MOLECULAR BASIS OF SUGAR SENSING IN *DROSOPHILA*

A Dissertation

by

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ABSTRACT

In contrast to mammals, which sense sweet tasting molecules through a single, dimeric sugar taste receptor, *Drosophila melanogaster* use at least eight sugar gustatory receptor (*Gr*) proteins to recognize a range of dietary sugars. We showed that the sugar *Gr* genes are expressed in partially overlapping fashion in the single sweet gustatory receptor neuron (GRN) to generate at least 8 different subtypes and that sugar receptors heteromultimeric complexes. We used a combinatorial strategy to examine the composition of functional sugar receptors using an "empty neuron" system that is based on an octuple mutant fly strain lacking all eight sugar *Gr* genes. By expressing all 28 possible pairwise *Gr* gene combinations in the "empty neuron" that express the Ca^{2+} sensitive GCaMP6 protein, we find that 18 of these combinations can reconstitute sugar responses to a subset of sugars. Remarkably, each of these combinations restores responses to wild type levels to at least one of the eight sugars that we tested, and some combinations restored high responses to two or three sugars. Some of these combinations were able to convey sugar responses to bitter GRNs when expressed under the control of the GAL4 driver for the bitter receptor *Gr33a*, indicating that bitter and sweet GRNs use the same signaling mechanism. To explore the possibility whether sugar receptor complexes are composed of more than two different subunits, we introduced triple combinations of sugar Gr genes in the "empty neuron". For two of the four different triple combinations, response profile revealed new sugar responses not observed with any of the three respective pairwise combinations, suggesting that for at

least some sugars, functional complexes are likely to contain three different Gr subunits. Taken together, our analysis reveals that sweet GRNs of wild type flies might have more than 20 different sugar receptor complexes, each tuned to subsets of sugars.

DEDICATION

This dissertation is dedicated to my family in Turkey; they have always encouraged me with their love.

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Contributors

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Chemosensation is essential for animals to locate food sources, avoid predators, find potential mates, and identify oviposition sites. Volatile molecules are sensed by olfactory receptor neurons whereas non-volatile compounds are sensed by gustatory receptor neurons.

The human and fly chemosensory system have similarities at the organizational level. Chemical ligands are recognized by the peripheral cells, and information is carried through afferent fibers to the designated part of the brain. The different taste modalities that can be discriminated by flies and humans show striking similarities, and ligands mostly attractive to flies are also attractive to humans. In fact, a human's response profile to sugars and sweeteners are more similar between *Drosophila* and humans than humans and other primates [1], despite the differences at the molecular level. In this introduction, I will describe the olfactory and gustatory system of flies and mammals in detail, as they pertain to my project.

Drosophila Olfactory System

The antenna is the main *Drosophila* olfactory organ (Figure 1). It consists of three segments. Two proximal segments of the antenna are responsible for hygrosensation, sound perception, and heat sensing [2]. Most distal, the third segment contains sensory sensilla that are involved in the detection of odorants [3]. These olfactory sensilla are

hair-like structures and subdivided into three types (basiconic, tricoid, and coeloconic) based on their size, shape, and spatial positioning on the third antennal segment (Figure 1) [4].

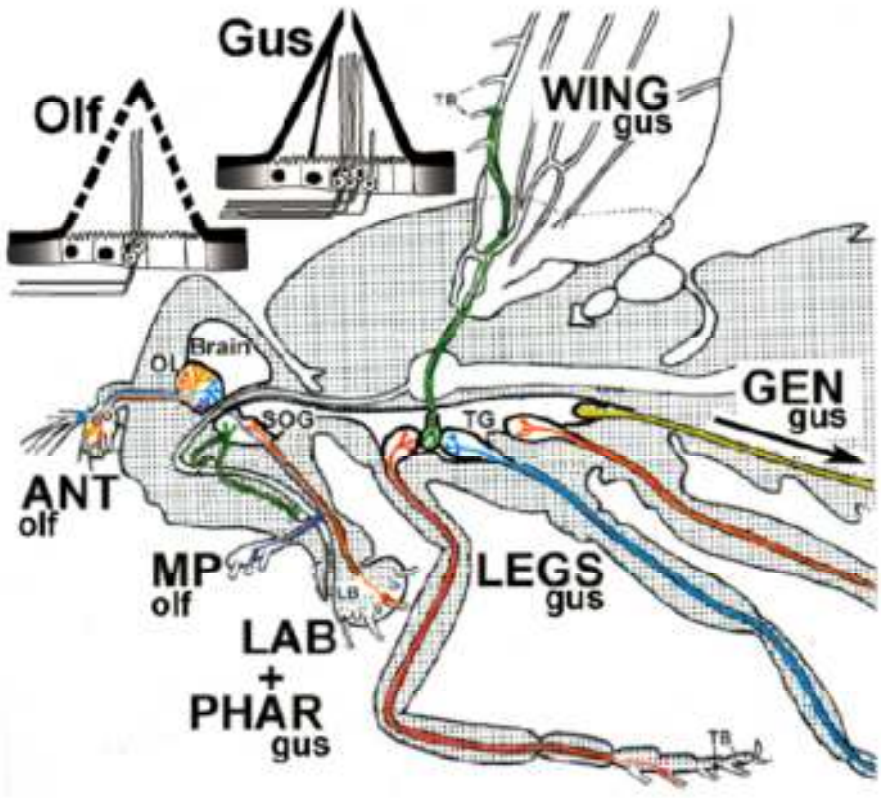

Figure 1 Diagram of Adult *Drosophila melanogaster* chemosensory neurons. Olfactory sensory (Olf) neurons are primarily located in the antennae (ANT) and the maxillary palps (MP); these neurons project to the olfactory lobe (OL) in the brain. Gustatory Neurons (Gus) send signals from the labellum (LAB), pharynx (PHAR), legs, wings, and genetalia (GEN) to the thoracic ganglion [5] or the subesophageal ganglion (SOG) in the CNS (Adapted from Stocker, 1994).

Two pairs of second, accessory olfactory organs reside in the maxillary palps, located on the proboscis (Figure 1). They house basiconic sensilla that are known to respond to olfactory stimuli [3]. In some insect species, the maxillary palps are used as a taste organ rather than an olfactory organ [6]. Shiraiwa proposed that the olfactory neurons on the maxillary palps in *Drosophila* are tuned to mediate odor-induced taste enhancements [7].

There are about 400 sensilla in each antenna and 60 in each maxillary palp. The pores and channels in the shaft of each sensillum allow odorants to access the dendritic extensions of the olfactory neurons that harbor the olfactory receptors. Each sensillum houses up to four neurons. They are encapsulated with accessory cells. Previously, researchers have tried to link sensilla type to a particular response (for example, basiconic sensilla houses neurons that respond to attractive odors). Yet, there is no defined specific response pattern based on sensilla type. However, there are certain patterns at the cellular level, which are directly dependent on the olfactory receptors they express. Specifically, each olfactory neuron of basiconic and trichoid sensilla expresses a specific member of the olfactory receptor (Ors) gene family, along with the obligate co-receptor gene *Or83b* (now renamed to *Orco*). While neurons of the coeloconic sensilla express predominantly three to four members of another chemoreceptor gene family, the Ionotropic receptors (Irs) genes [8, 9].

Olfactory sensory neurons (OSN) are housed at the base of the sensilla. They transduce the chemical message further to higher brain centers. OSNs extend their dendritic protrusions into the sensillum. There are about 1200 OSNs in each antenna and

120 in each palp sensilla. Each olfactory receptor neuron of basiconic and trichoid sensilla expresses *Orco* and one specific *Or* that defines that particular neuron, while each OSNs of coeloconic sensilla expresses up to four IRs. Carbon dioxide (CO_2) sensory neurons are the exception in the sense that they express Gustatory receptors (*Gr21a* and *GR63a*) [10, 11]. Axonal termini of each specific class of OSN form a synaptic connection with a dendrite of a projection neuron (PN). PNs send their axons to higher order processing centers in the lateral horn and the mushroom body calyx [12].

Olfactory Receptors

Three research groups independently reported the expression of a family of genes encoding seven-transmembrane domain proteins (classified initially as G-Protein coupled receptors, GPCRs) in the olfactory sensilla of *Drosophila*. [13-16]. These 60 olfactory receptors genes are extremely divergent, with an average amino acid identity of approximately 20% [17].

Later studies have elucidated the receptor ligand map and expression pattern for individual Ors in the antenna [18-20]. Vosshall et al. showed that one of these receptors, *Or83b* (*Orco*) is expressed in all Or neurons examined, suggesting its role as a co-receptor [21]. Later functional studies demonstrated that *Orco* is in fact a necessary partner to other *Ors* in forming ion channels and allowing for their proper dendritic localization [22].

Drosophila expresses 62 olfactory receptors encoded by 60 genes [18]. Each receptor has seven transmembrane domains, reminiscent of GPCRs. However the

membrane topology of Ors are inverse of the topology found in GPCRs. Split-GFP experiments have shown that the N-terminus of Ors is located outside, while the C-terminus resides inside the cell [22]. Additionally, Ors also demonstrate weak homology to known GPCRs [22]. Heterologous expression studies provide evidence for Ors to function as ligand gated ion channels, independent of G-proteins. In contrast to these studies, several other studies suggest the involvement of G-protein signaling. Kain and colleagues [23] showed reduced odor responses in G(q) alpha mutant flies in a behavioral assay and Deng and colleagues analyzed the role of G-proteins in olfactory neurons using electrophysiological recordings and demonstrated that G-protein mutant flies show reduced responses to the tested odors [24].

In light of this controversy, Galizia and colleagues proposed a model where Ors activate two parallel pathways; ion-channel and GPCRs [25]. Both the ligand selective Or and the co-receptor (Orco) were shown to contribute to the cation channel activity [26-29]. These results add an intriguing component to the currently elusive picture of the insect olfactory transduction system.

Ionotropic Receptors

A large number of Ionotropic Receptors (IR) genes was recently discovered in many insect species and found to encode a second, arthropod chemoreceptor protein family [9, 30]. IRs are related to the ionotropic glutamate receptor (iGluRs) family [9, 30].In *Drosophila melanogaster*, there are 61 IR genes and, but only 14 of them are expressed

in the antennal coeloconic sensilla [30-32]. IR8a and IR25a are broadly-expressed and assumed to be common subunits in many different types of IR complexes.

Another gene family that plays an important functional role in olfaction is the Odorant Binding Proteins (OBP) gene family. They encode 52 water soluble proteins that bind and transport hydrophobic odorants through the lymph surrounding the dendrite in the olfactory sensilla. Loss of *OBPs* leads to abnormal olfactory behavior. For example, lush (a well-characterized OBP) is required for cis-Vaccenyl Acetate (cVA) sensing in males. *Lush* mutant males fail to start copulation with females [33, 34].

The Odor Code

Several studies have characterized the response profiles of OSN using in vivo and in vitro systems. Carlson et al. used an empty neuron system strategy in the ab3A neuron (Or22a/b expressing), which responds to ethyl butyrate (EtHb)[35]. They first created Or22a/b mutant flies which loose sensitivity to EtHb and do not respond to any other odors tested. These researchers have expressed and tested the odor profile by using electrophysiological recordings for 13 *Ors* in this system [20, 35]. There are other studies that use mutant strategies to de-orphanize *Ors* [19, 36-41]. Together, these studies give us a clear picture of the odorant-receptor-neuron map. The list of odorants associated with specific receptors has increased over time is continuously updated and has been made available to researchers in a public database, DoOR2.0 [42]

The ectopic expression studies have led to detailed response profile for ORNs. The receptor determines the odor response spectrum, the mode of response (excitation vs

inhibition), the termination kinetics and the level of spontaneous activity [35]. Exchanging receptors between OSNs will swap their odor response profile. Heterelogously expressed receptors will thus define the response properties for the neuron [20].

In general, each receptor binds to multiple odorants and each odorant is bound by multiple receptors. Odors are encoded by the combined activity of multiple ORN types. Increasing the concentration of odorant will increase the number of neurons that are firing [20, 43].

Mammalian and Insect Gustatory System

Animals initially use visual and olfactory cues to locate a potential food sources but they depend on their gustatory system to make a final decision whether or not to consume a food. Mammalian and insect taste systems share a number of similarities. These include similar taste modalities, neuronal organization and some physical structures (sensilla and mammalian taste pegs). However, there are also major differences, most prominently the distribution of taste sensory structures. In mammals, all taste cells are located within the mouth, mostly on tongue, while insects have taste sensilla distributed over the body, in addition to the main taste structure, the labial palps located at the tip of the proboscis. These additional locations include all the legs, the wings and possibly the ovipositor in females [44].

Taste Modalities

Mammals and insect share taste modalities. The mammalian taste systems generally can perceive five basic taste qualities: sweet, bitter, salt, sour, and umami [44].Based on behavioral and electrophysiological experiments, five established taste modalities are shared between *Drosophila melanogaster* and mammals. Flies can sense sweet chemicals, bitter chemicals and can differentiate between high salt and low salt concentrations. The latest reports suggest flies can also taste three additional taste modalities: umami, sour, and fatty acids. [45-49]. Fatty acid perception has recently been discovered in *Drosophila* and the molecular mechanism is still under investigation, with the potential of aiding our understanding of mammalian fatty acid perception. Insects also have an additional taste modality not found in mammals: water taste [50, 51]. They can detect the presence of water and show increased preference when thirsty. Additionally, insects (flies) and certain mammals can detect carbonation and pheromones through the taste system [52-54].

Mammalian Taste System

The taste bud or papillae is the basic taste structure on the tongue epithelium, the main mammalian taste organ. Taste buds sample foods and liquids for their palatability or toxicity. The taste bud consists of up to 100 clustered taste receptor cells (TRC). TRCs organize into four main types [55]. Type I cells are the most numerous in any given taste bud. Current research suggests that type I cells have a glia-like function and are involved in salty stimuli detection. However, more research is needed to determine the type I cells

role in salt perception [56]. Type II cells makes up one third of all cells in a taste bud. They mainly express GPCRs encoded by two different gene families, T1Rs or T2Rs and detect sweet, umami and bitter stimuli (see below) [57, 58]. Type III cells mainly express ion channels and detect sour stimuli [59], while immature type IV cells are located at the base of the taste bud and function as a precursor that differentiate into mature type I-III cells. Average life span of taste bud is about 10 days and type IV cells constantly help renew the dying mature taste cells [60, 61].

When a ligand binds to a receptor on the apical tips of the TRC, it activates a downstream signaling transduction cascades which end up with TRC depolarization. The depolarized TRC releases ATP as a signaling molecule to relay the information to the dendrites of the taste neurons. The primary taste neurons are projected to the nucleus of the solitary tract from which, information is relayed to the gustatory cortex and ultimately mediates sensory perception that drives either ingestion or aversion [62].

Sweet and umami are appetitive while sour and bitter are repulsive. Salt has a bimodal response profile; while low salt concentrations are appetitive, high salt concentrations are repulsive. Recently lipid-detection is debated as the sixth taste modality [63, 64] .

Mammalian Taste Receptors

Taste receptors include various classes of receptors and channels, i.e. GPCRs and ion channels. The main mammalian taste receptors are class C GPCRs. There are two main subfamilies: taste receptor type 1 (T1Rs) which detect attractive molecules and taste

receptor type 2 (T2Rs) which detect various bitter tastants. Taste receptor type 1 member 2 (T1R2) and T1R3) form a heterodimer that detects sweet molecules [65]. This sweet receptor is well studied and was first identified in a screen for genes with a high level of expression in the mouse taste buds [66]. The sugar receptor genes were located to a locus previously identified as Sac that showed a sugar insensitivity phenotype. When T1R2-T1R3 are heterelogously expressed in HEK-293 cells, they confer sensitivity to sucrose, fructose, artificial sweeteners and some sweet amino acids [58]. The T1R1-T1R3 heterodimer is tuned toward umami tastants (amino acids). Mice that have a genetic ablation in *T1R2* or *T1R3* loose sensitivity to sugars and artificial sweeteners. A third, related receptor, T1R1, mediates umami taste in a distinct set of type 2 taste cells. This receptor forms a heterodimer with T1R3 and genetic deletion of either gene leads to loss of sensitivity to amino acids, suggesting that these genes are necessary and sufficient for sweet and umami taste [65, 67].

Taste receptor type 2s (T2Rs) genes are more diverse and range in number from 4 to 39 , with about 25 genes found in the human genome [57]. *T2Rs* show a partially overlapping co-expression pattern with each other in bitter TRCs. There are two classes of *T2Rs* based on response profile: specialists and generalists. Specialist receptors bind to a one or few ligands with high affinity (i.e. *T2R3* recognizes only one compound) while generalist receptors can bind to multiple ligands (i.e. *T2R14* can recognize up to 33 compounds), with low affinity. Heterologous expression analysis suggests that some T2Rs have redundant functions.

