pseudogene) [15]. So far, it is not known if these receptors detect sweet compounds or if they are even necessary for sweet taste detection, which is a critical gap in the field.

Using electrophysiological recordings from the labellum, we discovered that many of the long labellar taste sensilla in *A. gambiae* can detect a variety of sugars. Previously, we found that AgGr25 responds to fructose and other sugars when expressed in the *Drosophila* ab1C neuron (Chapter 2). We expressed each receptor of the *A. gambiae* sweet clade individually in the *Drosophila* ab1C

neuron and recorded the neural responses to a panel of sweet tastants that were detected by labellar sweet neurons. All seven receptors of the sweet clade can detect at least one sugar, and each receptor detects a unique subset of sweet compounds suggesting that the receptors of the sweet clade are involved in sugar detection.

Recent studies have shown that in *A. gambiae,* sucrose induced responses in a long labellar taste sensillum can be inhibited by bitter compounds in the absence of a canonical bitter neuron [10, 12]. It is crucial to understand the mechanisms involved in the detection of bitter compounds in the presence of sugar, since sugar baited lethal traps rely on the ingestion of adequate amount of toxins to control populations in many mosquito species [3, 5]. To see if this phenomenon is found in different mosquito species, and therefore evolutionarily conserved, we recorded from long labellar sensilla using mixtures of sucrose and bitter compounds in two mosquito species: *A. gambiae and A. aegypti*. In both mosquito species, labellar sweet neuron's response to sucrose was inhibited by bitter compounds. In chapter 4, we showed the each receptor of the *Drosophila* sweet clade is inhibited by bitter compounds. We wondered if, like the *Drosophila* sweet clade, receptors of the *A. gambiae* sweet clade could be inhibited by bitter compounds. To test this hypothesis, we expressed one of the receptors, AgGr15, in the *Drosophila* ab1C neuron and applied mixtures of sugar and bitter tastants. AgGr15 response to trehalose was inhibited by multiple bitter compounds

suggesting that bitter tastant mediated inhibition of the sweet clade is an evolutionarily conserved mechanism in insects.

## 5.2 Material and Methods

### 5.2.1 Mosquito maintenance

*A. gambiae and A. aegypti populations* were maintained on membrane blood feeding. They are fed 10% sucrose solution. They were on a 12hr light: 12 hr dark cycles in room with 80% humidity and 25°C. Each cage usually contained a mix of male and females with 50 mosquitoes. Before each experiment, 5 male mosquitoes were removed from the cage and placed in a plastic fly tube which was kept in a secondary container, an empty paper ice cream container with wet paper towels to increase humidity. Mosquitoes were used within 2 hrs of being outside of the humidified cage. Any mosquitoes, that had not been used, were killed via overnight in -20°C freezer.

### 5.2.2 Single sensillum taste recordings

Mosquitoes were tested between 3-5 days. Male mosquitoes were lightly anesthetized using ice and their legs and wings were removed with tweezers. A single mosquito was put onto a slide on top of a piece of mounting tape with the proboscis hanging about 0.5cm off the edge of the slide. The mounting tape was large enough that only the tip of the proboscis (~0.5cm) was not on the tape.

Thin slices of double sided tape were used to immobilize the body and proboscis, taking care not to crush or smash the mosquito or its proboscis. A reference electrode with buffer solution was placed in the abdomen. Tip recordings from taste sensilla were as described [17], using 30 mM tricholine citrate as electrolyte. Any mosquito that showed visible signs of damage to the labellum and hairs that showed visible damage (bent or broken hairs) or had debris were discarded. Mosquitoes that stopped moving or had any rhythmic twitching (death twitch) were removed as well. We limited recording from each prep to 30 minutes.

### 5.2.2 Fly maintenance

Flies were maintained on standard cornmeal–dextrose medium at 25 °C and were tested within 3-10 days after pupation. Full genotypes are in Table 5.1.

### 5.2.3 Olfactory recordings

Extracellular single unit recordings from the ab1C neuron were performed as described in[18]. Refer to Chapter 2.

## 5.3 Results

### 5.3.1 Multiple long hairs on the *A. gambiae* labellum respond to sugar.

*A. gambiae* have a stereotypical arrangement of taste hairs on the labellum with two hair types called trichoid 1 (T1) and trichoid 2 (T2) [11]. T1 are long hairs about 30-35 microns long with 13 hairs found on the dorsal, lateral and ventral side on each side of the labellum and have been shown to be innervated by four chemosensory neurons and a mechanosensory neuron (**Fig. 5.2A**). T2 are about 5 microns long, cluster about 40 microns from the opening of the labellum and are innervated by two neurons. Some of the T1 respond to water and sugar where each neuron was differentiated by spike amplitude [10, 11]. Some T1 sensilla are water insensitive. In T1 sensilla that respond to water, the water neuron had the largest spike amplitude and the sugar spikes had smaller spike amplitude. There appears to be no sex-specific differences between the number and placement of the long hairs on the labellum [10]. We decided to record from male *A. gambiae* to analyze sweet taste responses since males are exclusively nectar feeders, which will eliminate any shifts in sensitivity due to the reproductive cycle [6]. We used the panel of 9 sweet tastants that we previously tested on *D. melanogaster* (Chapter 3), because it includes compounds in food sources for both flies and mosquitoes and would facilitate comparison of sugar detection between the two species [7-9, 17]. From the 13 T1 hairs that are

present on the dorsal, lateral and ventral surfaces, we selected 3 sensilla for electrophysiological analysis (**Fig. 5.2A**). We recorded from 5L, 2L, and 1V which have not been previously recorded from [10, 11]. To survey the sugar mediated responses of the T1 hairs, we applied 100 mM sugar or 10% glycerol mixed with 30mM TCC and recorded neural activity. We discovered that the sweet neurons in these long hairs respond to multiple sugars, the strongest being m-glucoside, and sucrose (**Fig. 5.2B**). All of the other sugars activated these neurons to a lesser extent. The spike amplitude is uniform for all traces suggesting that only one neuron is firing. Of interest, fructose and glucose have much weaker response (<10 spikes per second) which is similar to what we found from labellar recordings of *D. melanogaster* (**Fig. 3.3C**) [17]. This suggests that despite the differences in feeding sources, there are some conserved features in sugar detection between fruit flies and mosquitoes. Our responses are similar to what Kessler *et al.* found from the V8 and D5 except 5L, 2L, and 1V had higher responses to trehalose and maltose [11].

### 5.3.2 All of the receptors of the *A. gambiae* sweet clade can detect different sweet compounds

There are eight AgGrs receptors that are thought to be involved in the detection of sweet compounds. Seven come from the sweet clade, and the other is AgGr25. AgGr25 is the ortholog of the *Drosophila* Gr43a which is an internal fructose sensor [19]. We found that AgGr25 conferred sensitivity to fructose and

other sugars when expressed in the ab1C neuron in the fly olfactory system (**Fig. 2.6**). Since the fly ab1C neuron was suitable for functional expression of AgGr25, we next wanted to test if other putative sweet receptors are capable of sweet tastant detection. Despite the fact that the *A. gambiae* and *D. melanogaster* sweet clades are related to each other and cluster together based on sequence similarity compared to other receptors in the Gr families, there are no one to one orthologs between the two sweet clades [15]. Because of this, ligand specificity determined for the *Drosophila* sweet Grs cannot be directly transferred to *A. gambiae* making predictions difficult. Instead, we theorized that since receptors of the sweet clade confer sugar detection to *Drosophila* labellar L-type sweet neurons that receptors of the sweet clade in *A. gambiae* might confer sensitivity to the labellar T1 hairs. As such, we chose to use the same panel as was previously shown to activate the labellar T1 hairs (**Fig. 5.2**). We expressed each receptor of the *A. gambiae* sweet clade individually in the ab1C neuron and recorded responses of each ab1C:AgGrX neuron to our sweet panel. We found that each AgGr conferred response to at least one sugar, confirming a role for members of this clade in sensing sweet compounds (**Fig. 5.3**). Individual tastants including trehalose, sucrose, and maltose activated multiple singly-expressed receptors, which we found to be true for the *D. melanogaster* sweet clade as well (Chapter 3). Unlike what we observed for the *Drosophila* receptors, the AgGr sweet receptors could not be separated into distinct functional groups based on their response profiles and phylogeny. For example, AgGr18 and AgGr15

respond to both trehalose, m-glucoside and maltose yet are more divergent than other receptors of the sweet clade (**Fig 5.1 &5.3 B**). AgGr18 and AgGr14 which are the most similar receptors of the sweet clade do not detect any of the same sugars when expressed in the ab1C neuron. When we previously recorded from receptors from the *D. melanogaster* sweet clade, we observed a significant degree of overlap in detection for what we called Gr5a-dependent sugars and Gr64a-dependent sugars but we do not find the same relationship in the *A. gambiae* sweet clade (**Fig 5.3 B & C**). For example, neurons expressing AgGr14 responded to melezitose, sucrose, and fructose. When ab1C neurons expressed AgGr15, they responded to trehalose, m-glucoside, and maltose. This suggests that the separation of sweet receptors into two groups based on sugar detection is unique to *D. melanogaster* and may be lacking in *A. gambiae*.