One bitter compound can activate multiple receptors. For example, quinine can activate up to nine T2Rs with varying response levels, while acetaminophen can activate only one receptor [68]. T2Rs show a high level of polymorphism, which may provide an organism to evolve and recognize new chemical threats. In fact, mouse and human counterpart T2Rs show very different response profiles. In contrast to sweet and amino acid receptors T2Rs are generally considered to function as monomers; however recent reports suggest that they may also function as homodimers or heterodimers[68] .

Sour taste is generally aversive and is recognized differently than sweet, bitter and umami. Dietary acid lowers the pH of type III cells so it is detected internally rather than externally. The search for the channel responsible for transducing sour taste has long been a challenge in the field. Early reports suggested that epithelial sodium channels (ENaCs) or acid-sensing ion channels (ASICS) play a role in sour taste [57, 69] . However, knocking out these genes in mice does not completely abolish sour taste in mice. Recent reports suggest an important role for the PKD2L1-PKD1L3 complex in sour perceptions. When heterelogously expressed in HEK293 cells and native taste cells, it can reconstitute sour taste response [70]. However, type III cells in the PKD2L1 mutant mouse are still responsive to sour stimuli [69, 71], and single or double knock out mice showed only reduced, but still extant responses acids.

Salt taste is even less understood molecularly than sour taste. The cells that respond to salts as well as the transduction mechanisms are still poorly characterized. Current research supports two different modes of action; amiloride sensitive and amiloride insensitive channels play a role in salt sensing. When amiloride is given to the

mouse before a taste assay, the mouse loses sensitivity to low salt concentration while still maintaining repulsive responses for high salt. Moreover sensitivity to other salts are maintained [57, 62, 69, 72].

Drosophila Gustatory System

The taste system of *Drosophila* is widely distributed along the body. The basic taste structures are called sensilla or bristle. They are located on the labellum, leg, wing margins and ovipositor.

The labellum is the primary organ of the *Drosophila melanogaster* gustatory system. It is located at the tip of the proboscis (Figure 1) and consists of a pair of symmetrical epithelia known as labial palps. Each palp is covered with 33-34 chemosensory sensilla (Figure 1) [73].These sensilla are the main drivers for the initial determination of food choice [74]. In addition to the external chemosensory sensilla, the labellum also possesses less well-characterized sensory structures called taste pegs. The taste pegs are small protrusions lacking long taste hairs and are only accessible when the labellum is open. They also typically contain fewer neurons than the taste sensilla and are thought to play a role in the detection of carbonation [75]. Several internal sensory organs, the dorsal sense organ (DCSO),the ventral cibarial sensory organs (VCSO) and the labral sensory organ (LSO) line the pharynx (Figure 1) [44, 73, 76-78]. Several functional roles have been proposed for the GRNs in the internal sense organs. Upon sugar feeding or artificial activation, they help maintain continuation of feeding [79, 80]. Murata et al. suggested that activation of these GRNs triggers food searching behavior

[81]. Joseph et al. showed that a different subset of internal GRNs inhibits feeding and prevents overconsumption of sugars [82].

Labial palp sensilla are classified into three groups; long (L-type), intermediate (I-type), and short (S-type), based on the length of the sensilla (Figure 1) [83]. There are approximately 9 L-type sensilla, ~11 I-type and ~12 S-type sensilla for each labial palp [73, 76, 84]. L- and S-type sensilla house four GRNs while the I-type sensillum houses two [85]. In addition to GRNs, each taste sensillum contains a mechanosensory neuron and several support cells [73, 76, 84] .

The tarsal segment of the leg plays an important role in the taste system [86] as they make first contact with any potential food source. There are more taste sensilla on the dorsal side of the males' leg. Additional sensilla on male's foreleg play roles in pheromone detection and mating [52, 87-89].

The Drosophila wing margin harbors about 40 sensilla proposed to contain taste neurons. Recent studies suggest that these sensilla can sense sweet, bitter and lipopolysaccharides from bacteria and they are linked to self-grooming behavior [90, 91]. The female ovipositor has 13 putative taste sensilla. The functional role of these sensilla is unclear, but potentially they play a role in the selection of egg laying sites [92].

Taste Sensilla

These gustatory sensilla are tuned to recognize non-volatile substances upon touch. Structurally, a taste sensillum is composed of a socket, shaft and support cells. Each

sensillum houses one mechanosensory neuron (MSN) and 2 to 4 gustatory receptor neuron (GRN) based on the type of the taste sensilla. MSN are structurally different from GRN, the GRN extend dendrites into bristle to the sensilla tip, whereas the MSN dendrite extends only to the root of the shaft (figure 1) [2].

Gustatory Receptor Neurons

In the canonical taste bristle containing 5 neurons, 4 of which are GRN and electrophysiology recordings suggest that each GRN is tuned specifically for sensing sugar/sweet molecules, bitter/high salt, low salt and water. The neurons are tuned towards one taste modality and activation of the sugar or bitter neurons exogenously by an inducible activator shows behavior similar to neuron identity suggesting, neurons works as a "labeled line". [54]. In the leg some specialized sensilla also have neurons tuned for pheromone perception.

Taste modality specificities have not been as well characterized for the GRNs in the internal taste organs, the LSO, VCSO, and LCSO located in the pharynx. Expression analysis using GAL4 reporters suggests that some of these neurons are tuned to bitter and sugar perception. Recent studies suggest that these neurons play a role in regulation of feeding duration [79, 80] ,selection of egg laying site [93] or feeding inhibition to prevent overconsumption [82].

Gustatory Receptors

Bioinformatics analysis revealed a new family of putative chemosensory receptors in 2000 (Figure 2) [94]. Expression of the 18 newly identified genes discovered in the taste tissues. These genes were not expressed in the p*ox-neuro*^{70} mutant flies, which lack chemosensory neurons in the taste sensilla [94, 95]. Functional studies performed in these receptors were recognized as putative gustatory receptors [96]. *Drosophila melanogaster* gustatory receptor family has 60 genes yielding 68 proteins (Figure 2) [97]. Gr proteins contain 7 transmembrane domains and are related to *Drosophila Ors*. This seven-transmembrane topology at first, suggested that *Grs* are G-protein coupled receptor (GPCR). However, molecular genetic studies indicated that *Drosophila* Grs have an inverted topology distinct from GPCRs, with an internal N-terminus and an extracellular C-terminus [28, 98, 99]. The *Gr* family genes have now been characterized extensively. The average homology between individual members is not high but there are some sub-families with higher similarity [52, 74, 97, 100].

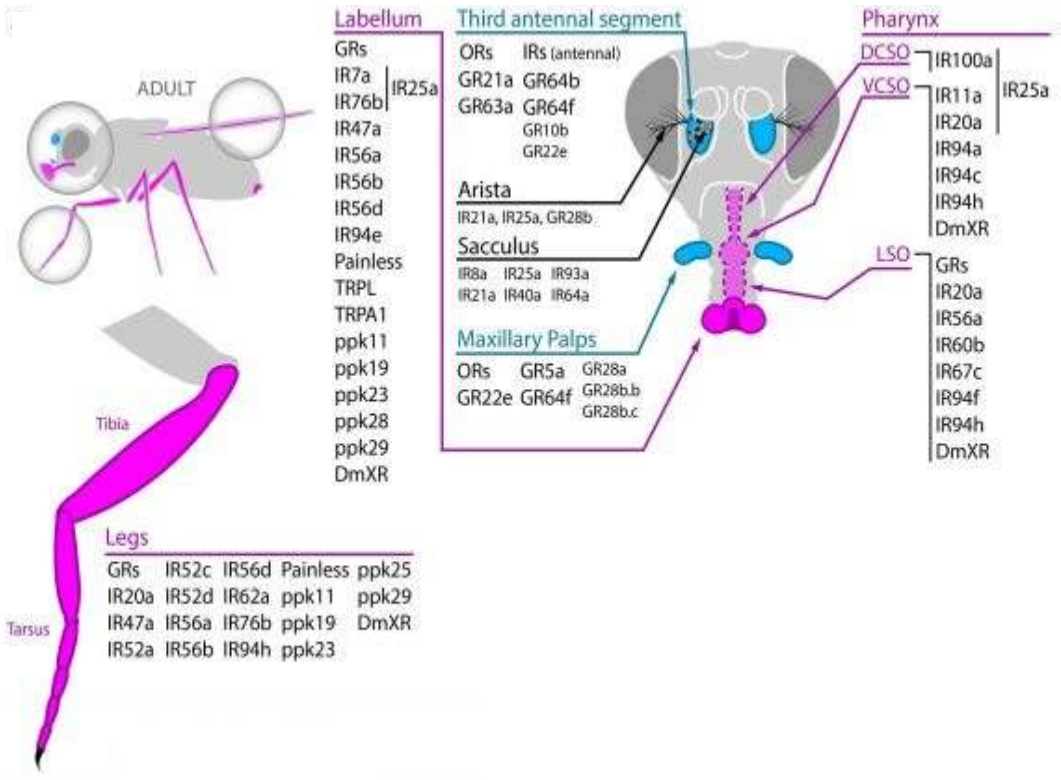

Figure 2 Receptor genes expressed in adult olfactory and gustatory organs.

Adult taste organs (magenta) are located on the proboscis's labellum, the tarsus and tibia of the leg, the anterior wing margin, the female genitalia (not shown), and in internal taste structures in the pharynx (DCSO, VCSO, and LSO). Within those taste organs, GRs, several members of the IR family, some members of the TRP family, ppk channels and the insect orphan receptor DmXR are present (Adapted from [101])

Two interesting members of the *Gr* family are *Gr21a* and *Gr63a*. Though these are named "gustatory" receptors with which they share sequence similarity, they are expressed only in the olfactory system in a subset of olfactory neurons that recognize CO_2. Jones et al. showed that deletion of either receptor gene eliminated CO_2 sensation,

suggesting they form a functional heterodimer. Kwon et al. exogenously expressed both receptors in *Or22a* neurons and, conferred CO_2 sensation [10, 11].

Several *Grs* have been shown to be required for pheromone sensing. Bray and Amrein showed that in males, *Gr68a* codes for a receptor recognizing a female pheromone and is required for proper mating behavior [52]. *Gr32a in* contrast is required in males to suppress the courting of other males and mated females, suggesting *Gr32a* recognizes a male-inhibitory pheromone. *Gr32a* was also found to be responsible for recognizing some bitter compounds [52, 53, 102, 103]. *Gr39a-d*, with have high similarity to *Gr32a* and *Gr68a,* have been shown to play a role in male courtship as well. Flies with mutated *Gr39a* or *Gr39a* knockdown with RNAi show reduced courtship levels toward females. Even tough, specific ligand for *Gr39a* has not been shown, it is likely that they play role in male courtship preference and pheromone sensing [104].

Bitter Gustatory Receptors

Based on bioinformatics and cellular expression analysis, the majority of *Gr* genes are expressed in bitter/high salt GRNs and thought to encode bitter receptors. These *Grs* are characterized by comparably little amino acid conservation between them, with the exception of members of small subfamilies, arisen through recent gene duplication events [105]. Sequence diversity among bitter *Grs* is consistent with the notion that bitter tasting compounds represent a vast array of diverse chemical structures [5, 106, 107]. *Gr33a* and *Gr66a* are expressed in most bitter GRNs and considered to be bitter neuron marker for these cells. Deletion of one or both affects sensitivity to several bitter

compounds but does not completely abolish bitter responses [5, 54, 106, 108]. *Gr33a*, *Gr66a*, and *Gr98a* are responsible for sensing various bitter molecules, and deletion of any one results in a decreased sensitivity for caffeine, and denatonium sensation. Earlier reports suggested that heterelogously expressing single or pairwise combinations of bitter receptor genes in the sugar GRN does not reconstitute bitter responses, suggesting that more than two Grs are necessary to form a functional receptor complex [5, 106].

Shim et al. showed that GR33a, GR66a and GR98b are required for L-Canavanine perception in bitter neurons. They also proposed that three bitter-sensing Grs are enough to recapitulate L-Canavanine sensing in sweet GRNs [109]. Lee et al claimed that three bitter receptors, Gr33a, Gr66a and Gr93a, are required and sufficient for coumarin sensing, based on recovery of responses in sweet GRNs when ectopically expressed. However, ectopic expression of three Grs did not rescue the response to the level of control neurons expressing these receptors endogenously. Their finding suggests that at least one more Gr is required for high level coumarin sensing [110].

Sugar Grs genes

The first functionally characterized *Gr* gene was *Gr5a*, identified initially as an X-linked Mutation (*tre*) that reduced sensitivity to trehalose [111, 112]. It was later associated with a point mutation in the coding sequence of *Gr5a* [113]. A clade of Gr genes most similar to *Gr5a* are predicted to be sugar receptors. Six members of this clade are tightly located on one locus; *Gr64*. Slone et al. showed that *Gr64* locus mutant flies are not

responsive to any sugar tested except for fructose, suggesting Gr64 family members play an important role in sensing a battery of sugars [74].

Earlier studies showed that *Gr5a* defective flies have reduced responses to trehalose but not to sucrose [113-115]. Further studies into sugar clade genes revealed functional roles for *Gr64a-f* and *Gr61a* in sugar perception [74, 97, 108]. Dahanukar et al (2007) first analyzed the expression of *Gr5a*, *Gr61a*, and *Gr64f* and concluded that they show a partially overlapping expression pattern in the labellum. They later investigated *Gr5a* and *Gr64a* and double mutant flies in cellular and behavioral assays. ΔGr5a flies showed reduced action potentials to trehalose, glucose, melezitose but not to other ten sugars tested in labellar GRN in L type sensilla. ΔGr64a showed reduced responses to panel of ten sugars tested. Interestingly, glucose, melezitose, and trehalose response was not affected. When they analyzed double mutant flies, they found no responses for any of the sugars they tested, and they concluded that *Gr5a* and *Gr64a* are the main sugar receptors. The behavioral responses to individual mutants were more restricted. Responses to a couple of sugars were reduced , i.e. ΔGr5a flies showed reduced response to glucose, melezitose, and methyl-a-D-glucopyranoside , ΔGr64a flies showed reduced response to stachyose and maltotriose. However, responses to most other sugars were not affected. The double mutant flies showed reduced responses to fructose, but response to other sugars were not tested. Authors also analyzed Gr61a mutant flies in cellular assay. They have observed that responses to most sugars were not affected and concluded that Gr61a plays no major role in sugar taste.

There are several oversights in the above paper. *Gr64a* mutations show defects in responses to many sugars including fructose, stachyose, maltotriose, maltose and sucrose. It is important to point out that the *Gr64a* mutant they used in this research deleted the upstream regulatory DNA of the locus, as well as the coding sequences of *Gr64a* and part of *Gr64b* gene. Considering how tightly this gene family of six genes are clustered (~ 200 bp between genes), it is possible or even likely that this ΔGr64a mutation also affected the expression of downstream genes (Figure 3). We later reported that Gr64a is not expressed in the labellum [116]. This strongly supports the idea that the observed responses were not due to Gr64a but due to reduction in expression of downstream Gr64 genes.