### 4.3.4 Labellar sweet taste neurons in both *An. gambiae* and *Aedes aegypti* are inhibited by bitter alkaloids

Previously, it has been shown that quinine, berberine and denatonium can inhibit the sweet taste neuron in *A. gambiae* labellar T1 hairs that do not respond to any tested bitter compounds [10]. A number of bitter compounds have been found to inhibit labellar sweet taste neurons in *D. melanogaster* (Chapter 4) [13, 14, 20]. We wanted to expand on these studies and determine whether bitter tastant mediated inhibition of sweet neurons is evolutionarily conserved in another mosquito species. We recorded from *A. gambiae* and *A. aegypti*. Both

species have multiple taste hairs in the labellum that are long and short [11, 21]. *A. gambiae* has 8 long labellar hairs on the ventral side and 5 on the dorsal side that are 30-35 microns. This lead to a total of 13 T1 per a labellar side and 26 total. Similar to *A. gambiae*, *A. aegypti* has short sensilla that are 5 microns long as well as long sensilla that are about 32 micron long [21]. The long sensilla are thought to contain chemosensory neurons. *A. aegypti* have 15 long sensilla on each labellar side and 30 in total. There are 10 on the ventral side and 5 on the dorsal side. We tested 3 of the long hairs for responses to sucrose alone and mixtures of sucrose with bitter compounds. We chose to record from 5L, 2L, and 1V on *A. gambia*e and 1D, 3D, and 2V on *A. aegypti* ( **Figure 5.4 A**). Our first step was to determine whether or not these taste sensilla responded to bitter compound by using tip recordings with bitter compounds (1 mM) alone. Similar to the *D. melanogaster* labellar L-type hairs, the long sensilla on the labellum of both mosquitoes did not respond to any tested bitter compounds (**Fig 5.4 B & C**). We then recorded with 100mM sucrose alone and mixtures of 100 mM sucrose and 1mM of bitter tastant. Both mosquitoes responded to 100mM sucrose (50-60 spikes/sec). When we recorded with mixtures of 100mM sucrose and bitter compound, we found that the response to sucrose was significantly reduced in both *A. gambiae* and *A. aegypti* (**Fig. 5.4 D & E**). Since none of the bitter compounds activated the long hairs in either species, it indicates that these sensilla lack a bitter neuron and that bitter compounds may directly inhibit the sweet neuron in mosquitoes. A similar mechanism is found in *Drosophila* as well,

suggesting bitter tastant mediated inhibition of the sweet neuron is likely conserved.

### 4.3.3 AgGr15 trehalose response is inhibited by bitter compounds

Both fly and mosquito sweet neurons are capable of being directly inhibited by bitter tastants. Previously in chapter 4, we discovered that receptors of the sweet clade can be directly inhibited by bitter compounds when expressed in the ab1C neuron (**Fig. 4.4**). We wondered if like *Drosophila*, the receptors of the *A. gambiae* sweet clade could be inhibited by bitter compounds as well. We chose AgGr15 because of its strong response to 100mM trehalose when expressed in the ab1C neuron. We recorded from *Drosophila* ab1C neurons expressing AgGr15 (ab1C:AgGr15) using 100mM trehalose alone and mixtures of 100mM trehalose and 5mM bitter alkaloid. We chose nine bitter compounds which included the four previously tested on labellar sweet neuron and includes others that had different degrees of inhibition for *Drosophila* sweet neurons (**Fig. 4.1**). Responses to the mixtures were compared with those obtained using 100 mM trehalose alone. Strychnine, lobeline, and quinine significantly inhibited neural responses to trehalose in ab1C:AgGr15 neurons, supporting the possibility that sweet taste receptors may be involved in inhibition by bitter compounds in *A. gambiae (***Fig. 5.5**). It also suggests that inhibition of the receptors of the sweet clade by bitter tastant is evolutionarily conserved and

sweet receptors from other mosquitoes such as *A. aegypti* may also be able to detect both sweet and bitter compounds.

### 5.4 Discussion

Before our analysis, there was little evidence showing that the *A. gambiae* sweet clade was capable of detecting sweet compounds. Here, we show that three of the T1 sensilla responded to multiple sweet compounds and that the sweet clade in *A. gambiae* was capable of detecting these same sweet compounds. This indicates that every receptor of the sweet clade is involved in ligand recognition of one or more sweet taste compounds. Similar to the *Drosophila* sweet clade, we found multiple receptors overlapped in the detection of the same sweet compounds, indicating that there may be more than one binding site for a sweet compound in a sweet receptor complex.

*A. gambiae* have a stereotypical layout of taste hairs on the labellum. We tested three long hairs on the labellum and found that they respond strongly to sugars and not to bitter compounds, which is similar to what has been previously published about *Drosophila* labellar long taste hairs [10]. This is interesting since labellar long taste sensilla in both *D. melanogaster* and *A. gambiae* responded strongly to sucrose and methyl α-glucopyranoside, while having weaker responses to fructose and glucose. In both fruit (*Drosophila* food source) and nectar (*A. gambiae* food source), the sugars with the highest concentrations are sucrose, fructose, and glucose. Both *A. gambiae*, and *D. melanogaster* long

labellar hairs have weak responses to fructose and glucose. It may be that different labellar sensilla or even sensilla on different taste organs such as the tarsi may be essential for the strong feeding preference for fructose and glucose in *A. gambiae* [4].

We did find differences in neural responses to sweet compounds between 1D, 2L and 5L and previously published responses from V8 and D5 T1 sensilla ( **Fig. 5.2**) [11]. Both V8 and D5 had weak responses to trehalose and maltose. In our study, 1D, 2L, and 5L had stronger responses to trehalose (~32 spikes per sec) and maltose (~52 spikes per sec). It may be that different sensilla have different responses to sweet compounds, though they appear to have the same morphology. Kessler *et al*. (2015) discovered that some of the long sensilla are water insensitive while others responded to hypoosmolarity. Both V8 and D5 are water insensitive while 2L has been previously shown to respond to water [10, 11]. It appears that though T1 sensilla look similar, they may respond differently and a comprehensive survey of all the taste sensilla is necessary. The other seven sugars tested in our panel had similar levels of responses as those for V8 and D5 suggesting that differences in sugar detection between T1 sensilla on the labellum may be limited.

When we individually expressed seven of the receptors of the *A. gambiae* sweet clade in the *D. melanogaster* ab1C olfactory neuron, all the receptors responded to at least one compound. Despite there being no one-to-one orthologs in the *D. melanogaster* and *A. gambiae* sweet clade, we did find

some examples of receptors that had more sequence similarities between the two insects responding to the same compound, although no one-to-one relationships emerge. For example, AgGr15 is the most closely related receptor to DmGr64e, DmGr64f, and DmGr5a and all of these receptors respond to trehalose and m-α glucopyranoside. Another example is AgGr20 which responds to sucrose, maltose, and maltotriose and is most similar to DmGr64a which also detects those same sugars. The sweet clade in *dipterans* is thought to come from a single ancestral gene. Multiple tandem duplications and deletions have led to the differences between mosquito and drosophila sweet clades [15]. The overlap in the detection of sweet compounds between both sweet clades could indicate that the binding site may have come from the same lineage in both mosquitoes and flies, though the receptors between both sweet clades are highly divergent.