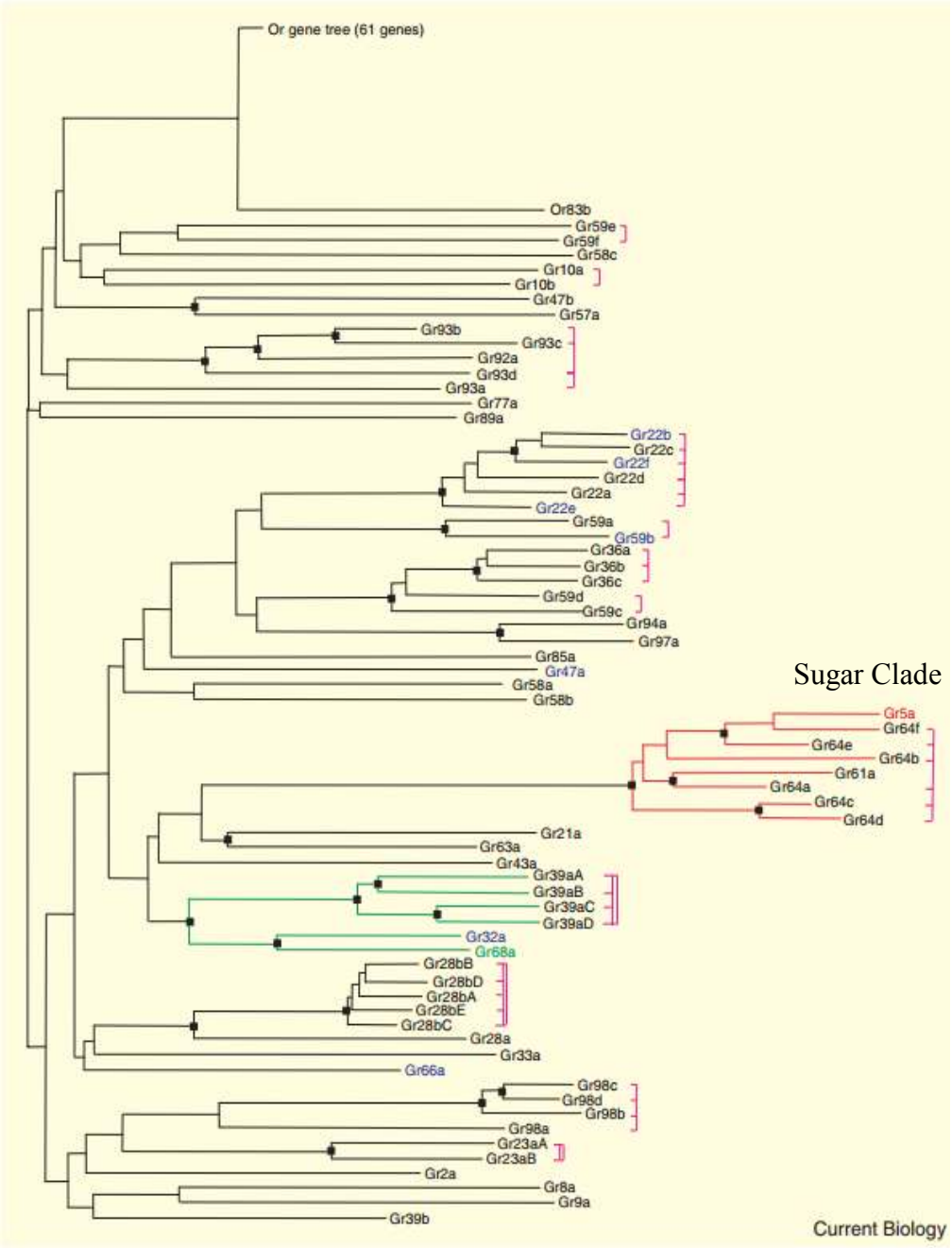

Figure 3 Tree of the insect gustatory family

The *Gr* genes are related to the *Or* genes. Genes that are clustered share significantly more similarity. Clustered genes are indicated by brackets. The sugar receptor gene family is shown in red. Pheromone receptor family is shown in green Black boxes indicate branches with 75–100% bootstrap support (Figure adapted from [44].)

Montell's group tried to identify functional roles of sugar Grs in a different way. His group used an mRNA tagging approach. They expressed FLAG tagged poly(A)-binding protein (FLAG-PABP) in sweet GRNs under the control of Gr5a-GAL4 and performed a pull-down of FLAG-PABP with anti-FLAG antibody, followed by RT-PCR to detect the presence of sugar Gr mRNAs . They reported that all 7 putative sugar Grs genes are expressed in $Gr5a^{GAL4}$ expressing neurons. They also created a Gr64 mutation by mobilizing a P-element which was inserted upstream of Gr64a, creating a deletion removing upstream regulatory DNA, all of Gr64a and half of Gr64b ($Gr64^{ab}$). They then analyzed sugar responses of this strain by electrophysiology and two-choice food preference assay and found that $Gr64^{ab}$ mutant flies shifted their preference from higher concentration of sucrose, glucose, maltose, trehalose, and arabinose to 2mM, fructose suggesting that Gr64a and Gr64f were required for this preference. They attempted to rescue this shifted preference behavior by either expressing UAS-Gr64a or UAS-Gr64b in sweet GRNs under the control of Gr5a-GAL4, and found rescue for the preference for sucrose, glucose, maltose and arabinose but not trehalose. Even combining Gr64a and Gr64b expression did not reconstitute trehalose preference. Similarly, tip recordings of $Gr64^{ab}$ flies showed that flies lost sensitivity to sucrose, glucose, maltose and trehalose, and providing expression of Gr64a through Gr5a-GAL4 only rescued sucrose to comparable levels to the control, while responses to glucose and maltose response were partially rescued and responses to trehalose were still lost [117]. This is consistent with the complex organization of the *Gr64* locus, as the deletions generated by the Montell

group, which is similar to that of the Carlson group, might have affected expression of downstream genes (see above).

Later, Montell and colleagues showed that *Gr64f* is required for behavioral and cellular responses to trehalose, and several other sugars.[118].

The observation that *Gr64f* is express in all sugar GRN and rescuing Gr64f expression in $Gr64^{ab}$ background rescues most sugar response led authors to proposed a model where Gr64f is the co-receptor for sugar perception.

Gr64a-f genes are assumed to play a role in sugar perception based on their similarity to Gr5a. The polygenic nature of these genes has been a problem in the field for studying their individual roles. The intergenic distance is on average less than 180bp. Definitive evidence came from Slone's work [74]. Authors created a deletion in the *Gr64a-f* locus, Δ*GR64*, which led to *flies* demonstrating a reduced PER to all sugars tested. Responses toward most tested sugars reduced to less than 20% PER. However, fructose and sucrose response levels were 50% and 30 % when tested with high concentration of sugars, respectively. Glucose and arabinose responses were also observed, albeit at a low level. These responses can be attributed to intact *Gr43a*, *Gr5a* and *Gr61a* in Δ*GR64 flies*. To conclusively prove the involvement of the *Gr64* family in sugar perception, Slone et al. used a genomic rescue in Δ*GR64* flies. The expression of UAS-Gr64abcd_GFP_f in sweet GRNs with a Gr5a-GAL4 driver was enough to rescue sugar perception to wild type level. The *Gr64e* gene was swapped with a GFP sequence in the UAS-Gr64abcd_GFP_f construct, so rescue flies are missing *Gr64e* and possibly

Gr64f. The expression of *Gr64a-d* along with *Gr43a*, *Gr5a* and *Gr61a* is enough to rescue sugar perception to wild type levels in Δ*GR64 flies*.

A robust technique to study neuronal responses by calcium imaging in the tarsal GRNs was developed by Miyamoto et al [119]. Dose-response profiles of denatonium and sucrose showed concentration-dependent Ca^{2+} responses of bitter and sweet GRNs, respectively. The authors further tested cellular responses of Gr33a-GAL4 and Gr61a-GAL4 neurons with a panel of sweet, bitters and, salt molecules, and showed that bitter GRNs and sweet GRNs respond to their expected ligands exclusively. Bitter GRNS in *GR33a mutant flies, GR33a*^{GAL4}, showed reduced responses to quinine, denatonium and lobeline. *Gr61* deletion flies, ΔGr61a, showed reduced response to glucose. Rescuing expression of *GR61a* in these neurons rescued reduced glucose response. Surprisingly, *Gr61a* rescue flies showed an enhanced response to sucrose. Mechanosensory and chemosensory sensilla shows structural differences; the former is straight while the latter is curved. Even though 5v-sensillum on the fifth tarsal segment is morphologically similar to mechanosensory sensilla, Miyamoto et al. showed functional evidence of the chemosensory nature of this sensillum. Another striking observation reported by the authors is the varied cellular response patterns of the sweet GRNs from three different sensilla in the 5th tarsal segment [119].

Even though Slone et al. provided evidence for involvement of the *Gr64* family in sugar perception; individual roles and expression pattern of these genes were unclear. In our Fujii et al. paper, we used a recently improved technique to create knock-in alleles [116]. Our knock-in flies had the coding region of individual *Gr64* family genes

swapped with GAL4/LexA. These flies provided us an opportunity to analyze the expression pattern and behavioral requirement of sugar *Grs*. We showed that sugar Gr genes are expressed in a combinatorial manner in eight distinct sets of sweet GRNs. Behavioral investigations show that individual sugar *Gr* mutations affect taste responses to a small number of sugars [116].

Gr43a is expressed in the leg, internal sense organs, proboscis and brain and functions as a fructose receptor. Miyamoto et al. also reported that the brain *Gr43a* neurons functions as internal nutrient sensor [120].Mishra et al. showed that *Gr43a* is also expressed in the larval internal taste organ and function as the main larval sugar receptor [121].

Sugar Grs in Other Insects

The *Drosophila* sister species with published genomes have similar numbers of sugar *Gr* genes as *D. melanogaster*. There are six sugar *Gr* genes in *D.pseudoobscua/permilis* with *Gr5a* missing and *Gr64e* as a pseudogene. *D. grimshawi* also has 6 sugar *Gr* genes with *Gr5a* and *Gr64a* pseudogenized. *D. virilis /mojovensis* have 7 sugar *Gr* genes with *Gr64d* missing. Even more distantly related diptera, such as the mosquitoes *Anopheles gambiae* and *Aedes aegypti* have 8 and 7 sugar *Gr* genes, respectively, while *Culex pipiens* has 14, including one possible pseudogene [122]. However, outside diptera, the number of *Gr* genes varies substantially. The red beetle *Tribolium castaneum* has 16 sugar *Gr* genes. There seems to be correlation between total number of Grs and number of sugar clade genes (figure 4). In contrast, eusocial insect species have low numbers of

sugar *Gr* genes. Both *Apis mellifera* and the wasp *Nosonia vitripennis*, as well as the ant species have *Pogonomyrmex barbatus* have only two predicted sugar Grs have only two sugar *Gr* genes. Surprisingly, no sugar *Gr* orthologs were found in human louse and locust [123, 124]. Finally, *Bombyx mori* has five sugar *Gr* genes.

Well conserved *Gr43a* is also present in this species. A recent report showed that similar to *Drosophila Gr43a* ortholog *BmGr9* is expressed in the brain. Surprisingly, a sugar clade gene *BmGr6* is also present in this brain neuron suggesting non-canonical roles for these genes.

A limited number of functional studies have been carried out on the function of sugar *Gr* genes of some insect species. The general approach taken is to look at the expression pattern in chemosensory tissues for bioinformatically predicted genes. Relevant tissues are dissected and RNA is extracted followed with RT-PCR to detect expression of genes. In addition, several heterologous expression studies have been done with the *Bombyx mori* sugar *Gr* genes [125-127]. Recently, Jung et al. expressed the bee sugar receptors AmGr1 and AmGr2 in *Xenopus* oocyte, showing AmGr1 by itself can recognize several sugars while AmGr2 individually cannot recognize any of the sugars tested. Lastly, expressing *AmGr2* along with *AmGR1* altered the specificity of the response profiles observed for the single A*mGr* genes [128].

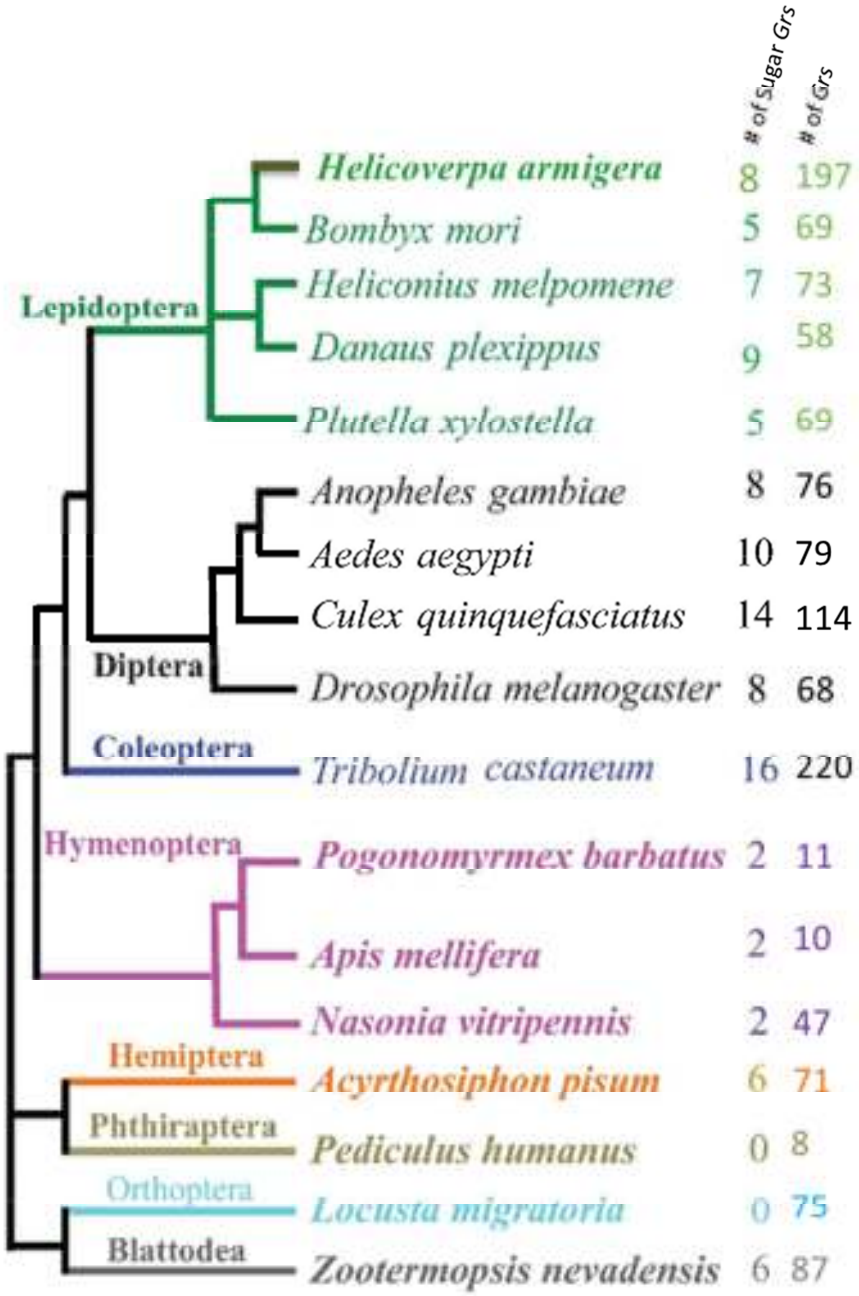

Figure 4 Number of GRs and predicted sugar GRs from various insect species. (Adapted from [129] .)

Non-Canonical Functions of Grs

Gustatory receptors are functionally diverse and the majorities of them are expressed and function in the gustatory system. However, there are some exceptions. As mentioned previously, *Gr21a* and *Gr63a* function as CO_2 receptors in olfactory system [11]. *Gr43a* and *Gr64a* are expressed in brain neurons and function as internal taste sensors [116, 120]. Many members of the *Gr28* subfamily are expressed in various non chemosensory organs of both the larvae and adult flies [130]. *Gr28B(b)* is described to function as UV light sensor in the larvae [131] and surprisingly *Gr28B(d)*, expressed in the aristae functions as a heat sensor [130, 132, 133]. Finally, a large number of *Gr* genes is expressed in the GI tract, but here, the respective receptors are likely to have a chemosensory function to monitor ingested food chemicals [134, 135].

Other Gene Families in the Gustatory System

Ionotropic Receptor Family

The Ionotropic receptor (IR) family consists of about 60 members, some of which have recently been shown to have chemosensory function [89, 136-138]. Some members are expressed in the antenna and recognize volatile molecules, such as ammonia and amines [9, 139]. However, other *IR* genes are expressed in gustatory system [9, 89]. Zhang et al. showed that *Ir76b* is required for low salt preference, with Ir76b mutant flies showing a severe defect in low-salt attraction, while high salt aversion remained unaltered. These investigators further analyzed GRN responses in the labellum using electrophysiological

recording from the L4 and L6 sensilla, showing that, *Ir76b* mutant neurons, compared to those of *wild type* neurons, had a reduced firing rate upon stimulation with 50mM NaCl, while that to 500 mM NaCl was not affected. Koh et al. identified 16 *IR* genes from the *IR20a* clade being expressed in gustatory neurons, and several of these were co-expressed with either *Gr5a* or *Gr66a*. However, *Ir52a* and *Ir52b* were expressed in non-sweet and non-bitter GRNs in a sex-specific manner. Interestingly, males with mutations in these genes showed delayed copulation, suggesting a possible role as male pheromone receptors [89].

Another gustatory role for *IR* genes was proposed for the detection of polyamines. These molecules are released by dead and decaying organisms including fruits. Polyamine levels in the media affect female's choice for oviposition. In the laboratory, wild type females will not lay eggs on agarose media adulterated with polyamines. To identify the chemosensory organ responsible for this choice behavior, Hussain et al. tested flies with their antenna, wings, front legs, hind legs or labellum removed. Only flies with their labellum removed fail to avoid polyamines in a two choice oviposition assay. The authors next screened for chemosensory genes and neurons responsible for this avoidance using RNAi knockdown and neuronal silencing with and Kir2.1. They found that *Ir76b* and bitter neurons were required for this oviposition preference [137]. Lastly, other, non-chemosensory roles have been suggested for some *IR* genes. *Ir21a*, *Ir25a*, *Ir93a*, and *Ir40a* have been shown to play an important role in thermosensation and hygrosensation in the fly antenna [140, 141].