In *Drosophila*, the receptors could be separated into two categories, by both sequence similarities and which sweet compounds they could detect. Four of the receptors responded to trehalose and were more closely related to each other than the receptors that did not respond to trehalose (Chapter 3). We did not see a similar phenomenon in the *A. gambiae* sweet clade and the amount of overlap and differences could not be explained by sequence similarities. Furthermore, it appears that the receptors of the sweet clade in *A. gambiae* have less receptor-sugar interactions than is found in *Drosophila* sweet clade. Based on our sweet panel that we used for both the *D. melanogaster* and *A. gambiae* receptors, there is 27/72 ( 37.5%) Gr-sugar interactions in the *D. melanogaster*

sweet clade and 18/ 63 ( 27%) Gr-sugar interactions in the *A. gambiae* sweet clade. It may be that each Gr of the sweet clade is more specialized to detect each of the sweet compounds instead of the higher degree of overlap found in *Drosophila*. One extreme example is that AgGr17 only responded to sucrose and not any of the other compounds in our panel. Another possibility is that our panel may not be suitable for *A. gambiae* and there are other likely mosquito specific sweet ligands. This would probably be due to the differences in feeding sources. While flies feed on fermented fruits, mosquitoes feed on nectar, extrafloral nectar, and honeydew [2, 4, 7, 22, 23]. Nectar sources contain multiple complex oligosaccharides and sugar alcohols, which were not included in our panel [2, 24]. Since we specifically chose sugars that activated both fly and mosquito labellar sweet neurons, our panel may be insufficient. A larger panel would be able to discover mosquito specific sweet ligands and find Gr-ligand interactions that do not exist in the *Drosophila* sweet clade. We would then be able to conclusively determine if receptors of the *A. gambiae* sweet clade are more narrowly tuned than in *Drosophila*. Furthermore, the *A. gambiae* sweet clade expression has not been mapped to any of the sweet neurons. Not only do we not know how broadly or narrowly tuned each receptor is, we do not know how many or which sweet receptors are expressed in a sweet neuron. Future experiments mapping the expression of each Gr, and comparing both the response profile of the labellar sweets neuron and the response profile for each sweet Gr expressed in each neuron would be useful in determining the

composition of a sweet receptor complex in each neuron. Then, mutant analysis could be used to validate these results and determine the role of each sweet Gr. One advantage of comparing responses between these two insects is because of how highly divergent the receptors of the sweet clade in *Drosophila* and *A. gambiae* are, we could make predictions of possible sugar binding sites. For example, multiple receptors can detect trehalose: AgGr15, AgGr16, AgGr18, DmGr5a, DmGr64b, DmGr64e, and DmGr64f. If we assume that the binding site for trehalose is similar in all of these receptors, we could find similar potential binding sites in all of them and perform site specific mutagenesis to find the trehalose binding site.

Both *A. gambiae* and *A. aegypti* long labellar hairs responded to sucrose and this response could be inhibited by bitter compounds. Inhibition of sweet taste neurons occurred in sensilla that did not respond to any tested bitter stimuli, suggesting that it is independent of bitter neuron activity. Interestingly, recent studies have shown that quinine does not inhibit blood feeding behaviors in female *A. gambiae* to the same level as sugar feeding, and their hypothesis was that inhibition of blood feeding by quinine is primarily due to activation of the bitter neuron [12]. The heightened sensitivity to bitter compounds during sugar feeding may be due to both direct inhibition of sweet taste neurons and bitter neuron activation. This dual mechanism may be necessary during sugar feeding since many plants produce deterrent chemicals to discourage feeding from vital areas of plants [25]. Furthermore, insect honeydew may contain alkaloids when

the producing insect may have consumed them when feeding on plants, potentially increasing the amount of contact with alkaloids during sugar feeding [26]. We only tested 4 bitter compounds for bitter tastant mediated inhibition of the sweet neuron, yet we did see variation in the degree of inhibition by bitter compounds. Caffeine weakly inhibited *A. aegypti* sweet neurons while lobeline greatly inhibited both the *A. gambiae* and *A. aegypti* sweet neuron. As of now, it is unknown if mosquito sweet taste neurons can be directly inhibited by a large variety of bitter compounds as seen in the fly, or if the inhibition is more conservative (**Fig. 4.3**). The same thing could be said of bitter neuron activation. There has been no reports of recordings from labellar bitter neurons in *A. gambiae* and shown that they respond to broad number of bitter compounds, as has been performed on *Drosophila* labellar bitter neurons [27]. In *Drosophila*, there is great deal of overlap between the bitter compounds that can activate labellar bitter neurons and inhibit labellar sweet neurons (**Fig. 4.3**). It is unknown if bitter neuron activation and sweet neuron inhibition by bitter compounds in *A. gambiae* have similar degree of overlap in detection as was discovered in *D. melanogaster.*

We tested mixtures of sweet and bitter tastants on one of receptors of the sweet clade, AgGr15, when it was expressed in the *Drosophila* ab1C neuron and found that AgGr15 can be directly inhibited by three bitter compounds: denatonium, lobeline, and strychnine. This supports the possibility that other receptors of the mosquito sweet clade are involved in bitter tastant mediated

inhibition. If that is true, then inhibition of a sweet Gr by bitter compounds may be evolutionarily conserved and other *Dipterans* may have a similar mechanism. It is thought that sweet Grs existed before bitter Grs [15, 28]. This might be how ancient ancestors avoided ingestion of toxic compounds.

Though we have focused on sweet Grs, mosquitoes express putative bitter receptors. The *A. gambiae* Gr family has 76 receptors where: 3 are carbon dioxide receptors, 9 are sweet receptors (including AgGr19P), and 64 may be bitter receptors [15, 16]. Except for DmGr28/ AgGr33, there doesn't appear to be any one to one orthologs between bitter receptors in *A. gambiae* and *D. melanogaster*. It would be interesting to see if there exists any overlap in detection of bitter compounds between the bitter Grs in *A. gambiae* and *D. melanogaster.*

### 5.5 Abbreviations

*Anopheles gambiae (A. gambiae)* Pg. 163

*Aedes aegypti (A. aegypti)* Pg.163

Methyl-alpha glucopyranoside (m-glucoside) Pg.165

Trichoid 1 (T1) Pg. 169

Trichoid 2 (T2) Pg.169

Ab1C neurons expressing AgGr15 (ab1C:AgGr15) Pg. 174

## 5.6 References


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Figure 5.1

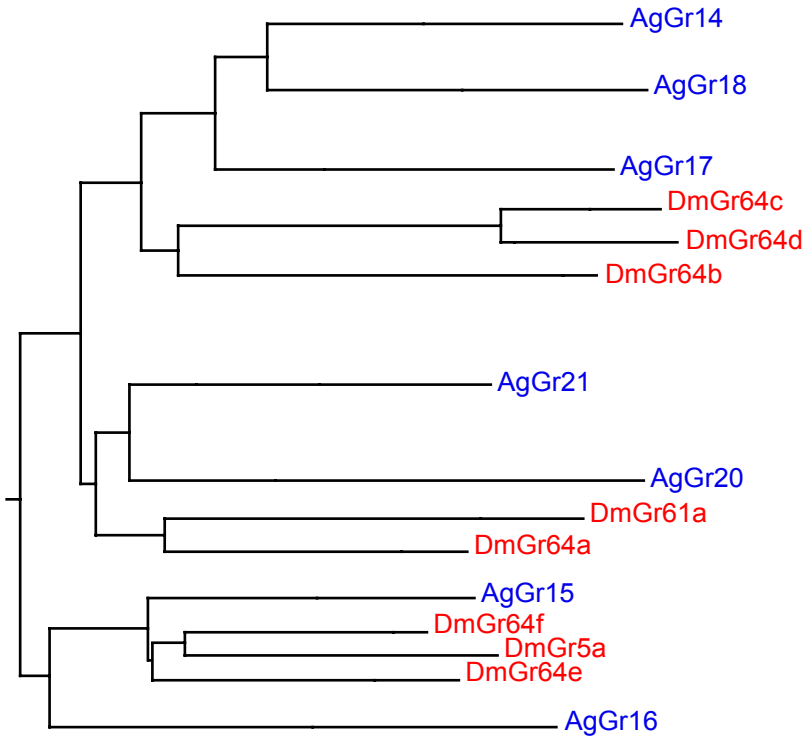
AgGr14
AgGr18
AgGr17
DmGr64c
DmGr64d
DmGr64b
AgGr21
AgGr20
DmGr61a
DmGr64a
AgGr15
DmGr64f
DmGr5a
DmGr64e
AgGr16

**Figure 5.1 Phylogenetic tree of the sweet receptor clade from *D. melanogaster* and *A. gambiae***. Adapted from [15].

# Figure 5.2

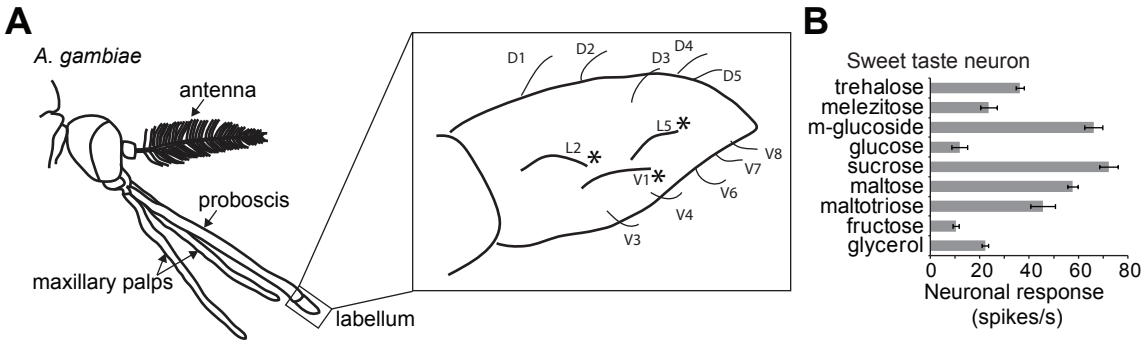
A
A. gambiae
antenna
proboscis
maxillary palps
labellum
D1
D2
D3
D4
D5
L5
L2
V1
V8
V7
V6
V4
V3
B
Sweet taste neuron
trehalose
melezitose
m-glucoside
glucose
sucrose
maltose
maltotriose
fructose
glycerol
0
20
40
60
80
Neuronal response
(spikes/s)