The Pickpocket Family

Members of other genes families, such as the pickpocket (*ppk*) genes were shown to be expressed in the gustatory organs.. Liu et al. claimed that *ppk11* and *ppk19* are the salt receptors in the larvae. Based on expression data and limited functional evidence they also proposed that these genes function as salt receptors in adults [142]. However, recent studies convincingly repudiated this claim [136, 143]. Cameron et al. and Wang et al. independently showed that pp*k*28 is responsible for sensing water and osmolality [50, 51]. Finally, other studies suggested that ppk family members function as pheromone receptors (i.e.*ppk23*, ppk25 ,and ppk29)[88, 143-147].

TRP channel family

Finally, a few members of the transient receptor potential (TRP) channel family are expressed in some taste organs. Anzi et al. showed that *TrpA* is expressed in bitter GRNs and mediate avoidance behavior to isothiocyanate and aristolochic acid [148] [149]. Soldano et al showed that *TRPA* in the *Gr66a* expressing neurons is necessary and sufficient for lipopolysaccharide avoidance [150]. And Zhang et al showed that *TRPL* is required for an aversive response to camphor. When *TRPL* was misexpressed in the *Gr5a* neurons, they become responsive to camphor but not to other bitter molecules. [151].

Projections to CNS

Labellar GRNs are bundled together in the labial nerve and project to sub-esopheageal zone (SEZ). The SEZ is the primary taste center of the fly brain. Internal taste organs also project to the SEZ through the pharyngeal/accessory pharyngeal nerve (Figure 5). The majority of leg GRNs project to the ventral nerve cord. According to their tarsal location (fore-, mid- or hindleg), they terminate in the pro-, meso- and metathoraric ganglia, respectively (Figure 5C). Surprisingly, some leg GRNs directly project to SEZ. Thoma et al. reported that Gr43a+ 5s sensilla sugar neurons directly project to this region (Thoma, Knapek et al. 2016) . Carlson recently described the projection map of GRNs from all organs. Axons from the same organ project to similar regions, regardless of GRN type. However their projection layer is different. For example, bitter or sugar GRNs from internal sense organs project to anterior SOG (Kwon, 2014). However, labelar sweet GRN projections are more sagital (Figure 5).

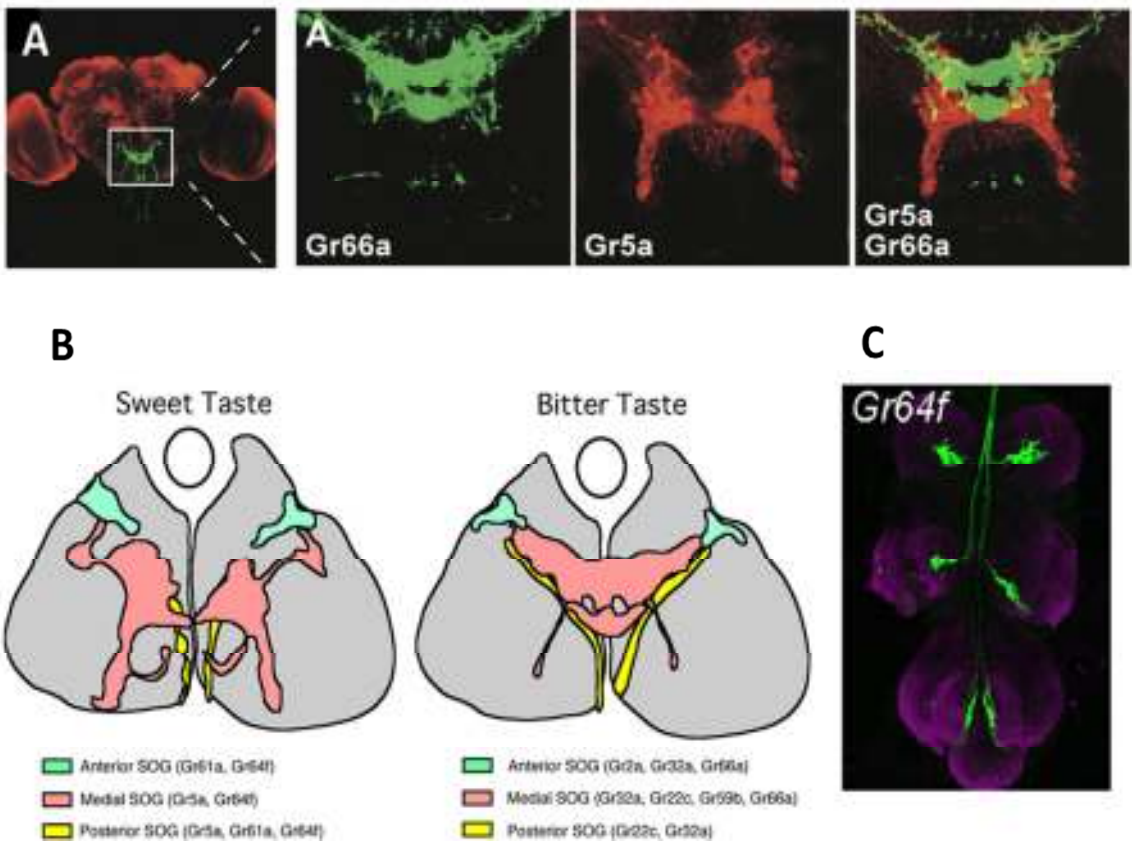

Figure 5 Projection patterns in the SOG and thoracico-abdominal ganglia. (Adapted from [86, 152, 153].)

Higher Order Gustatory Neurons

Second order gustatory neurons have not been characterized in detail. However, recent studies from several laboratories identified candidate second-order neurons, but each group reported a different set and have not shown a complete set that are sufficient and necessary for transmission of each taste modality more effort needed here to resolve the issue [154-157]

Drosophila as a Model Organism

Drosophila has been an important model system for genetic studies since Thomas Morgan used flies to identity chromosomes as the basis of heredity over a hundred years ago. Studies conducted on *Drosophila* have advanced our understanding in several fields including development, sex determination and dosage compensation, immunology, neurogenetics and many aspects of behavior. Benzer's screen for mutations that led to behavioral defects set the basis for the discovery of many genes that control, circadian rhythms, memory and courtship [158-162], many of which have ortologs in mammals. Additional advantages include short life cycle, ease of maintenance, large number progeny/female, and availability of many molecular genetic tools. Also, *Drosophila* displays many complex behavioral patterns, yet is has a relatively simple nervous system. Lastly, 40% of the genes have orthologs in humans' [163], and therefore, the discoveries made in *Drosophila* can be relevant to mammals .

The Binary Expression Systems (GAL4/UAS and LexA/LexAop)

One of the most powerful tools used in *Drosophila* is the GAL4/UAS binary expression system. [164](see figure below) This system has been complemented recently with similar bi-partite expression systems, such as LexA/LexAop and QF/QUAS . Their application allows detailed analysis of many genes with regard to cell type, co-expression, but they also allows the functional characterization of virtually any set of cells or neurons for which specific drivers are available (Figure 6A). Generally, the endogenous promoter of a gene of interest fused to the coding sequence of a

transcription factor (GAL4, LEX, QF), and flies containing such a driver is then crossed to a fly carrying a corresponding reporter gene (UAS-x, LEXop-y, QUAS-z), whereby x, y and z can simply be a visible marker (GFP, LacZ), a coding sequence of a *Drosophila* gene, a Ca^{2+} sensor, a toxin, or any other type of functional protein (Figure 6B) [165-167].

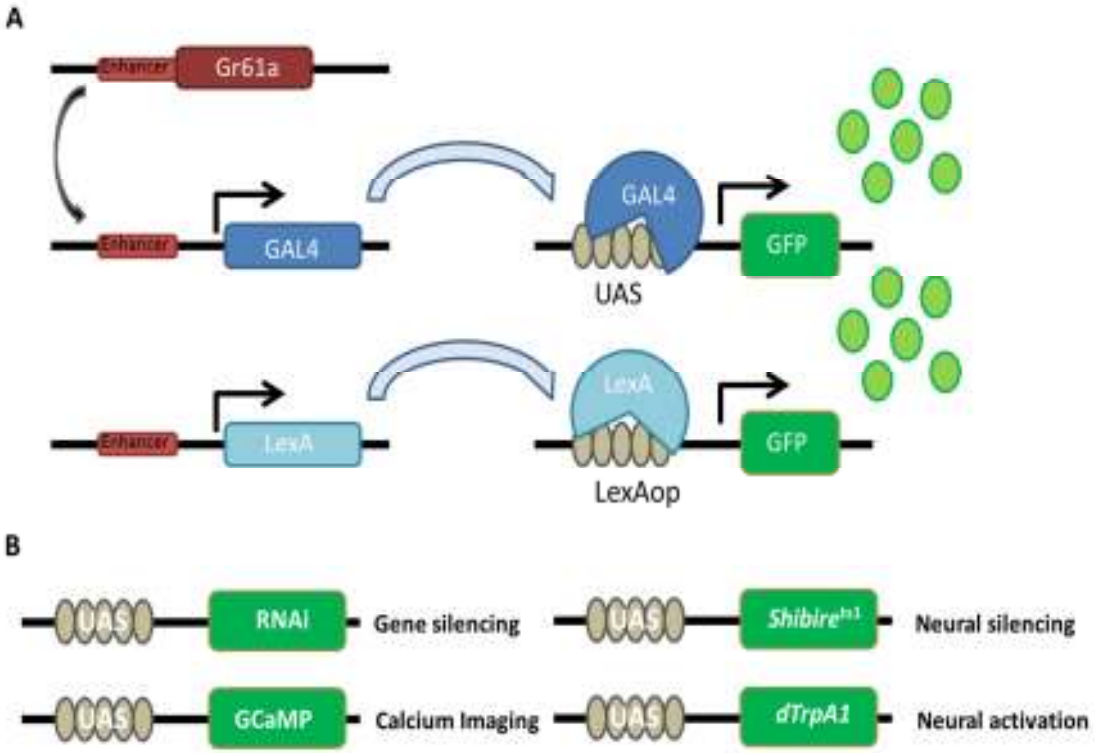

Figure 6 Binary Expression systems in Drosophila [166]

CHAPTER II

DROSOPHILA SUGAR RECEPTORS: HOW FLIES PERCEIVE SWEET TASTE, REVISITED*

Introduction

Identification of and discrimination between nutritious and non-nutritious chemicals is a critical task during feeding. In most mammals, including humans, receptors for nutritionally valuable compounds, such as sugars and amino acids, are detected by single heterodimeric receptors, T1R2/T1R3 and T1R1/T1R3, respectively [65], while non-nutritious, often harmful and toxic compounds are recognized by bitter taste receptors encoded by about 30 *T2R* genes [168-170]. T1R2/T1R3, T1R1/T1R3 and T2Rs are expressed in distinct sets of taste cells located in the lingual and other orosensory epithelia, thereby providing a basis for sweet, umami and bitter taste perception [171].

Anatomically, arthropod taste systems differ significantly from those of mammals [44, 172-174]. In *Drosophila*, taste sensilla are considered the functional equivalents of mammalian taste buds and constitute the sensory structures for the detection of soluble chemicals. Taste sensilla cover not only the labial palps (i.e. the fly "tongue") at the tip of the proboscis, but reside also on all legs (tarsi), especially the

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*Parts of this chapter are reprinted with permission from Fujii, S., A. Yavuz, et al. (2015). "Drosophila sugar receptors in sweet taste perception, olfaction, and internal nutrient sensing." Curr Biol **25**(5): 621-627.

most distal 4th and 5th segments of the tibia [2]. Numerous taste sensilla- like bristles are also located on the anterior edge of the wing [2, 175], albeit a function for these in chemosensation has yet to be established. Taste sensilla are composed of a single mechanosensory neuron and four (or in some cases two) chemosensory neurons [2]. Yet, similar to individual taste cells in mammals, each of the chemosensory neuron in a sensilla (referred to as gustatory receptor neuron, or GRN) is thought to be tuned to a specific group of taste stimuli, such as sugars, bitter-tasting chemicals/high salt, low salt solutions and water, respectively [172]. Two main classes of taste stimuli, sugar and bitter compounds, are recognized by members of the gustatory receptor (Gr) protein family. In *Drosophila melanogaster*, this family is encoded by sixty-eight genes, which fall into distinct subfamilies. While mutations for a few *Gr* genes have been characterized, a generic role in bitter or sugar sensing is generally inferred from their expression profiles. Specifically, many *Gr* genes are expressed in subsets GRNs that are activated by bitter chemicals [108, 153], and three of them, *Gr66a, Gr33a* and *Gr93a* are necessary for detecting caffeine and quinine [5, 102, 106]. Likewise, members of another small family of putative *sugar Gr* genes - *Gr5a*, *Gr61a*, *Gr64a-f* - are expressed in GRNs responding to many different sugars [108, 153, 176]. The first characterized member of this group, *Gr5a*, encodes a receptor required for detection of trehalose [111, 113-115, 177], and flies lacking the entire *Gr64* locus fail to respond to all sugars, except fructose and sucrose, both in behavioral and electrophysiological assays [74, 118]. Interestingly, a few *Gr* genes have been associated with roles not related to taste perception. *Gr68a* and *Gr32a*, while expressed in taste neurons, appear to sense non-

volatile pheromones, as they are necessary to promote and suppress male courtship behavior, respectively [52, 178-180]. *Gr21a* [96] and *Gr63a* are expressed in the olfactory system where they are necessary to sense CO_2 [10, 11], and *Gr43a,* which, in addition to the taste system, is expressed in the brain, functions as an internal sensor for hemolymph fructose [120]. Lastly, members of the *Gr28* gene family are expressed in many sensory and brain neurons [130], and have been implicated in light avoidance sensing in larvae and temperature sensing in flies [131, 132].

Despite a general agreement that the sugar *Gr* subfamily collectively encodes sweet taste receptors, the molecular and cellular underpinnings of how these proteins mediate recognition of diverse nutritious and non-nutritious sugar chemicals have remained elusive. Based on electrophysiological and behavioral analyses of *Gr5a* and *Gr64* mutant flies, two groups proposed that behavioral and cellular sugar responses mediated by the labellum is largely or exclusively dependent on *Gr5a* and *Gr64a* alone [117, 176]. However, given the complexity of the *Gr64* locus and the nature of the deletions used in these studies, it is as likely that the severe phenotype reported by these investigators are the consequence on gene expression of *Gr64* genes not structurally affected by these mutations (i.e. *Gr64c*, *Gr64d*, *Gr64e* and *Gr64f*).

Gr5a, *Gr61a* and *Gr64f* were established as early markers for sweet taste neurons [74, 176]. More recent, extensive expression studies using a complete collection of *Gr-GAL4* transgenes identified two additional *Gr64* genes expressed in the labellum and legs (*Gr64c* and *Gr64e*) [107, 181]. Lack of expression was reported for *Gr64a-GAL4*, along with *Gr64b-GAL4* and *Gr64d-GAL4*, in both labellum and tarsi, albeit spliced

transcripts for these genes are detected by RT-PCR [74, 117, 176]. Most likely, the difficulty for obtaining reliable expression data for these GAL4 drivers originates in the structural organization of the *Gr64* locus: the six genes are separated by only ~ 200 base pairs, but encompass more than 12 kilobases (Figure 7). Thus, regulatory DNA elements essential for appropriate expression are likely scattered both far up- and downstream of the locus and therefore might be absent in these *Gr64-GAL4* drivers. Further complicating the analysis of the *Gr64* genes is the observation that at least some are expressed as bi- or multicistronic transcripts [74, 176].

Here, we present a comprehensive expression analysis of the sugar *Gr* genes using a GAL4/LEXA gene knock-in strategy that successfully targeted five of the six *Gr64* genes. We observe broad, sweet neuron specific expression only for *Gr64b*, *Gr64c* and *Gr64f*, while *Gr61a*, *Gr64a* and *Gr64e* are expressed in different sub populations of sweet GRNs, and *Gr5a* is not specific for sweet neurons. Notably absent is expression of *Gr64a* in the labellum. The compartmentalization of *Gr* gene expression generates at least eight distinct types of sweet neurons in taste sensilla of the main taste organs. Behavioral analysis of single sugar *Gr* knock-in alleles revealed that each mutant reduces taste sensitivity to a small subset of sugars, suggesting that all sugar *Gr* genes contribute to the detection of nutritious carbohydrates. These observations are not compatible with the propagated view of a few *Gr* genes being essential and sufficient for sensing all sugars, but they favor a model in which all sugar *Gr* genes contribute to sweet taste.

Lastly, we also observe non-canonical sugar *Gr* expression. The $Gr5a^{LEXA}$, $Gr64b^{LEXA}$ and $Gr64f^{LEXA}$ alleles are all expressed in olfactory sensory neurons, and $Gr64a^{GAL4}$ is expressed in the brain, suggesting non-gustatory functions for several sugar receptors.

Results

Expression of Gr5a and Gr64a–Gr64f Knock-in alleles

In contrast to most other *gustatory receptor (Gr)* genes, the *GAL4/UAS* expression system has failed to report expression for several *Gr64* genes [74, 107, 176, 182], presumably due to the distribution of regulatory elements within and up and downstream of this large locus (Figure 7A). Therefore, we generated a tool set for both expression and functional analyses by engineering gene-targeting constructs for all six *Gr64* genes and *Gr5a*. Successful homologous recombination [183, 184], followed by flippase recognition target (FRT)-mediated excision of the white maker gene, produced six new Gr alleles—$Gr5a^{LEXA}$, $Gr64a^{GAL4}$, $Gr64b^{LEXA}$, $Gr64c^{LEXA}$, $Gr64e^{LEXA}$, and $Gr64f^{LEXA}$—in which the Gr coding sequences were replaced with that of *LEXA::VP16* (referred to as *LEXA*) or *GAL4* (Figure 7A). Although we cannot rule out that removal of small introns present in the Gr coding sequences leads to incomplete reporting of endogenous gene expression, this strategy is the most likely one to reveal accurate *Gr64* gene activity.

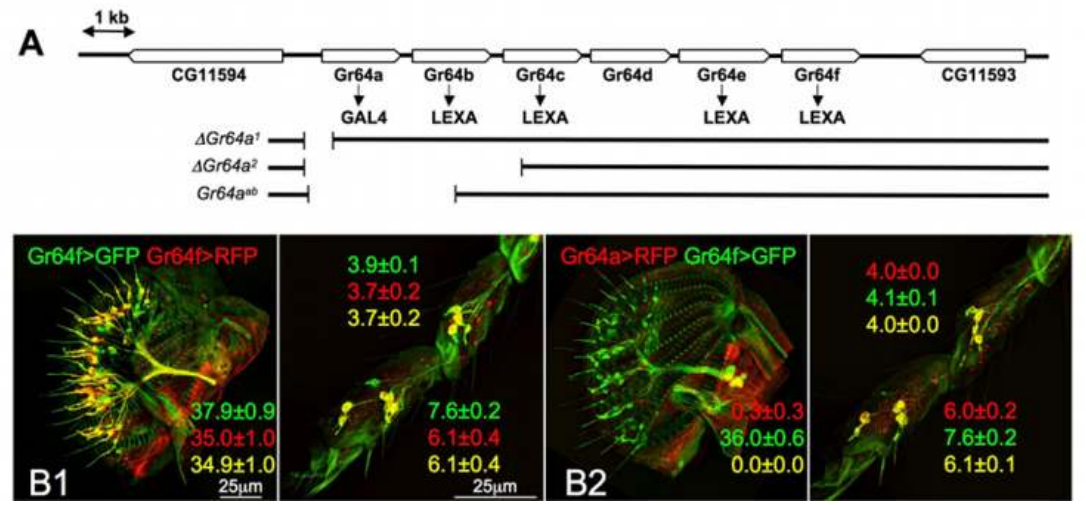

Figure 7 Expression of Sugar Gr$^{LEXA/GAL4}$ Alleles and Gr61-GAL4 in Labial Palps and Tarsi

Organization of the Gr64 locus and replacement of individual coding sequences by GAL4 and LEXA. Approximate extent of deletionsused in [117, 176]is shown below.(B1–B8) Double stainings of each knockin allele with Gr64f-GAL4 or Gr64f^{LEXA} are shown for the labial palps (left) and fourth and fifth tarsal segments of the foreleg (right). One reporter-gene copy (UAS-RFP and lexAop-GFP) was present in all genotypes, except for (B5), where two copies were present due to weaker expression of the Gr64e^{LEXA} allele. The drivers (one copy or allele) were Gr64f^{LEXA} and Gr64f-GAL4 (B1),Gr64f^{LEXA} and Gr64aGAL4 (B2), Gr64b^{LEXA} and Gr64f-Gal4 (B3), Gr64c^{LEXA} and Gr64f-Gal4 (B4),Gr64e^{LEXA} and Gr64f-Gal4 (B5), Gr5a^{LEXA} and Gr64f-Gal4 (B6), and Gr64f^{LEXA} and Gr61a-Gal4 (B7). Numbers in green (GFP), red (RFP) and yellow (merged) indicate averaged positive number (6SEM) of cells expressing lexAop-GFP and UAS-RFP reporters, as counted in respective channels. (B8) (enlargement of square in B6)shows multiple Gr5a^{LEXA}-expressing neurons (arrowheads) in a single sensillum. 6 % n % 10, except for (B2), where n = 3.

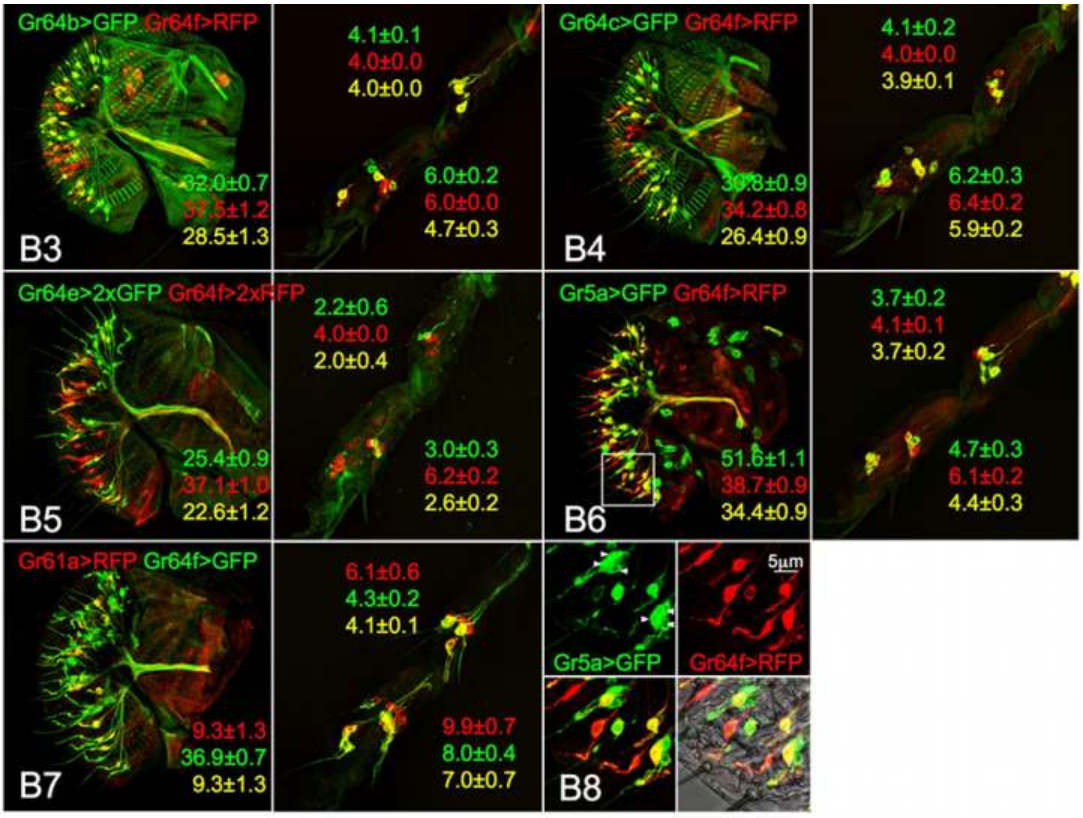

Figure 7 Continued

Expression Profiles of Gr64$^{LEXA/GAL4}$ Alleles Define Eight Distinct Subsets of Sweet GRNs

Combining the *Gr64f-GAL4* transgene, previously shown to label all sweet gustatory receptor neurons (GRNs)[176], with *Gr64f^{LEXA}* and corresponding reporters (*UAS-RFP* and *lexAopGFP*) revealed extensive, if not complete, overlap in a single GRN of each taste sensillum (Figure 7B1). Thus, the *Gr64f-GAL4* transgene provides a reliable tool to

evaluate co-expression with the remaining *Gr64*^{LEXA} alleles in sweet GRNs of the main taste organs (Figures 7B and 8). In the labial palps, all knock-in alleles were specifically expressed in the sweet neuron of at least some sensilla, with the exception of *Gr64a*^{GAL4} and *Gr5a*^{LEXA}. Surprisingly, *Gr64a*^{GAL4} expression was completely absent (Figure 7B2), while *Gr5a*^{LEXA} expression was present not only in sweet neurons (Figures 7B6 and 7B8; see below) but also in either the low salt and/or water neuron [108, 153]. Based on co-expression with *Gr64f-GAL4* or *Gr64f*^{LEXA} and association with particular taste sensilla (Figure 7B, left; Figure 8A), we defined at least four categories of sweet neurons in the labial palps (Figure 9). The most complete set of sugar *Gr* genes was observed in the GRN of 7 of the 31 sensilla in each palp, expressing all but the *Gr64a* gene. At the other end of the spectrum was a somewhat heterogeneous group of sweet GRNs associated with six centrally located S-type sensilla, all expressing *Gr64f* and *Gr5a* and, in a stochastic fashion, *Gr64b*, *Gr64c*, and *Gr64e* (Figure 9). Lastly, we observed robust co-expression of *Gr5a*, *Gr64c*, and *Gr64f* in six neurons associated with taste pegs and co-expression of *Gr61a*, *Gr64a*, *Gr64b*, and *Gr64f* in four neurons in the labral sense organ (Figure 8B). We next investigated expression of the same pairwise *Gr* combinations in taste sensilla of the fourth and fifth tarsal segments (Figure 7B, right panels). As the major difference to the labellum, *Gr64a*^{GAL4} expression was observed in all sweet neurons. Tarsal sweet GRNs also fell into four groups (Figure 9), with the most complete set expressing all sugar *Gr* genes (f5s, f4b) and the least complete set expressing only *Gr61a*, *Gr64b*, and *Gr64f* (f5a). Of note, the sweet GRNs of the f5v sensilla expressed *Gr61a*, *Gr64a*, *Gr64c*, and *Gr64f*, as well as the previously identified

fructose receptor gene *Gr43a [119, 120]*. Lastly, we examined GRN projections to the brain. As expected, the partial overlap observed in taste organs between knock-in alleles and *Gr64f-GAL4* was reiterated in the subesophageal zone, to which all labellar and many tarsal GRNs projected (Figure 10).

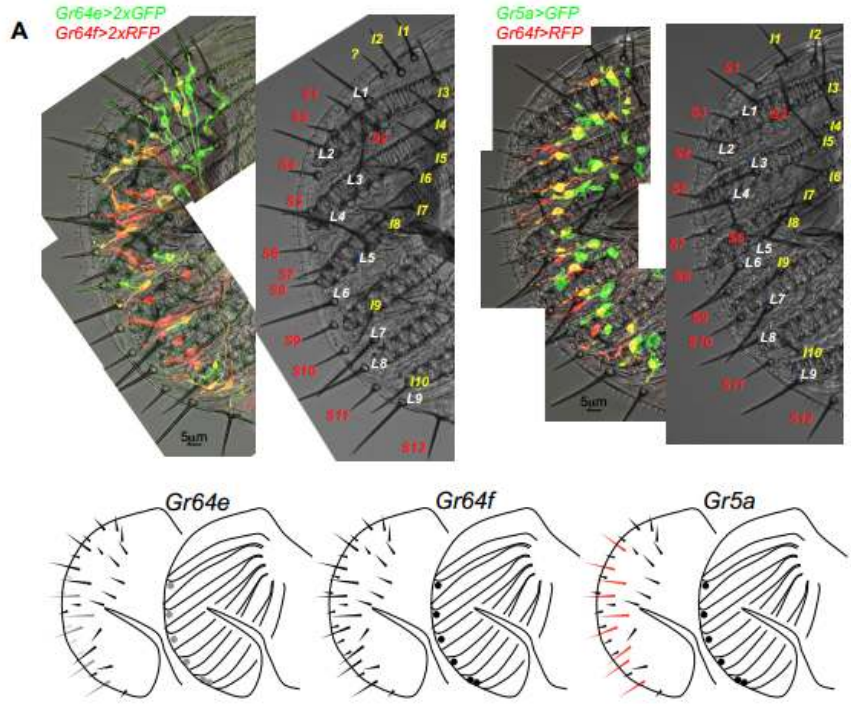

Figure 8 Mapping of sweet GRNs to specific labellar and internal taste sensilla

A) Association of Gr64f-GAL4, Gr5a^{LEXA} and Gr64e^{LEXA} expressing GRNs with specific labellar sensilla. Association of sweet neuron cell bodies with L- S- and I-type sensilla in the labellum is shown by overlay of bright field photomicrograph with fluorescent signals obtained through the green/red channels. Genotypes are lexAop-mCD8GFP UAS-mCD8RFP/+; Gr64f-GAL4/+;Gr64e^{LEXA}/lexAop-rCD2GFP UAS-mCD8RFP (left) and lexAop-mCD8GFP UAS-mCD8RFP/Gr5a^{LEXA}; Gr64f-GAL4/+;lexAop-rCD2GFP UAS-mCD8RFP/+ (right). The bottom shows a diagram of the expression data seen in the photomicrograph. Grey bristles and pegs

indicate sensilla containing sweet neuron with stochastic expression of Gr64e, and red bristle indicates sensilla that harbor more than one Gr5a expressing neuron. B) Expression of $Gr64f^{LEXA}$ in taste pegs (top) and internal taste organs (bottom). $Gr64f^{LEXA}$ is expressed in six neurons associated with taste pegs (arrowheads), located between each rows of pseudotrachea (white arrowheads, p1-6). We regularly observe 2 pegs between the 5th and 6th pseudotracheal rows and occasionally one peg located dorsally of the first pseudotrachae (yellow arrowheads, p0). Red triangles indicate mechanosensory bristles. * indicates that 1 or 2 positive cells are occasionally observed with these alleles. Genotype: lexAop-mCD8GFP UAS-mCD8RFP/+; +/+;$Gr64f^{LEXA}$/ +. Example of sugar Gr gene expression in the labral sense organ (LSO). MP denotes maxillary palp. Genotype: lexAop-mCD8GFP UAS-mCD8RFP/+; Gr61a-GAL4/+;$Gr64f^{LEXA}$/+. For expression summaries, see tables.

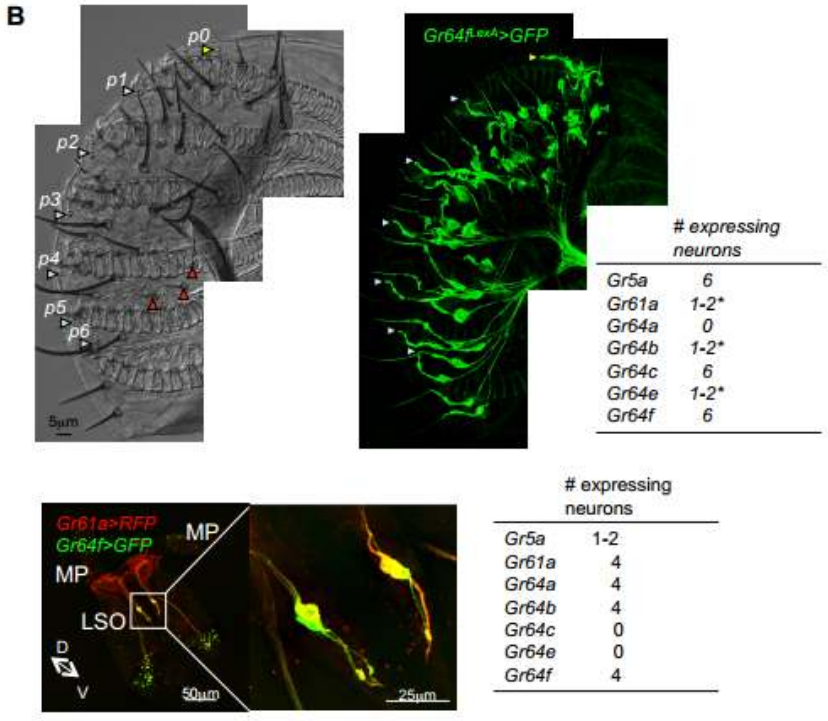

Figure 8 Continued

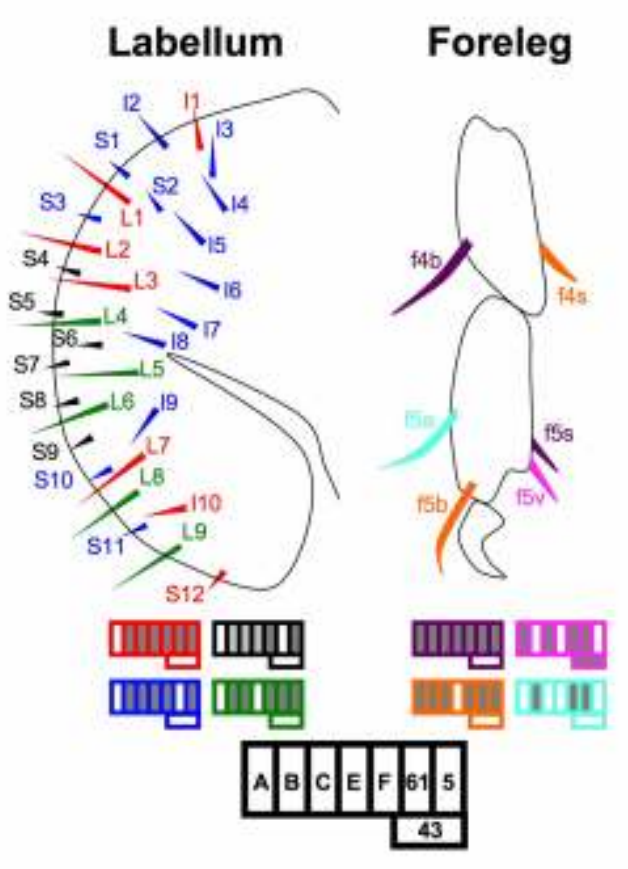

Figure 9 Expression Code for Specific Sweet Neurons in Labial Palp and Tarsal Sensilla

Schematic diagram of a labial palp and the foreleg illustrates the different expression profiles of the sweet-sensing neurons in the short (S), intermediate (I), and long (L) sensilla of the labellum [48] and in the sensilla of the foreleg [182]. Expression profile of the eight identified codes are shown below. A, B, C, E, F, 61, 5, and 43 refer to *Gr64a*, *Gr64b*, *Gr64c*, *Gr64e*, *Gr64f*, *Gr61a*, *Gr5a* and *Gr43a*. Note that expression of *Gr64b*, *Gr64c* and *Gr64e* in sweet GRN of S4 to S9 is heterogeneous (i.e., observed in some, but not all, flies), indicated in dotted gray pattern. Solid gray indicates gene expressed; white indicates gene not expressed.