**Figure 5.2 *A. gambiae* labellar sweet taste neurons detect multiple sugars.** (**A**) Schematic of a mosquito labellum. * mark the taste hairs that are tested. Adapted from [11] (**B**) Mean responses of labellar sweet taste neurons to indicated stimuli. Sugars are tested at 100mM and glycerol at 10%(vol/vol) (n=6)

# Figure 5.3

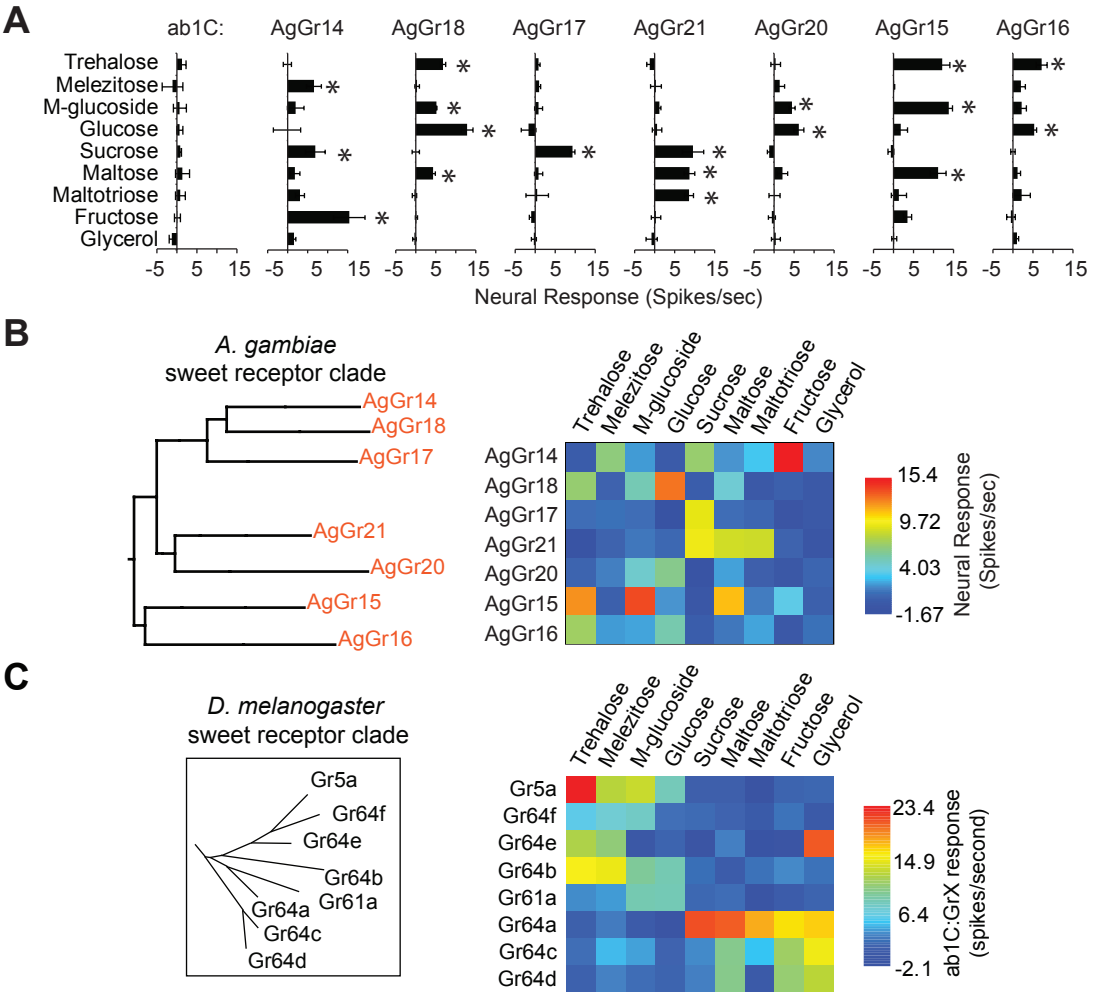
A
ab1C:
AgGr14
AgGr18
AgGr17
AgGr21
AgGr20
AgGr15
AgGr16
Trehalose
Melezitose
M-glucoside
Glucose
Sucrose
Maltose
Maltotriose
Fructose
Glycerol
-5 5 15
Neural Response (Spikes/sec)
B
A. gambiae
sweet receptor clade
AgGr14
AgGr18
AgGr17
AgGr21
AgGr20
AgGr15
AgGr16
Trehalose
Melezitose
M-glucoside
Glucose
Sucrose
Maltose
Maltotriose
Fructose
Glycerol
15.4
9.72
4.03
-1.67
Neural Response (Spikes/sec)
C
D. melanogaster
sweet receptor clade
Gr5a
Gr64f
Gr64e
Gr64b
Gr61a
Gr64a
Gr64c
Gr64d
23.4
14.9
6.4
-2.1
ab1C:GrX response (spikes/second)

**Figure 5.3 *An. gambiae* sweet receptor clade detects sweet tastants**. (**A**) Mean responses of ab1C:AgGrX neurons to panel of sweet stimuli. Sugars are tested at 100mM and glycerol at 10%(vol/vol) (n=6-10). * mark responses that are statistically significant (*P<0.05) (**B**) Phylogenetic tree of sweet Grs in *A. gambiae* adapted from [15] (Left) and heat map of mean neuronal responses of ab1C: AgGrX neurons to indicated sweet tastants. Data are the same as in Fig. 5.3A. Heat map was made with PAST (**C**) Phylogenetic tree of sweet Grs in *D. melanogaster* adapted from [17] (Left) and heat map of mean neuronal responses of ab1C:GrX neurons to indicated sweet tastants. This figure is same as Fig. 3.2C. Data are the same as in Fig. 3.2A. Heat map was made with PAST