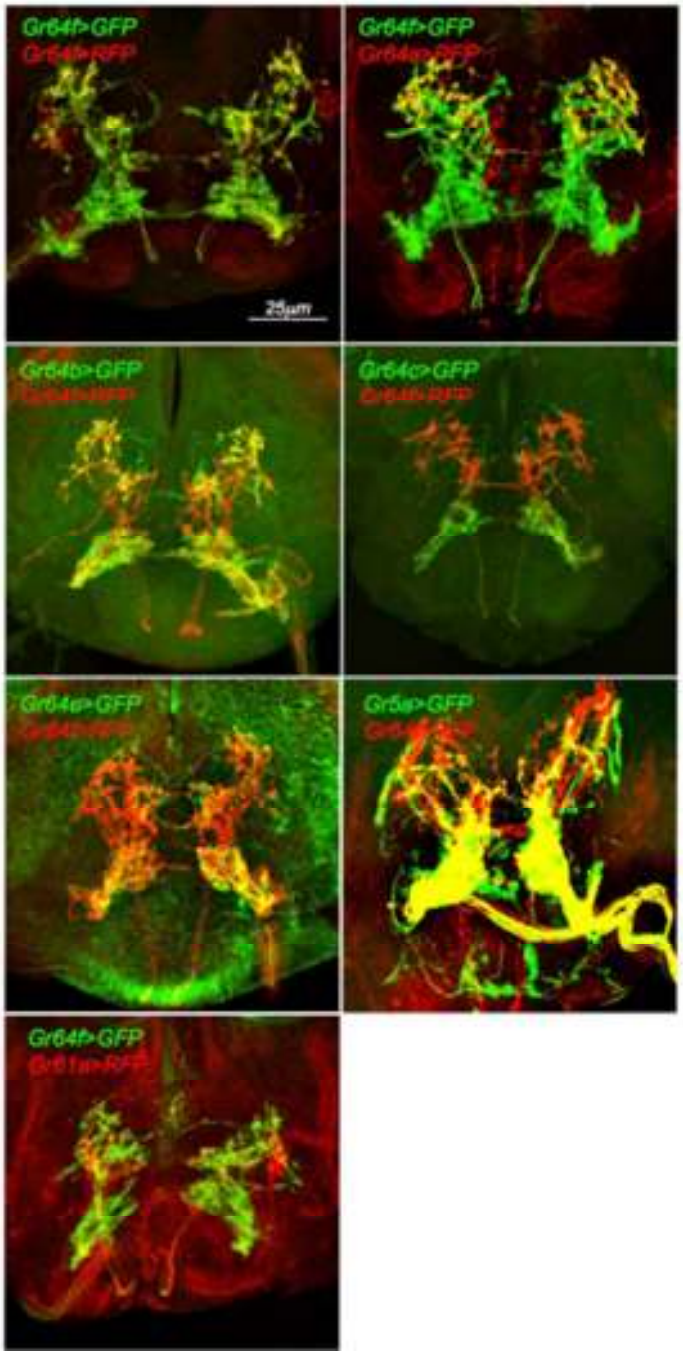

Figure 10 Projections of GRNs of flies with two different sugar Gr knock-in alleles
The images show the subesophageal ganglion of the brain. For more details of genotypes, see Figure 7.

Effects of Individual Knock-in Mutations on Proboscis Extension Reflex Responses

The *Gr64*$^{LEXA/GAL4}$ alleles also represented loss-of-function mutations. Thus, we generated homozygous mutant flies and examined their ability to sense seven sugars and glycerol, using the proboscis extension reflex (PER) response assay. PER reflects feeding motivation and is induced when labial or tarsal taste sensilla are stimulated with a sugar solution. As some Gr genes show major expression difference between tarsi and labellum (i.e., *Gr64a*), PER analysis was carried out by stimulating each taste organ separately (Figure 11). We normalized PER responses of Gr mutant files to w1118 flies, the strain used to generate the knockin alleles (Figure 11 and 12). Most mutations significantly affected PER responses to two or three sugars. For example, homozygous *Gr5a*^{LEXA} flies exhibited the strongest PER phenotypes, including a severe reduction to maltose and trehalose, regardless of which organ was stimulated, and to several additional sugars when tarsi were stimulated. Homozygous *Gr64f*^{LEXA} flies exhibited approximately a 50% PER reduction to arabinose, glucose, and trehalose in tarsi, while labial PER was reduced to a lesser extent for the former two sugars, as well as for fructose (Figure 11). Homozygous *Gr64c*^{LEXA} flies showed significant PER phenotypes to arabinose (leg and labellum), sucrose, maltose, and glycerol (leg only), while homozygous *Gr64b*^{LEXA} and *Gr64e*^{LEXA} flies showed severe PER reduction only to glycerol. Finally, consistent with the expression profile, *Gr64a-GAL4* mutant flies exhibited a tarsal-specific PER phenotype only to two sugars, fructose and maltose. Taken together, our observations show that (1) each sugar *Gr* gene contributes to the

detection of at least one sugar and (2) PER response to every sugar, except melezitose, is affected by more than one sugar *Gr* mutation (Figure 11C).

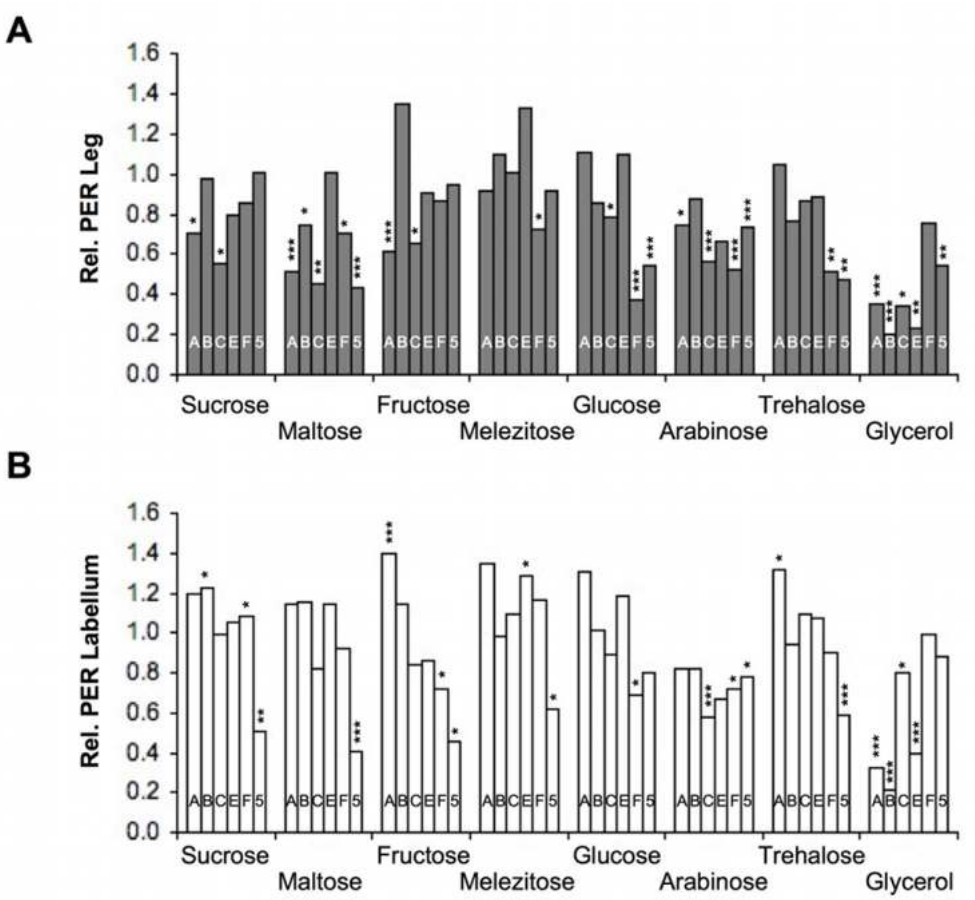

Figure 11 Proboscis Extension Reflex Behavior of Sugar Gr Mutant Flies

(A and B) Probability of proboscis extension reflex (PER) response of homozygous sugar $Gr^{LEXA/GAL4}$ mutant flies, as a fraction of the response of w1118 control flies, when legs (A) or labial palps (B) were stimulated. Genotypes were $Gr64a^{GAL4}/Gr64a^{GAL4}$ (indicated by A), $Gr64b^{LEXA}/Gr64b^{LEXA}$ (indicated by B), $Gr64c^{LEXA}/Gr64c^{LEXA}$ (indicated by C),$Gr64e^{LEXA}/Gr64e^{LEXA}$ (indicated by E), $Gr64f^{LEXA}/Gr64f^{LEXA}$ (indicated by F) and $Gr5a^{LEXA}/Gr5a^{LEXA}$ (indicated by 5). Asterisks indicate significant differences between control flies (w1118) and Gr knockin mutant flies, using Student's t test: *p <

0.05, **p < 0.01 and ***p < 0.001. Number of experiments for control flies (w1118) was, depending on sugar, between 10 and 20 (15 < n < 20 for leg; 10 < n < 15 for labellum). Number of experiments for mutants was between 6 and 12 (8 < n < 12 for leg; 6 < n < 10 for labellum). For PER of w1118 flies, see Figure 12. Values > 100% to select sugars may arise through increase in taste receptor complexes for these sugars, caused by removing competing Gr partners or by altering expression of other Gr genes. Sugar concentrations (100 mM for fructose, sucrose, melezitose and maltose; 500 mM for glucose, trehalose, and arabinose; and 10% for glycerol) were chosen so as to effectively reveal phenotypic differences between wild-type and Gr mutant strains. PER was conducted as described by Chen and Amrein [48].

(A) Summary of PER responses. Max score was 8 (corresponding to w1118).Scores with statistically significant decrease to 62.5% or less (see Figures 11A and 11B) are highlighted in red. Increases in PER were not considered since they most likely represent indirect effects of a mutation. Low response to glycerol is likely due to effects of the $Gr64a^{GAL4}$ mutation on other genes in the locus.

C

Mut\sugar		Suc	Mal	Fru	Mel	Glu	Ara	Tre	Gly
Wild type	leg	8	8	8	8	8	8	8	8
	lab	8	8	8	8	8	8	8	8
$Gr64a^{GAL4}$	leg	8	5	5	8	8	6	8	3[1)]
	lab	8	8	8	8	8	7	8	3[1)]
$Gr64b^{LEXA}$	leg	8	6	8	8	7	7	7	2
	lab	8	8	8	8	8	7	8	2
$Gr64c^{LEXA}$	leg	5	4	6	8	7	5	8	3
	lab	8	7	7	8	8	5	8	7
$Gr64e^{LEXA}$	leg	7	8	8	8	8	6	8	2
	lab	8	8	7	8	8	6	8	4
$Gr64f^{LEXA}$	leg	7	6	7	6	3	5	5	7
	lab	8	8	6	8	6	6	8	8
$Gr5a^{LEXA}$	leg	8	4	8	8	5	6	4	5
	lab	5	4	4	5	7	7	5	7

Figure 11 Continued

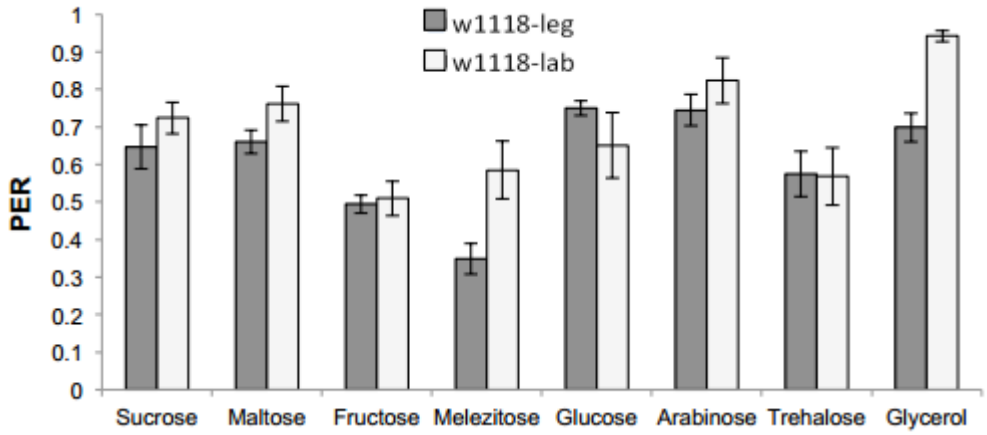

Figure 12 PER analysis of w1118 (control) flies

Legs and labellum were stimulated separately and the frequency of PER was recorded for seven sugars and glycerol. The value obtained for these control flies was used to determine relative PER of *Gr5a* and *Gr64* mutant flies, shown in Figure 11.

Non-canonical Expression of Sugar Gr Genes

Several studies have uncovered noncanonical expression of *Gr* genes *[96, 135, 185, 186]*. Thus, we examined sugar *Gr* gene expression in olfactory organs and the brain of adult flies containing each $Gr64^{LEXA/GAL4}$ allele. Indeed, $Gr5a^{LEXA}$, $Gr64b^{LEXA}$, and $Gr64f^{LEXA}$ were all expressed in olfactory organs (Figure 13A). $Gr5a^{LEXA}$ showed the most restricted expression, confined to a few neurons located in the maxillary palps. Expression of $Gr64b^{LEXA}$ was observed only in a subset of olfactory neurons in the antenna, while $Gr64f^{LEXA}$-expressing neurons were found in both olfactory organs. Projections of $Gr5a^{LEXA}$ neurons converged to a single glomerulus (VA7l; Figure 13B), while those of $Gr64b^{LEXA}$ neurons labeled four glomeruli (DM3, DA1, VA6, and VM2)

in each antennal lobe. Not surprisingly, many glomeruli were labeled in *Gr64f*^{LEXA} flies (Figure 13D). Association of *Gr*-expressing olfactory neurons with specific glomeruli was also confirmed in brains of flies lacking specific olfactory organs (Figure 14A). Lastly, we combined the *Gr64b*^{LEXA} allele with the broadly expressed olfactory neuron marker *Orco-GAL4* and found that the *Gr64b*-expressing neurons also express *Orco* (Figure 14B).