# Figure 5.4

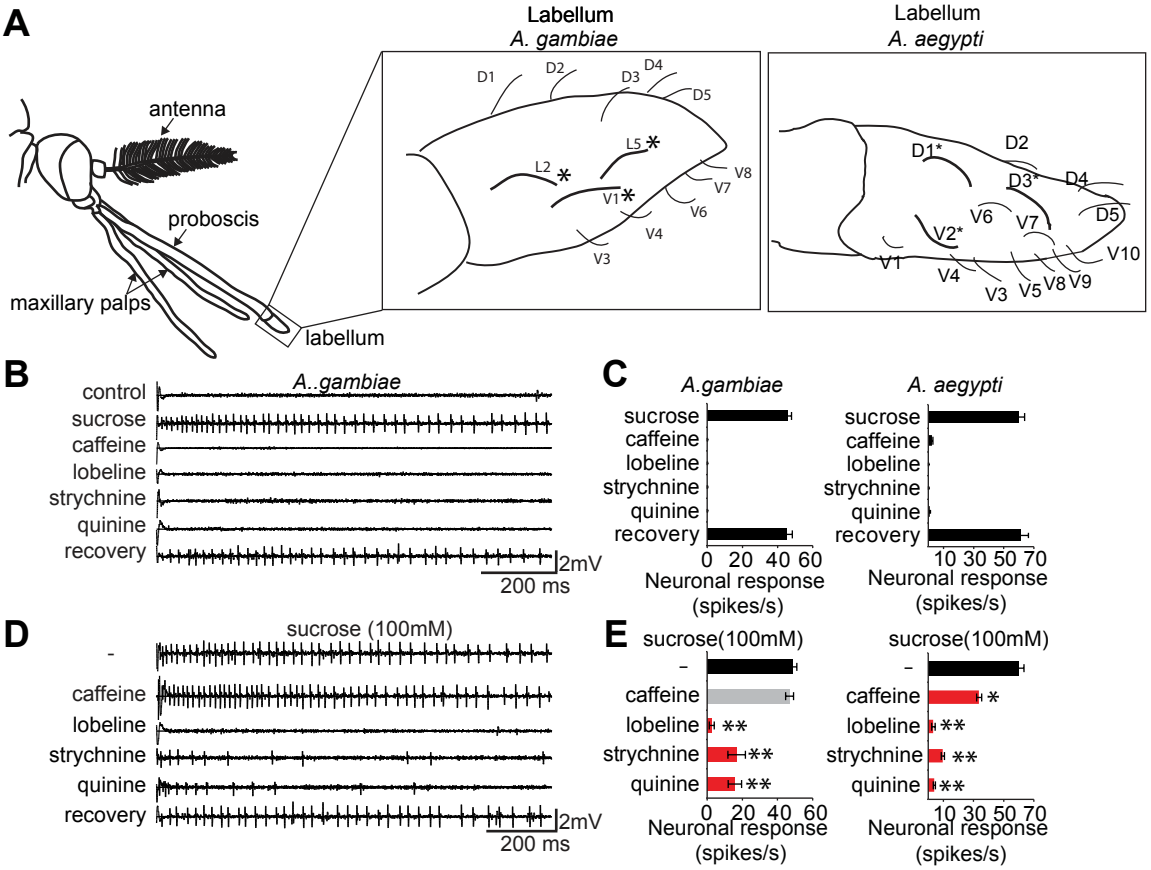
A
Labellum
A. gambiae
Labellum
A. aegypti
antenna
proboscis
maxillary palps
labellum
D1
D2
D3
D4
D5
L2
L5
V1
V3
V4
V6
V7
V8
D1*
D2
D3*
D4
D5
V2*
V6
V7
V10
V1
V4
V3
V5
V8
V9
B
A..gambiae
control
sucrose
caffeine
lobeline
strychnine
quinine
recovery
2mV
200 ms
C
A.gambiae
A. aegypti
sucrose
caffeine
lobeline
strychnine
quinine
recovery
0 20 40 60
10 30 50 70
Neuronal response
(spikes/s)
D
sucrose (100mM)
-
caffeine
lobeline
strychnine
quinine
recovery
2mV
200 ms
E
sucrose(100mM)
-
caffeine
lobeline
strychnine
quinine
0 20 40 60
10 30 50 70
Neuronal response
(spikes/s)

**Figure 5.4 Mosquito sweet taste neurons are inhibited by bitter tastants.**. (**A**)Schematic of *A. gambiae (Left) and A. aegypti (Right)* * mark the taste hairs that are tested. Labeling of *A. gambiae* T1 hairs is adapted from [11] and labeling of A. aegypti is adapted from [21]. (**B**) Sample traces from *A. gambiae* response to 100mM sucrose and 10mM bitter compound alone. (**C**) Mean responses in sweet taste neurons in *A. gambiae* (left) and *A. aegypti* (right) to 100mM sucrose and 10mM bitter compound alone. (n=6-10) (**D**) Sample traces from *A. gambiae* response to 100mM sucrose alone (-) and mixtures of 100mM and 10mM bitter. (**E**) Mean responses in sweet taste neurons in *A. gambiae* (left) and *A. aegypti* (right) to 100mM sucrose alone (-) and mixtures of 100mM and 10mM bitter. (n=6-10) Red bars indicate significance. $P<0.05$ Dunnett's t-test vs. sucrose alone.

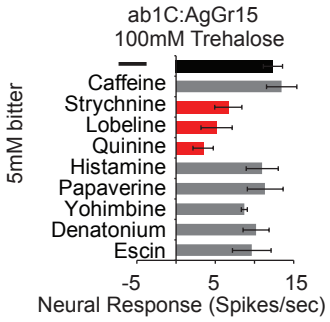
ab1C:AgGr15
100mM Trehalose
5mM bitter
Caffeine
Strychnine
Lobeline
Quinine
Histamine
Papaverine
Yohimbine
Denatonium
Escin
-5
5
15
Neural Response (Spikes/sec)

Figure 5.5

**Figure 5.5 AgGr15 is inhibited by bitter tastants**. Mean responses of the ab1C neuron expressing AgGr15 to 100mM trehalose (-) and mixtures of 100mM trehalose and 5mM bitter compounds. (n=6-10) Red bars indicate significance. $P<0.05$ Dunnett's t-test vs. trehalose alone.

**Table 5.1 List of Genotypes used in chapter 5**

| Line name | Genotype/ source | Figures |
|---|---|---|
| *wild type* | *w[1118]* | 5.3 |
| Gr63a-GAL4 on III | BDSC (# 9942) | |
| UAS-AgGr14 | Dahanukar Laboratory | |
| UAS-AgGr15 | Dahanukar Laboratory | |
| UAS-AgGr16 | Dahanukar Laboratory | |
| UAS-AgGr17 | Dahanukar Laboratory | |
| UAS-AgGr18 | Dahanukar Laboratory | |
| UAS-AgGr20 | Dahanukar Laboratory | |
| UAS-AgGr21 | Dahanukar Laboratory | |
| UAS-AgGr22 | Dahanukar Laboratory | |
| ab1C: | *w[1118]* | 5.3 |
| ab1C: AgGr14 | UAS-AgGr14-2;UAS-AgGr14-2; Gr63a-GAL4/Gr63a-GAL4 | 5.3 |
| ab1C: AgGr15 | UAS-AgGr15-3;UAS-AgGr15-3; Gr63a-GAL4/Gr63a-GAL4 | 5.3, 5.5 |
| ab1C: AgGr16 | UAS-AgGr16-1;UAS-AgGr16-1; Gr63a-GAL4/Gr63a-GAL4 | 5.3 |
| ab1C: AgGr17 | UAS-AgGr17-1;UAS-AgGr17-1; Gr63a-GAL4/Gr63a-GAL4 | 5.3 |
| ab1C: AgGr18 | UAS-AgGr18-2;UAS-AgGr18-2; Gr63a-GAL4/Gr63a-GAL4 | 5.3 |
| ab1C: AgGr20 | UAS-AgGr20-1;UAS-AgGr20-1; Gr63a-GAL4/Gr63a-GAL4 | 5.3 |
| ab1C: AgGr21 | UAS-AgGr21-2;UAS-AgGr21-2; Gr63a-GAL4/Gr63a-GAL4 | 5.3 |
| ab1C: AgGr22 | UAS-AgGr22-1;UAS-AgGr22-1; Gr63a-GAL4/Gr63a-GAL4 | 5.3 |

## 6.0 Conclusion

### 6.1 Summary of results

The purpose for this study was to determine the role insect sweet Grs play in the detection of both sweet and bitter compounds. To that end, we designed a system to heterologously express gustatory receptors (Gr) from insects to study ligand recognition (**Chapter 2**). Using that system, we set out to determine which receptors of the *Drosophila* sweet clade are involved in ligand detection (**Chapter 3**), characterize bitter tastant-mediated inhibition of both the *Drosophila* sweet neuron and sweet receptors (**Chapter 4**), and deorphanize receptors of the *A. gambiae* sweet clade (**Chapter 5**).

We expressed individual sweet and bitter Grs from *D. melanogaster* and *A. gambiae* in the fly ab1C neuron and recorded using different types of stimuli. By doing so, we discovered that there are evolutionarily conserved mechanisms between *D. melanogaster* and *A. gambiae* and some mechanisms that are unique to *D. melanogaster*. For example, all the receptors in both the *D. melanogaster* and *A. gambiae* sweet clade are capable of detecting sweet compounds. Both fly and mosquito labellar sweet neurons are inhibited by bitter compounds without input from a canonical bitter neuron. Furthermore, receptors from both insects sweet clades can be inhibited by bitter compounds as well. One difference we found is that in *D. melanogaster* the receptors can typically be separated into two groups that detected nonoverlapping subsets of sweet

compounds. Moreover, when comparing sequence similarity versus sweet tastant detection, more similar receptors responded to the same sweet compounds while more distant receptors responded to different nonoverlapping sweet compounds in *D. melanogaster*. The fly sweet receptor clade can be loosely separated into two groups based on ectopic functional analysis: one that detects Gr5a dependent sugars and another that detects Gr64a dependent sugars. We did not find the same thing in the *A. gambiae* sweet clade, and in some cases, more distant receptors respond to the same sweet compounds though each receptor detected a unique subset of sugars.

We validated response profiles of some of the sweet receptors from *D. melanogaster* that were derived from ectopic expression analysis by recording from available corresponding individual Gr mutants. For every response conferred by a Gr in the ectopic expression system, we found that there is a correlating loss of response in the corresponding Gr mutant. We did find cases where a sweet receptor is necessary for a neural response to a sweet compound but does not confer sensitivity when ectopically expressed. Together, these results suggest a model where multiple receptors may be required for a sugar response in the endogenous taste neuron, but not all of the receptors are directly involved in recognition of that ligand.