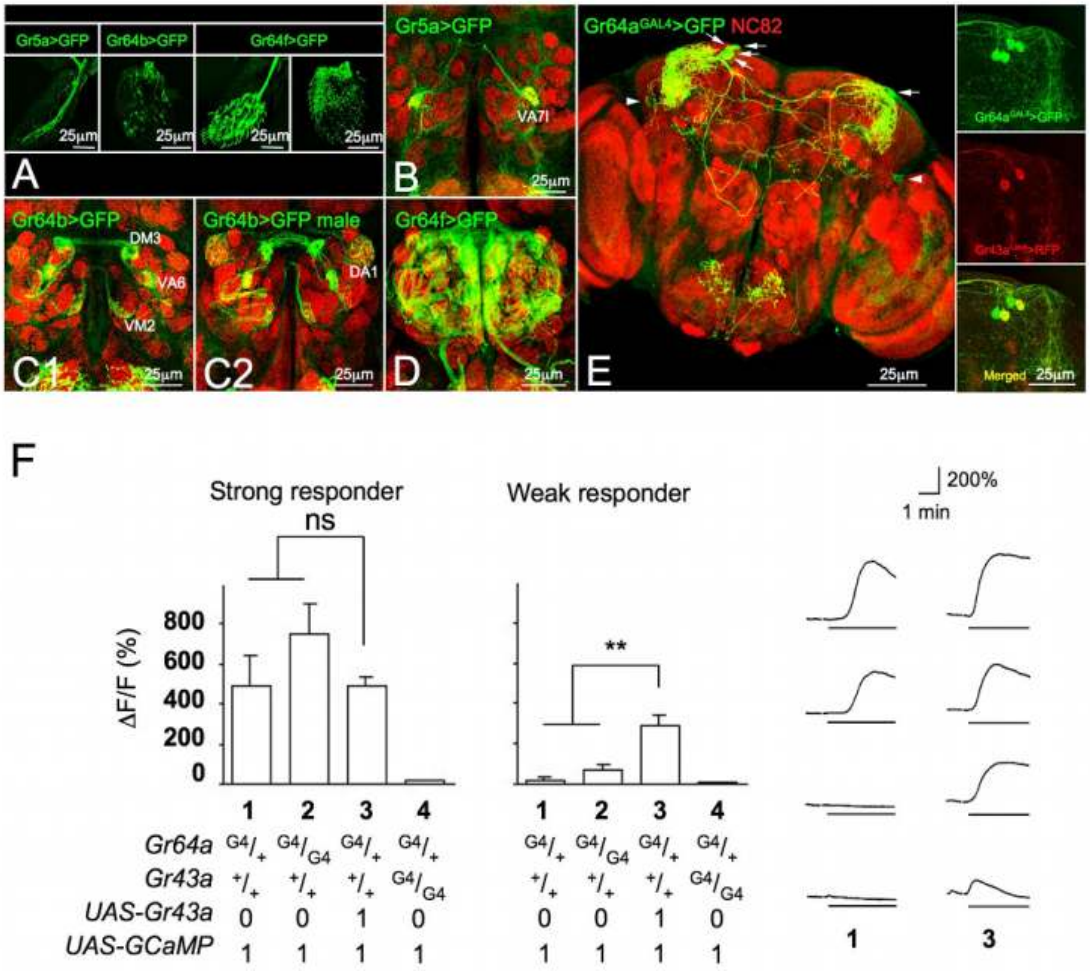

Figure 13 Non-canonical Expression of Sugar Gr Genes

(A) *Gr5a*^{LEXA} (left) and *Gr64b*^{LEXA} (middle) expression is restricted to the maxillary palp and antenna, respectively, while *Gr64f*^{LEXA} (right) shows broad expression in both. (B–D) Projections of neurons expressing *Gr5a*^{LEXA} (B) and *Gr64b*^{LEXA} (C) project to a single (VA7l) or a few (DA1, DM3, VA6, and VM2) glomeruli, while those expressing *Gr64f*^{LEXA} (D) label a large number of glomeruli. Note that DA1is sexually dimorphic, i.e., larger in males (C2) than in females (C1). All preparations were from females, except for (C2). The reporter in all cases was *lexAop-rCD2GFP*. (E) *Gr64a*^{GAL4} is expressed in four neurons of the posterior superior lateral protocerebrum(large image; arrows) and one neuron in the anterior dorsolateral region of the brain (arrowheads). The *Gr64a*^{GAL4}-expressing neurons also express the nutrient sensor *Gr43a*, evident from co-localization of GFP and RFP in small images on the right. Antibody staining (antimcherry and anti-GFP) of *Gr43a-LEXA*, *lexAoprCD2RFP/UAS-mCD8GFP; Gr64a*^{GAL4}/+ brain is shown.(F) Ca2+ imaging of Gr64a^{GAL4} posterior brain neurons in wild-type (lanes 1 and 3: *Gr43a+/+;Gr64a*^{G4}/+) flies, *Gr64a* mutant (lane 2: *Gr64a*$^{G4/G4}$) flies, and *Gr43a* mutant (lane 4:*Gr43a*$^{G4/G4}$*; Gr64a*^{G4}*/+)* flies. Two neurons in wild-type and Gr64a mutant brains (1 and 2 in left graph) always respond strongly, while the two other neurons respond very poorly (1 and 2 in right graph). No response was observed in Gr43a$^{G4/G4}$ mutants (lane 4), and imaging data were randomly assigned to low responders and high responders. In wild-type brains containing a *UAS-Gr43a* transgene (lane 3 in each panel), responses in all four cells were similarly robust: the two cells with higher ΔF/F value were assigned to the strong responder group, while the two cells with the lower ΔF/F value were assigned to the weak responder group. Concentration of fructose was 10 mM. ns indicates no statistical significant difference between indicated groups;**p < 0.01; two-tailed Student's t test (n = 9 to 10). The traces on the right show the four cells from a wild-type (lane 1) and a Gr43a-overexpressing brain (lane 3).

(G) Live GCaMP and RFP signals in *Gr64a* brain neurons of *Gr43a-LEXA/lexAop-RFP/Gr64a*^{GAL4}*/UAS-GCaMP6m* (top) and GCaMP signals before 10 mM fructose administration (middle) and at 2 min after the administration (bottom, pseudo colored). RFP-positive neurons give stronger response than RFP negative neurons. ***p < 0.001; two-tailed Student's t test (n = 4)

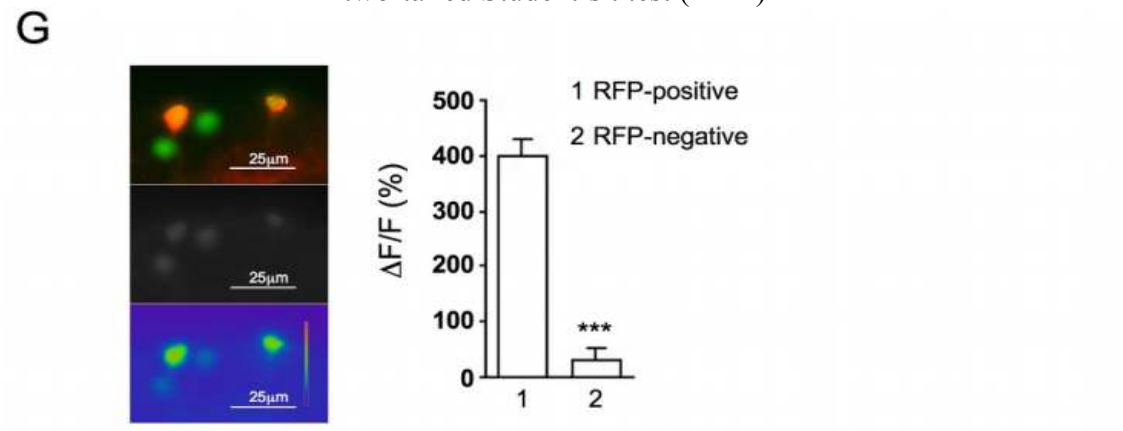

Figure 13 Continued

Expression of Sugar Gr Genes in Brain Nutrient-Sensing Neurons

To explore whether any of the sugar *Gr* genes is expressed in the brain, we performed antibody staining of all knock-in strains with appropriate reporters. Intriguingly, *Gr64a*^{GAL4} produced an expression pattern strikingly reminiscent of *Gr43a*, a sensor for hemolymph fructose [120]. To determine whether these two Gr genes are co-expressed, we combined *Gr64a*^{GAL4} with a *Gr43a-LEXA* transgene [185] and found that respective reporter genes labeled the same cells (Figure 13E). *Gr64a*^{GAL4} is consistently observed in four neurons per brain hemisphere, and two to three of these cells also express *Gr43a-LEXA*. We previously showed that *Gr43a*-expressing neurons respond to fructose [120]. To examine whether all four *Gr64a*^{GAL4}-expressing neurons respond to fructose and whether *Gr64a* plays a role in this response, we performed Ca^{2+} imaging experiments in homozygous and heterozygous *Gr64a*^{GAL4} flies (Figure 13F and 4G). We found that two of the four *Gr64a*^{GAL4} neurons were strongly activated by fructose (strong responders), while the other two neurons exhibited only a muted response (weak responders; Figure 13F). These responses were not dependent on *Gr64a* but required *Gr43a*. Intriguingly, we observed that the weak responder neurons in *Gr43a+/+;Gr64a*^{GAL4}*/+* flies were transformed into strong responder neurons when supplied with a *UAS-Gr43a* transgene (Figure 13F).

Lastly, association of *Gr43a* expression levels and fructose sensitivity was confirmed using flies in which expression for *Gr43a* and *Gr64a* was independently monitored (Figure 13G).Taken together, these data show that the nutrient-sensing *Gr43a* neurons in the posterior superior lateral protocerebrum fall into two groups based on

their fructose sensitivity (i.e.,*Gr43a* expression level) and that they express a second sugar receptor gene, *Gr64a*. Given that fructose sensing is not dependent on *Gr64a*, we suggest that these neurons sense yet another substrate, possibly another sugar.

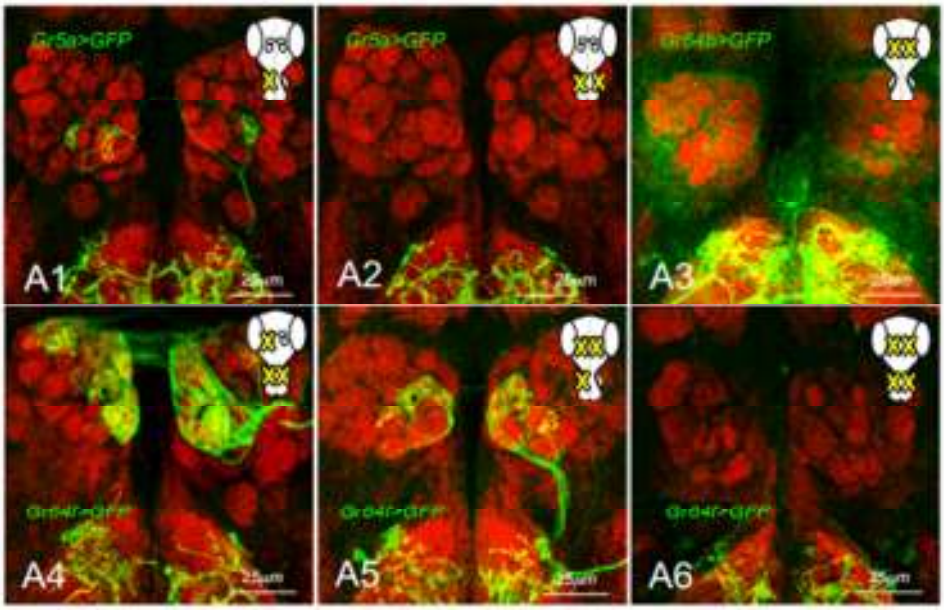

Figure 14 Sugar *Gr* gene expression in olfactory neurons

A) VA7l staining in Gr5a^{LEXA} flies was dependent on the presence of at least one maxillary palp (A1 and A2). In Gr64b^{LEXA} flies lacking both antennas, staining in the antennal lobe completely disappeared (A3), but unaffected when maxillary palps were removed (data not shown). In Gr64f^{LEXA} flies with all but a single antenna removed, GFP staining becomes restricted to glomeruli in the dorso-medial region of the antennal lobe (A4), while in flies with a single maxillary palp, staining is restricted to the ventro-medial region (A5). However, all staining is lost in flies lacking all four olfactory appendages (A6). The reporter in all experiments was lexAop-rCD2GFP. B) Double staining of olfactory organs and brains to visualize expression of Gr64b^{LEXA}knock-in alleles and Orco-GAL4 (previously known as Or83b-GAL4), a maker for neurons of basiconic and trichoid sensilla (top panel), and their projections to the antennal lobes (image at the bottom). Genotype: Orco-GAL4/+;Gr64b^{LEXA}/UAS-mCD8RFP,lexAop-rCD2GFP.

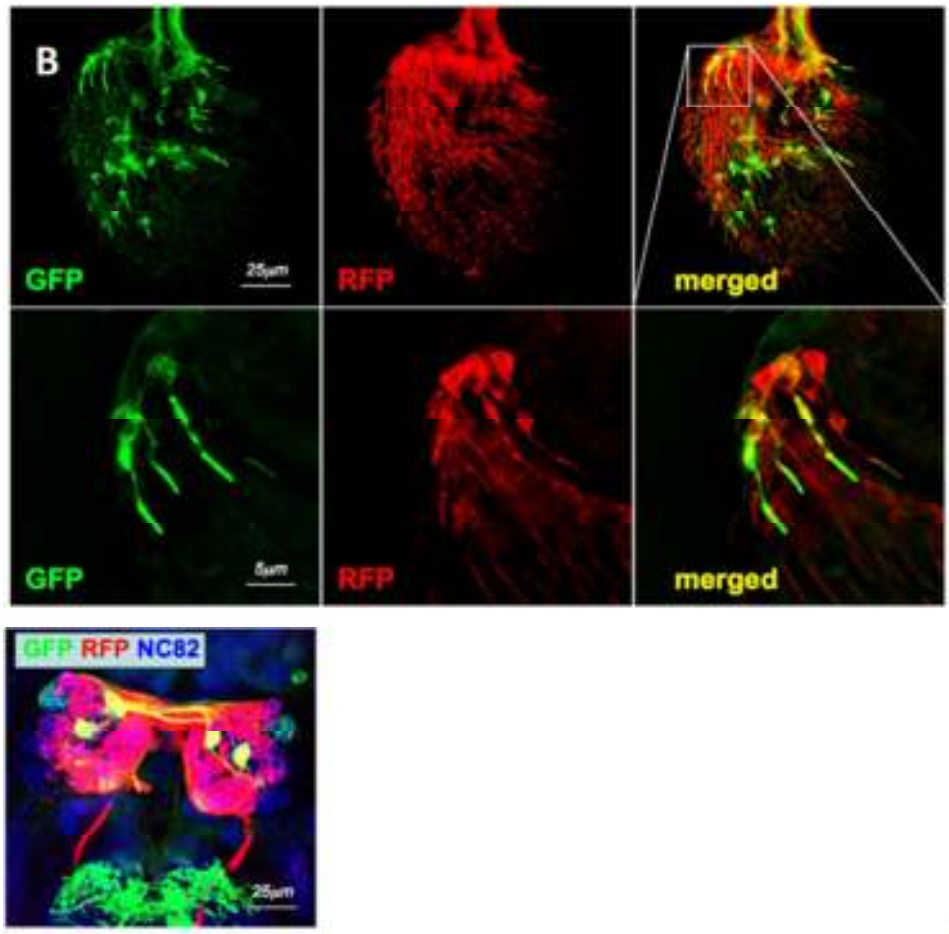

Figure 14 Continued

Conclusion

Sugars, the main group of chemicals underlying sweet taste, provide essential nutritional value for many mammals and insects. Yet, diverse molecular strategies have been implemented for their recognition during animal evolution [171, 186].

Why does *Drosophila* employ such a large array of sugar receptors, while mammals can cope with a single heterodimeric pair of G protein-coupled receptors [65], and honey bees and wasps appear to be served well with a single pair of sugar *Gr* genes [187, 188]? We suggest two possible rationales for this phenomenon. First, in *Drosophila*, a genus with close to 1,500 species, many of which share the same habitats, sugar *Gr* gene expansion may accommodate different food preferences. In a frugivore generalist, such as *Drosophila* melanogaster, functional expression of many sugar *Gr* genes may be advantageous for the detection of diverse sugar chemicals from different fruit sources. In specialists such as *Drosophila sechellia* and *Drosophila erecta*, which feed on a single type of fruit, expression of a few, exquisitely tuned sugar *Gr* genes may allow for more robust physiological and behavioral responses; thus, selective gene expression may complement non-neutral evolution proposed to occur in chemoreceptor genes of different *Drosophila* species[189]. A second possible reason for sugar receptor gene expansion may rest within the properties of the *Gr* proteins themselves. Both expression and functional analyses have implicated these proteins in diverse sensory contexts, including sensing of CO_2 [10, 11], internal nutrients [120], gut content [134, 190], temperature [132], and light [131]. Thus, Gr proteins may be highly adaptable, and, hence, expansion of these genes may be beneficial in conquering new ecological niches.