Compared to *D. melanogaster*, little is known about sweet taste detection in *A. gambiae.* Sugar detection is important considering all mosquitoes feed on nectar [1]. Here, we recorded from 3 of the long labellar hairs using a panel of

sweet compounds and discovered that they can detect multiple sweet compounds, all of which are also detected by *D. melanogaster*. Since labellar sweet neurons responded to multiple sweet compounds, we then individually expressed each receptor of the *A. gambiae* sweet clade in *Drosophila* ab1C neurons and recorded responses to the same sweet panel. All receptors of the mosquito sweet clade functioned in the ab1C neuron and responded to at least one sweet compound, suggesting all the receptors of the sweet clade are involved in ligand detection.

Recent studies have found that sweet neurons in *D. melanogaster* can be inhibited by bitter compound without input from a bitter neuron and sensitivity to bitter tastant mediated inhibition is dependent on OBP49a [2, 3]. We validated that the sweet neuron can be inhibited by bitter compounds and OBP49a is required for inhibition of the sweet neuron by bitter tastants. Then, we recorded from labellar taste neurons on both *wild type* and OBP49a mutants using mixtures with higher concentrations of bitter and sweet compounds. We found that at higher concentrations, labellar sweet neurons can be inhibited by multiple types of bitter compounds even in the absence of OBP49a. Furthermore, we discovered an instance where a bitter compound could inhibit a sweet neuron but does not activate labellar bitter neurons. For the most part, we found that the bitter compounds that activated labellar bitter neurons also inhibited labellar sweet taste neuron suggesting a great amount of overlap between these two mechanisms. Bitter tastant mediated inhibition of the sweet neuron may be

evolutionarily conserved in *dipterans* since labellar sweet taste neurons in both *A. gambiae* and *A. aegypti* can be inhibited by bitter compounds without any bitter neuron input.

Sweet taste receptors can be directly inhibited by bitter compounds. When we expressed each receptor from the *D. melanogaster* sweet clade, except Gr61a, individually in the ab1C neuron and recorded using mixtures of sweet and bitter compounds, we found that bitter tastants can inhibit sugar induced neural activity. The ab1C neural responses to its endogenous ligand, $CO_2$, is immune to inhibition by bitter compounds and the presence of bitter tastants in the electrolyte solution did not affect the neural response to $CO_2$, suggesting inhibition by bitter compounds is due inhibition of the expressed sweet Gr. Each receptor of the *Drosophila* sweet clade is inhibited by a unique panel of bitter compounds with different levels of sensitivities, indicating bitter tastant mediated inhibition of the sweet neuron may be dependent on inhibition of the sweet Grs found in a sweet receptor complex expressed in a sweet neuron. Additionally, we demonstrated that a receptor from the mosquito sweet clade can be directly inhibited by bitter compounds. Inhibition by bitter compounds is unique to the sweet clade since both Gr43a and its mosquito ortholog, AgGr25, neural responses to fructose was immune to inhibition by bitter compounds. Both of these receptors are fructose receptors that are outside of the sweet clade supporting the possibility that the receptors of the sweet clade in both insects have evolved as dual sensors for sweet and bitter compounds.

## 6.2 Discussion

### 6.2.1 *Drosophila* sweet clade

All of the sweet receptors can confer detection of multiple ligands when expressed in the ab1C neuron. We find no evidence for a single obligate co-receptor in the sweet Gr clade that might function in all sweet taste neurons, consistent with observations that the loss of a single sweet Gr never leads to the loss of all taste responses (**Fig. 3.2 & 3.6**) [4-9]. Rather, our findings support a model in which each receptor contributes to detection of sweet compound, an idea that is consistent with previous observations that endogenous taste response to a sugar depends on the combination of receptors expressed in the neuron [5-7, 10-12]. Previous studies have shown that deletion of the Gr64 clade eliminates all responses to sweet compound, with the exception of any cell that expresses Gr43a [5, 12]. Gr43a expressed in a neuron devoid of other sweet Grs responds to both sucrose and fructose [12].

The loss of two receptors, Gr5a and Gr64a, leads to the loss of all sweet responses in labellar sweet taste neurons, suggesting that at least one of these receptors is necessary to maintain some sugar detection by the labellum [10]. One possible reason could be that Gr5a and Gr64a form separate receptor complexes with all the other receptor. Without expression of either Gr5a or Gr64a, no functional receptor complexes could form on the membrane of sweet

neurons. Gr5a and Gr64a are necessary for the detection of separate and nonoverlapping subsets of sugars, suggesting they may not interact with each other (**Fig 3.2 & 3.3**). While not all receptors have been tested (Gr64b, Gr64c, and Gr64d), the loss of either Gr64f or Gr64e leads to reduction of neural responses in labellar sweet neurons to subsets of sugars that overlap both Gr5a and Gr64a dependent sugars, suggesting these receptors function with both Gr5a and Gr64a. Gr64e mutants show reduced responses to trehalose, melezitose, and m α glucopyranoside (Gr5a dependent) plus maltose, maltotriose, and glycerol (Gr64a dependent). Gr64f mutants show reduced responses to trehalose, melezitose, m α glucopyranoside, and glucose (Gr5a dependent), plus sucrose and maltose (Gr64a dependent). It should be noted that both functional studies and expression analysis indicate Gr43a is not expressed in the labellum and it has been shown that artificial expression of a sweet Grs in a Gr43a expressing neuron devoid of other Grs rescues ligand sensitivity to multiple sweet tastants [6, 12]. This would argue that Gr5a, Gr64a, or Gr43a are necessary to maintain some sugar detection in the fly.

Another possibility is that Gr5a, Gr64a, or Gr43a are necessary to ensure other Gr members can reach the membrane in the proper orientation. The lack of either Gr64a or Gr5a could make it so no other Grs can reach the membrane of labellar sweet neurons. To determine if either Gr64a or Gr5a are necessary for other Grs proteins to be at the cell membrane, Gr64f or Gr64e protein could be myc-tagged in both *ΔGr5a;ΔGr64a* flies and *wild type* flies. Then, we could track

protein expression and movement in the dendrites of sweet neurons. If either Gr64e or Gr64f protein is found at the sweet neuron cell membrane in *ΔGr5a;ΔGr64a* flies, it suggests that Gr5a or Gr64a is necessary for some function other than Gr protein transportation to the membrane. If it isn't, then it suggests that either Gr5a or Gr64a is necessary for trafficking Gr64e or Gr64f to the cell membrane or to prevent degradation of Gr proteins.

There is evidence that contradicts our hypothesis that either Gr5a or Gr64a are necessary for sugar detection by labellar sweet neurons. Expression of two receptors in an empty sweet taste neuron can rescue some responses [12]. Coexpression of Gr64b and Gr64e rescues glycerol detection in a sweet neuron devoid of other sweet Grs, albeit at a reduced response compared *wild type* sweet neurons. In our analysis, loss of Gr64a leads to a complete loss of glycerol detection (Ch.3). Fujii *et al.* (2015) found Gr64b is necessary for glycerol detection by using *Gr64b-LexA*$^{ki}$ flies [11]. One possibility is that Gr64b is necessary for glycerol detection as part of a receptor complex but Gr64b does not have a binding site for glycerol. Another possibility could be Gr64b may not be expressed in labellar taste hairs. To solve this issue, a comprehensive map for the expression of all sweet taste receptors in the labellum would be useful. Some labs have tried to accomplish this but some problems have arisen that have led to controversy in the field [5, 10, 11]. One problem is that to map endogenous expression of proteins in the fly with a high degree of confidence is usually done by *in situ* hybridization, but this technique has been largely

unsuccessful for Gr proteins [10, 13, 14]. The lack of success is thought to be due to Gr proteins being expressed at low concentrations [10, 14]. Instead, most of the *D. melanogaster* Gr family has been mapped by using promoter expression analysis [11, 14-18]. Usually this is done by amplifying several kbs in front of the transcription site and inserting the fragment upstream of a GAL4 or LexA coding sequence and then driving expression of a marker [16-18]. One caveat of using this system is that it is dependent on the promoter region that was selected. If too large or too small a section is used, then you may get off target sites or no expression in cells where Gr protein is endogenously expressed in. Another approach is to using gene knock-in and replacing the gene of interest with GAL4 or LexA. This strategy can be used to both remove the gene and express a marker. Even this technique has limitations where deletion of introns and untranslated regions of gene which could potentially alter expression analysis. Because of the limitations of both techniques, there are still questions about the expression patterns of the sweet Grs. For example, Gr64a has been found to be expressed in the labellum by functional analysis and not by expression analysis [10, 19]. This has led to questions if Gr64a is actually expressed in the labellum or is involved in sugar detection at all [11]. Resolving these questions requires a two-fold approach, one to map expression and another approach to determine whether Gr64a is necessary for sugar detection. It is crucial to create a new Gr64a mutant using smaller deletions that won't disrupt introns or untranslated regions to reduce the possibility of off target

effects, but will disrupt functional expression of Gr64a protein. Using Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR), we could create a small deletion that would lead to a frame shift and potential loss of Gr64a [20]. To validate loss of only Gr64a and not Gr64b, we would have to sequence both Gr64a and Gr64b and use RT-PCR to show no expression of Gr64a and normal expression of Gr64b. To determine whether Gr64a is expressed in labellar hairs, Gr64a gene could be tagged using knock in myc tag directly on Gr64a gDNA [21]. By tagging the endogenous protein, we could then stain directly for Gr64a. This two-fold strategy could also be used to map expression of the other sweet Grs and create mutants for Gr64b, Gr64c, and Gr64d.

We created a technique to express receptors individually and study each receptor's properties, but there are some limitations for using this technique and in this study. Two limitations of the system entailed determining the amount of a stimulus reaches the ab1C neuron and coexpression receptors of in the ab1C neuron. To deliver sweet and bitter compounds, we used diffusion of our stimuli from our recording electrode which has a pore with a diameter of a few micrometers. While the recording electrode may have 100mM tastant, the concentration that reaches the ab1C neuron is thought to be significantly lower (**Fig.2.1**). Though we showed this process was capable ensuring a sufficient amount of our compounds reached the ab1C neuron, the neural responses of the ab1C neuron were weak compared to labellar sweet taste neurons. Adding dye

to the solution would be useful in visualizing diffusion to determine the amount of our compound that reaches the neuron. We considered using higher concentrations of our sweet compounds to increase neural responses but this was difficult since changes in viscosity of the electrolyte solution could affect the extracellular recordings. As such, changes in viscosity of the electrolyte solution prevents higher concentrations from being used so receptors that have low sensitivity for a specific compound may be missed using this system.

Recent studies as well as our own results provided evidence that receptors of the sweet clade are subunits that function in sweet taste neuron in a receptor complex [5, 11, 12]. Artificial expression of Gr64f in sweet labellar neuron in a Gr64 mutant rescued trehalose detection where only Gr5a and Gr61a are endogenously expressed [5]. In the same study, expression of Gr64a and Gr64f in the same sweet labellar neuron in a Gr64 mutant rescued sucrose and maltose detection. We also found instances where a single sweet Gr is necessary for a specific sugar response but did not confer sensitivity for it when that sweet Gr was expressed in the ab1C neuron (**Fig. 3.4**). Based on previous studies and our results, it indicates that sweet Grs function together in a complex to detect sweet compounds. When we coexpressed sweet receptors with Gr5a in the ab1C neuron, we expected to see increased sensitivity to sweet compounds. We did not find any new or significantly increased responses to sweet compounds (**Fig. 3.2**). Based on our results, it was unclear whether the receptors function together or if they function separately when expressed in pairs in the

ab1C neuron. It could be that the endogenously expressed Gr21a and Gr63a interfered with the sweet Grs forming a complex with each other. We did find one example where there was evidence of interactions between two sweet receptors when both were expressed in the ab1C neuron. Coexpression of Gr64a and Gr64e in the ab1C neuron had stronger response to glycerol than when either receptor was expressed alone (**Fig. 3.5**). Secondly, ab1C:Gr64a +Gr64e neurons response to glycerol was inhibited by 2-amino 1,3 propanediol (67%) to a similar degree as in ab1C:Gr64e neurons (72%) while ab1C:Gr64a neurons were unaffected, supporting the possibility of functional interactions (**Fig. 3.5**). To validate interactions between sweet receptors, we could remove all sweet receptors from a sweet taste neuron and then add them back in different combinations [5, 12]. Recent studies have shown some success using these techniques [5, 12]. In one study, using a tarsal sweet neuron devoid of sweet Grs, they found that expressing pairs of Grs conferred detection of sweet compounds [12]. Based on single Gr mutant analysis and our ectopic expression analysis, there are a few novel combinations that would be worthwhile to test (**Fig. 3.2&3.3**). Single mutants for Gr61a, Gr64e, Gr64f, and Gr5a all have reduced responses to trehalose, plus Gr5a, Gr64e, Gr64f, and Gr64b confer trehalose detection in the ab1C neuron. Based on the multiple receptor combinations that can detect sucrose ( Gr43a [12], Gr64a+Gr64f+Gr5a+Gr61a [5], Gr64a+Gr64e [12]), we hypothesize that there would be multiple combinations of Grs to detect trehalose. By expressing combinations of receptors

that can detect or are necessary for trehalose detection, we could discover which receptor combinations form a functional trehalose receptor complex. The difficulty with using such a strategy is combining multiple mutants and transgenes in a single fly. For example, the sweet receptors are found on the first and third chromosome and would require a minimum of three deletions (Gr5a, Gr61a, Gr64). Next, using a binary system to express these receptors in the sweet neuron would require at least one GAL4 promoter and upstream activation sequence (UAS)-GrX for each Gr. While it is possible to combine multiple mutations and transgenes on the same chromosome for each combination, it may be time prohibitive to test all potential combinations and it would be prudent to be selective about which combinations to test. For example, since responses to trehalose are severely reduced in Gr5a mutants, most combinations should include Gr5a to begin with such as: Gr5a +Gr64f, Gr5a+Gr64e, and Gr5a+Gr64b+Gr64e+Gr64f. Another possibility is to use a different taste neuron such as a bitter or salt neuron. Since bitter Grs can form a functional receptor in a sweet taste neuron [22], it may be worthwhile to see if the opposite is true as well.

Both flies and mammals can detect many of the same sweet tastants yet they appear to be using two different types of receptors [23]. Mammals such as humans or rodents have two sweet receptors, T1R3 and T1R2, and these receptors function together as a heterodimer and are G protein coupled receptors [24]. Comparatively, *D. melanogaster* expresses nine sweet Grs. While reduction

G protein subunits in taste neurons can affect sweet neuron responses [4, 25-27], Grs are most likely not G protein couple receptors since one of the taste receptors has been shown to be a ligand gated ion channel, suggesting the others may be as well [28]. There are some similarities between both insect and mammalians sweet receptors. Based on structure function analysis; it was shown that both mammalian receptor subunits have discrete binding pockets for sweet tastant detection [29, 30]. Similarly, every *D. melanogaster* sweet receptor can confer detection of sweet tastants suggesting that every receptor is involved in ligand recognition. Moreover, different taste receptors subunit can form receptor complexes with other receptor subunits in both the mammalian and fruit fly taste system. T1R1 + T1R3 function together to confer umami taste detection in mammals [23] and multiple pairs of sweet Grs in *D. melanogaster* can function together to confer sweet tastant detection [5, 12]. Still, expression of at least one sweet Gr can maintain some sweet detection and multiple receptor pairs can confer sensitivity to the same sweet tastants in the fly. Both T1R2 and T1R3 are necessary for sweet detection in the mammalian system, suggesting these receptors function differently.

The olfactory receptor (Or) family are the most closely related receptors to the gustatory receptors. In fact both receptor types have the same inverted topology where the N termini are in the cytoplasm [31, 32]. Ors function as heterodimers with an obligate co-receptor (Orco) [33]. While Orco is necessary for signal transduction, the other Ors are solely responsible for ligand detection.

Orco does not respond to any known natural ligands [34]. Conversely in the sweet receptor clade, all of the Grs are capable of ligand detection. There also appears to be no obligate coreceptor and the sweet receptors appear to be more promiscuous than Ors, since multiple combinations can confer sensitivity to sweet tastants [12, 19]. Furthermore, Grs can function as multimers. Expression of three bitter Grs (Gr8a, Gr98b, and Gr66a) confers sensitivity to L-canavanine in sweet taste neurons [22]. Ors are ligand gated ion channels where loss of G protein subunits also can affect olfactory neuron sensitivity to odors [35-37]. Similar things have been discovered for the sweet Grs as well. One of the sweet Grs, Gr43a, has been shown to be a ligand gated ion channel and loss of G protein subunits affects sensitivity to sweet tastants [4, 27, 28]. It may be that both Ors and Grs function similarly.

### 6.2.2 Bitter tastant mediated inhibition of sweet neuron and sweet Grs

When we recorded from labellar L-type sensilla using mixtures of sweet and bitter compounds, we found that bitter compounds could inhibit sweet neurons' response to sugar (**Fig. 4.1, 4.2, &4.3**). We tested 20 bitter compounds on both the sweet neuron and sweet Grs in *Drosophila*. All of the bitter compounds inhibited at least one sweet Gr. Not all the bitter compounds inhibited the sweet taste neuron but we only tested using mixtures of 5mM bitter compound and 100mM sucrose (**Fig. 4.3**) and did not test higher bitter concentrations. Our next step is to repeat recordings from labellar sweet neurons

using higher concentrations of bitter compounds. This is based on the fact that each bitter compound inhibited at least one sweet Gr at 10 mM, suggesting that all of these bitter compounds are capable of inhibiting sweet neurons (**Fig. 4.5**).

Multiple bitter compounds can inhibit sweet neuron and they come from multiple structurally diverse classes indicating that there may be many more bitter compounds from the 4000 known bitter alkaloids, plus aversive amino acids and other plant defense chemicals that have the potential to inhibit the sweet neuron [38, 39]. In fact, there may be more bitter compounds able to inhibit sweet taste neurons as compared to the 35 sweet compounds that have been shown to activate labellar sweet taste neurons [8, 10]. There are insects such as the honey bee that do not appear to have a canonical bitter neuron yet still avoid bitter compounds [40]. One possibility is that sweet neurons and potentially the sweet Grs, themselves, in honey bees are inhibited by bitter compounds, thus avoiding ingestion of toxic compounds.

We wonder if inhibition of sweet neurons by bitter compounds is enough to prevent consumption of bitter compounds alone and does preference for sugar/bitter mixtures shift when detection of bitter compounds relies solely on sweet neuron inhibition. To answer both of these questions, we could silence bitter neurons using *Gr89a-GAL4;UAS-Kir* flies and compare changes in preferences for sucrose mixed with different concentration of bitter compounds between control and bitter neuron silenced flies using binary feeding assays [17]. This would allow us to see changes in preferences and allow us to determine if

there are changes in sensitivity to bitter compounds. Since many of these bitter compounds are toxic and could make a fly ill, short assays would be best since the help to minimize any post ingestive effects.

There is a great amount of overlap of the compounds that can be detected by both bitter neuron activation and by inhibition of the sweet neuron. One possibility for why may be to heighten sensitivity to bitter compounds. Increasing the number of mechanism to avoid bitter compounds could increase prevention of consumption. Another possibility is that the detection of bitter compounds is important outside the context of sugar feeding and activation of bitter neurons is necessary for these behaviors. Other behaviors where detection of bitter compounds might be useful are during mating and egg laying [41-43]. In fact, some of the compounds that are used in courtship and mating behavior activate bitter receptors [43-45].

When comparing inhibition of the sweet neuron and the sweet Grs by bitter compounds, we found no obvious correlation between the number of receptors that are inhibited by a specific bitter compound or the % inhibition of each sweet Gr and the level of inhibition in the endogenous taste neuron. We tried ranking bitter compounds by the number of receptors they inhibited at 5mM and compareing it to percent inhibition of the sweet neurons response to 100mM sucrose by 5 mM bitter compound but we found no correlation between the number of receptors inhibited and % inhibition of the sweet neuron. For example, lobeline, denatonium and strychnine are the strongest inhibitor of the sweet

neuron but at 5mM, they inhibited 2, 5, and 1 sweet Grs respectively (**Fig. 4.3 &4.5**). Theophylline, a weak inhibitor of the sweet neuron, inhibited all of the sweet Grs when they were expressed in the ab1C neuron. Furthermore, we did not find any sweet Gr whose bitter tastant inhibition profile was similar to that of the sweet neuron which argues against one sweet Gr instead of multiple receptors being essential for bitter tastant inhibition of the sweet neuron. For example, Gr64a and Gr64f were inhibited by the largest number of bitter compounds at 5mM, yet neither receptor was inhibited by strychnine, the strongest inhibitor of the sweet neuron. One reason we may not find a direct correlation could be due to studying each receptor individually when expressed in the ab1C neuron. It may be that when sweet Grs are together in a sweet receptor complex, they could be modifying or blocking binding sites for bitter compounds. There is precedent for such a phenomenon in Grs [46]. Delventhal *et al*. (2016) found that misexpressing a bitter receptor in a new bitter neuron or removing a bitter receptor from its endogenous neuron could both confer new ligand sensitivity and inhibit responses [46]. Thus, it is possible that a sweet Gr could inhibit another sweet Gr from detecting a bitter compound in a sweet receptor complex, reducing the % inhibition of the sweet neuron by a bitter compound. When we coexpressed Gr5a with either Gr64e or Gr64f in the ab1C neuron, we did find examples where the inhibition of the ab1C neuron by a specific bitter compound had the same % inhibition as when one of Grs was expressed alone in the ab1C neuron. For example, ab1C:Gr5a +Gr64f and ab1C:Gr5a trehalose

mediated responses were 57% and 63% inhibited by yohimbine respectively while ab1C:Gr64f neurons were not inhibited by yohimbine (**Fig. 4.6**). This supports the possibility that like activation by sweet compounds, inhibition of the sweet neuron by a bitter compound is dependent on the sweet Grs expressed in a receptor complex.

The binding sites for both bitter and sweet compounds on sweet Grs is unknown. Based on the vast structural differences between sweet and bitter compounds, we hypothesize that the bitter and sweet compounds have different binding sites on sweet Grs. We still don't know if there are multiple binding sites for sweet compounds and bitter compounds or if there is only one large binding pocket. More is known about mammalian taste receptors and their bitter and sweet receptors have examples of either a single binding site or multiple binding sites on a single receptor [29, 47, 48]. Sweet mammalian taste receptors have multiple binding sites for the detection of different sweet compounds [29, 48]. On the other hand, mammalian broadly tuned bitter receptors have one large binding site to detect a broad selection of bitter compounds with multiple affinity points within the binding site to increase specificity [47]. Because we have multiple examples where one bitter compound is detected by multiple sweet Grs, we could compare the protein structure of the sweet Grs and find similar potential binding sites that are conserved between sweet Grs. Then, we could use site specific mutagenesis to disrupt detection of a bitter compound. If bitter tastant mediated inhibition of a sweet Gr is due to allosteric modulation, we could

potentially disrupt inhibition of a sweet Gr by a bitter compound while not compromising sugar detection.

### 6.2.3 Evolutionarily conserved mechanisms of the fly and mosquito sweet clade

Since mosquito receptors can function in the fly, it suggests that there are conserved mechanisms between the Gr families in *D. melanogaster* and *A. gambiae*. This is further supported by both flies and mosquito labellar sweet neurons responding to sugar and being inhibited by bitter compounds. Like the fly, all of the receptors of the *A. gambiae* sweet clade respond to unique subsets of sweet tastants. Less is known about the mosquito and the lack of single Gr mutants makes it harder to draw conclusions. Still, these results suggest that there is no obligate co-receptor for sweet tastant detection and all of the sweet Grs are involved in ligand recognition. The sweet taste receptors may also be involved in the direct inhibition of the sweet neuron by bitter compounds since ab1C neurons expressing AgGr15 were inhibited by bitter compounds.

Future experiments would require comparing ectopic expression gain of function and loss of response in corresponding single mutants. With the advancement of the CRISPR system in multiple insects, it makes it possible to create mutations for each sweet Gr in *A. gambiae*. Another critical experiment is to map expression of each sweet Gr, which will allow us to make predictions

about potential sweet receptor complexes. Furthermore, a comprehensive survey of the taste hairs on the labellum of *A. gambiae* has not been done. The panel in this study is limited and does not cover all the sugars found in multiple feeding sources [49-52]. Using a large panel like has been used on the *D. melanogaster* labellum would be beneficial [10]. Also, only 4 bitter compounds were tested for bitter tastant mediated inhibition of labellar sweet neurons in mosquitoes as compared to the 20 compounds we used on *D. melanogaster* labellar sweet neurons. Mapping expression of sweet Grs and a survey of all labellar taste neurons for responses to a more comprehensive panel is critical for understanding sweet and bitter detection in mosquitoes.

### 6.3 Concluding remarks

In summary, we have shown that all the sweet Grs in both *D. melanogaster* and *A. gambiae* are capable of sugar detection and found no evidence of an obligate co-receptor. Furthermore, we designed a system that can express insect Grs individually so we can study the individual properties of each Gr. We found that inhibition of the sweet neuron by bitter compounds is evolutionarily conserved in *D. melanogaster*, *A. gambiae* and *A. aegypti* and sweet Grs can function as dual sensors of sweet and bitter compounds. Our results provide a platform to deorphanize both bitter and sweet Grs in insects as

well as find evolutionary conserved mechanisms that may exist in multiple insects' Gr families.

### 6.4 Abbreviations

Gustatory receptors (Gr) Pg. 196

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) Pg. 204

Upstream activation sequence (UAS) Pg. 207

Olfactory receptors (Or) Pg. 208

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