Sugar Receptor Expression Codes for Sweet Neurons

Non-quantitative PCR using RNA isolated from taste organs suggested that all six *Gr64* genes are expressed in labial palps [74, 117, 176], whereas systematic analysis of Gr-GAL4 lines did not detect expression of *Gr64a*, *Gr64b*, and *Gr64d* [107, 182]. Our study has shown that each targeted $Gr64^{LEXA/GAL4}$ allele is expressed in sweet neurons, and it establishes an expression map that defines at least eight different sweet neuron types (Figure 9). This complexity may increase further if *Gr64d* is also expressed in a distinct subpopulation of sweet neurons and if additional combinations of sugar *Gr* genes are expressed in sweet neurons of mid-leg and hind-leg. Using electrophysiological recordings, large differences were observed in responsiveness between the sweet neurons of f5a and f5s/f5b tarsal sensilla to virtually all sugars tested, the former generating far fewer spikes per second than the latter [182].This is consistent with our observation that f5a-associated sweet neurons express only three of the eight sugar *Gr* genes (*Gr61a*, *Gr64b*, and *Gr64f*), while those of the two other sensilla express at least six sugar Gr genes. In the labellum, electrophysiological recordings using sugar stimuli have been restricted to a few L-type sensilla [117, 176]but even among those, modest differences in tuning profiles were observed. We predict that labial sweet neurons will also vary substantially in their response profiles.

Gr proteins are likely to function as heterodimeric or multimeric complexes [10, 11, 74, 102, 117, 176]. Indeed, Jiao and collaborators have provided evidence for a heterodimeric composition of a functional trehalose receptor [118]. Thus, combinatorial expression of six or more *Gr* genes in a sweet neuron (observed for about ¼ of all sweet

GRNs) may generate at least 15 different varieties of sugar receptor complexes. Expression of pairs of *UAS-Gr* transgenes in flies mutant for all sugar *Gr* genes, combined with behavioral and physiological assays, can address which of these combinations constitute functional sugar receptors. Two reports suggested bicistronic and polycistronic transcription in the *Gr64* locus [74, 176], which would predict coexpression within the same neurons. However, the data presented here indicate that each of the Gr64 genes is regulated, at least in the main taste organs, independently of its neighbors. Nevertheless, polycistronic transcription observed for *Gr64a* and *Gr64b* [74] is consistent with the finding that the corresponding knockin alleles are co-expressed with *Gr64f* in four neurons of the labral sense organ (Figure 8B).

Alternatively, mRNAs containing multiple *Gr64* coding sequences might simply reflect inefficient termination of transcription, which is consistent with the absence of a conserved transcription termination signal (50-AATAAA-30) between *Gr* coding sequences [74]. Further studies will be necessary to establish the functional significance of polycistronic *Gr64* gene transcription.

Gr64a Is Not a Major Sugar Receptor

$Gr64a^{GAL4}$ is not expressed in sweet GRNs of labial taste sensilla, and *Gr64a* mutant flies exhibit normal labial PER responses to all sugars (Figures 7B and 11B). Yet, it has been proposed by two groups that sweet taste perceived through the labial palp is dependent on *Gr5a* and *Gr64a* [176{Jiao, 2007 #95] [117, 176]. Using electrophysiological recordings from L-type sensilla of $\Delta Gr64a^{2}$ mutant flies, in which

coding sequences of *Gr64a*, *Gr64b*, part of *Gr64c*, and 250 base pairs of upstream regulatory DNA were deleted, Dahanukar and colleagues reported a large reduction in spiking activity after stimulation with maltose and sucrose and a complete loss after stimulation with fructose [176].In contrast, Jiao and coworkers found that labial sweet neurons of homozygous $Gr64^{ab}$ flies (a deletion similar to $\Delta Gr64a^{2}$, but leaving *Gr64c* intact; Figure 7A) lacked any spiking activity when stimulated to all sugars tested, including glucose and trehalose [117], sugars that elicit normal spiking activity in $\Delta Gr64a^{2}$mutant flies. Rescue of these phenotypes (except for trehalose in [117]) through *Gr64a* overexpression led both groups of investigators to conclude that *Gr64a* is an essential component for sensing these sugars. However the more severe electrophysiological phenotype observed in $Gr64^{ab}$ flies (deleting *Gr64a* and *Gr64b*) compared to Δ*Gr64a2* flies (disrupting in addition *Gr64c*) is difficult to reconcile with *Gr64a* being the causative gene. These contradictory observations and the data presented in this study (Figures 7B,11B, and 11C) are inconsistent with *Gr64a* playing a major role in sweet taste mediated by the labellum. The following reinterpretation of these previous studies, however, is compatible with our data: the removal of upstream regulatory elements in $\Delta Gr64a^{2}$and $Gr64^{ab}$ (see Figure 7A), but retention of variable intergenic sequences, might affect expression of downstream *Gr64* genes differently, leading to the distinct phenotypes. Furthermore, given the functional redundancy implied by the subtle PER phenotypes of all homozygous *Gr64* alleles (Figure 11), overexpression of *Gr64a* may compensate for lack of expression of other *Gr64* genes and rescue a phenotype incorrectly associated with *Gr64a* (Figure 11).Another *Gr64*

gene, *Gr64f,* was implicated in trehalose sensing [118]. Here, labellar sweet neurons of Δ*Gr64* mutant flies (lacking all six *Gr64* genes) lost all spiking activity when stimulated with this sugar, similar to ΔGr5a mutants. This phenotype was rescued by overexpression of *Gr64f*, but not *Gr64e* or *Gr64d*. Our PER analysis supports a role for *Gr64f* in trehalose sensing, when elicited from tarsi. However, functional redundant trehalose receptors must exist too, as overall PER upon labellar stimulation was severely affected in homozygous $Gr5a^{LEXA}$, but not $Gr64f^{LEXA}$, flies.

Sugar Receptors Are Expressed in Olfactory Neurons

Gr21a and *Gr63a* are expressed in the olfactory system, where they function as a carbon dioxide sensor [10, 11]. However, these receptors are not expressed in the taste system, and, hence, CO2 sensing is probably their sole function. Expression of *Gr5a*, *Gr64b*, and *Gr64f* suggests a role for sugar receptors in both olfaction and taste. We can envision at least three roles for these *Gr* genes. First, *Gr5a*- and *Gr64b*-expressing neurons may also express *Gr64f*, and, hence, they may form sugar receptors in olfactory neurons. In many insects, antennae serve as both olfactory and taste organs, and expression of sugar taste receptors could reflect similar functions for olfactory neurons in flies. Indeed, a role for the *Drosophila* maxillary palps has been reported in sugar perception via multimodel sensory integration [7]. A second possibility is that these *Gr* proteins have acquired novel roles in olfaction, for example, in combination with *Ors*. Or proteins interact with Orco [21],and sweet *Grs* are also proposed to function as heterodimers or heteromultimers [74, 117, 118, 176]. Thus, it is intriguing to speculate that members of

these two related protein families cooperate to generate ‘‘hybrid’’ receptors with new ligand specificities. Lastly, it is possible that the sugar *Gr* proteins are expressed in cellular structures (i.e., axons) that are exposed to the hemolymph. Thus, rather than sensing external ligands, these *Grs* might sense an internal chemical that leads to modulation of olfactory responses (see also below).

Internal Nutrient Sensing through Sugar Taste Receptors

We recently showed a role for *Gr43a* as a hemolymph fructose receptor in neurons of the brain [120]. Our Ca2+ imaging studies indicate that these four cells fall into two functional pairs, with distinct fructose sensitivities. The presence of *Gr64a* suggests a broader role for these neurons in *internal nutrient sensing. While sensing fructose does not require Gr64a, this receptor may* modulate neuronal output. Alternatively, *Gr64a* may be activated by another sugar. Glucose or trehalose, the main sugars in the fly hemolymph, do not induce Ca2+ changes, but it remains to be investigated whether this reflects limitations of our assay, or whether *Gr64a* is activated by another hemolymph compound. In summary, non-canonical expression broadly widens the putative functions of insect sugar receptors to olfaction and internal nutrient sensing.

Experimental procedures

Molecular cloning of knock-in constructs

Targeting constructs for ends-out homologous recombination were based on the CMC-loxP-Gal4 [120] and CMC-loxP-LexA::VP16 vectors. CMC-loxP-LexA::VP16 was obtained by first adding loxP sites into the AvrII the BstEII sites of the CMC vector [21]. From the resulting plasmid ("CMC-loxP"), we cloned the LexA::VP16 sequence into the SpeI and AvrII sites yielding the targeting vector CMC-loxP-LexA::VP16. To generate gene-specific targeting construct, PCR fragments flanking the gene being targeted were cloned into the TOPO-XL vector (Life Technologies) and then subcloned into the upstream and downstream multiple cloning sites of CMC-loxP-Gal4 (*Gr64a*) or CMC-loxP-LexA::VP16 (*Gr5a*, *Gr64b*, *Gr64c*, *Gr64e*, and *Gr64f*).

Whole mount antibody staining

UAS-mCD8::RFP and *lexAop-mCD8::GFP* were driven by the *Gr64f-GAL4* transgene and each of the Gr^{LEXA} alleles or by $Gr64a^{GAL4}$ and $Gr64f^{LEXA}$ alleles for double staining in labial palps, tarsi and the brain. Mouse anti-GFP (1:2000), rabbit anti-mCherry (1:200), goat anti-mouse-Alexa 488 (1:200) and goat anti-rabbit-Alexa 555 (1:200) were used for all double stainings. Rabbit anti-GFP (1:5000), mouse anti-nc82 (1:20), goat anti-rabbit-Alexa 488 (1:200) and goat anti-mouse-Alexa 647 (1:200) were used for brain staining. Confocal images were captured with a Nikon A1R Confocal Microscope System.

Ca^{2+} Imaging of the brain neurons

Dissected whole brains were placed in a glass bottom dish with 57ul of a sugar-free ringer solution [120] and 3ul of ringer solution containing 200mM fructose were administrated. Images used for data analysis were acquired for 60 s before and 180-720 s after application (1 frame/3 s) with a Nikon eclipse Ti inverted microscope [120]. We determined the strong and weak responder cells as follows; if response of 4 cells in a hemisphere were recorded, two strong responded cells were called strong and other two were weak. If response of 2 or 3 cells were recorded, the strongest one was called strong and the weakest one was called weak.

Proboscis Extension Reflex (PER) Assay

PER assays were essentially carried out as described [48, 74] with minor modifications. Briefly, 4 to 10 (6) day old flies were starved for 22 to 24 hours in vials with a water-saturated Whatman paper. Flies were chilled on ice and mounted on their backs on a microscope slide using double-sided scotch tape. In PERs elicited from labial palp stimulation, all legs were secured to the tape to avoid accidental contact with tarsal neurons. Flies were allowed to recover for 1 to 2 hours after mounting, and they were allowed to drink water thoroughly prior to testing. A PER was recorded when a fly fully extended the proboscis after a ligand application. Test solutions were delivered by a 20 µl pipette on legs or by wet Kimwipe on the labellum. Each test solution was applied three (legs) and two (labellum) times per fly, and water saturation was achieved

again before the next application. Probability of PER for a single experiment was determined from 18 to 36 (legs) and 16 to 20 (labellum) test solution applications, using 6 to 12 (legs) and 8 to 10 (labellum) flies. At least six (legs) and four (labellum) independent experiments were used to establish a PER value for each mutant. Error bars represent +/- the standard error of the mean (SEM), and statistical significance was calculated using Student t-test, assuming unequal variance (one-way ANOVA with post hoc Bonferroni correction).

CHAPTER III

A GENETIC TOOL KIT FOR CELLULAR AND BEHAVIORAL ANALYSES OF INSECT SUGAR RECEPTORS*

Introduction

Detection of sugars and other calorie-containing compounds and their discrimination from other chemicals are critical behavioral tasks that enable animals to feed from nutritious food sources. These processes are embedded in the gustatory system, a hallmark of which is the cellular segregation of receptor proteins that detect different groups of chemicals such as sugars, proteins and bitter-tasting compounds. In all characterized animal model systems, food chemicals stimulate different types of taste receptor cells than chemicals with no nutritional value or harmful and toxic compounds by virtue of cell-specific expression of cognate receptors (labeled lines) [171].

In *Drosophila*, taste sensilla constitute the sensory structures for the detection of all soluble chemicals. Taste sensilla, which are the functional equivalents of mammalian taste buds, are found in several major body parts, especially the labellum and the legs. Most taste sensilla contain four gustatory neurons (GRNs; some sensilla contain only two GRNs), as well as a mechanosensory neuron [2]; the four neurons are thought to be dedicated to different taste modalities, which have been associated with three appetitive promoting (sweet, modest salty and water) and one aversive (bitter/high salty)

*This chapter is reprinted with permission from Yavuz, A., C. Jagge, et al. (2014). "A genetic tool kit for cellular and behavioral analyses of insect sugar receptors." Fly (Austin) **8**(4): 189-196.

modalities. Additionally, flies are also known to respond with acceptance behavior when provided with amino acids or fatty acids [45, 47, 191], the cellular mechanism of which is not well understood.

Given that most *Drosophila* species are frugivores, sweet taste plays a central role in flies' feeding behavior. Sweet sensation is mediated by the sweet GRNs present in most if not all sensilla of the two main taste organs, the labial palps and the distal most segments of the tarsi. Each of these sweet GRNs is thought to express members of a *Gr* gene subfamily composed of eight sugar *Gr* genes (*Gr5a*, *Gr61a* and *Gr64a* to *Gr64f*) [74, 108, 117, 153, 176]. A ninth *Gr* gene, *Gr43a*, was recently shown to be critical for sensing internal (brain hemolymph) fructose [120]. In the taste system, *Gr43a* is expressed in only a pair of tarsal taste sensilla, and its contribution to sucrose and fructose sensing is secondary to receptors formed by sugar Gr proteins [120].
While *Gr5a* and *Gr61a* have been characterized in some detail and shown to play a critical role in trehalose and glucose sensing, respectively [113, 176, 177, 192], the specific functions of each of the *Gr64* genes are less defined, other than the fact that as a whole, this gene cluster is essential for sugar responses both at the behavioral and cellular level [74, 118]. This paucity is due to lack of specific mutations in single *Gr64* genes, a consequence of the densely clustered organization of the *Gr64* locus (see Figure 15). Regardless, specific roles have been assigned for some of these genes based on phenotypes of deletion and insertion mutations. However, these types of mutations are likely to alter expression of structurally unaffected genes within the locus, which is exemplified by the more severe phenotypes ascribed to the *Gr64ab* mutation (deleting

Gr64a and Gr64b) than the more subtle phenotype observed in *Gr64a*^{2} (deleting *Gr64a, Gr64b and Gr64c*) [117, 176]. Thus, lacking defined mutations and comprehensive expression profiles, it is not possible to determine the specific roles of the six *Gr64* genes in sweet taste or the composition and tuning profiles of receptor complexes to specific sugars.

Here, we report the generation of powerful genetic tools that allow us to address these and several additional questions about insect sugar receptors. We constructed a number of *Gr* mutations via homologous recombination that revealed detailed expression of five of the six *Gr64a* genes {Fujii, 2015 #139}. Moreover, we created a sugar-blind strain in which all eight *sugar Gr* genes were deleted. We use this strain to show, contrary to a recent report [193], that functional sugar receptors are composed of at least two sugar Gr protein subunit. Lastly, we identify two functional receptor complexes for recognition of the sugars maltose and sucrose, as well as glycerol.

Results

The genetic tools presented in this paper will overcome three major impediments that have slowed progress in our understanding of sweet taste in *Drosophila*. First, there is a lack of precise and useful mutations for the six densely clustered genes in the *Gr64* locus. Second, expression for many sugar *Gr* genes has not been established, and hence, the role of such genes in sweet taste remains speculative. And third, we currently lack a tool that unequivocally associates specific sugar chemicals with Gr proteins. The tools presented here will help overcome these obstacles, and they provide not only a path to a

clear understanding of the role of each *sugar Gr* gene in sweet taste, but will also aid in elucidating the composition of specific insect sugar taste receptors.

Gene Targeting of Gr5a and Gr64a-f loci

The *GAL4/UAS* expression system has been successfully employed in many studies for analysis of many *Gr* genes [96, 108, 153, 175]. However, the success rate for *Gr64-GAL4* transgenes has been poor, and for half of the genes, no cellular expression profile has ever been reported with this system. Therefore we generated a series of sugar *Gr* knock-in alleles through homologous recombination [183, 194]. We generated seven transgenic fly strains containing a *LEXA* or *GAL4* targeting construct on the second chromosome, consisting of 5' and 3' non-translated sequences of all six *Gr64* genes and *Gr5a* (Figure 15). While null alleles for both *Gr5a* and *Gr61a* are available [113, 176, 177], the former, but not the latter was included in this study because two independently generated *Gr5a* transgenes were found to be expressed not only in sweet GRNs, but additional taste neurons [97, 116]. With the exception of the *Gr64d* construct, all transgenes were successfully recombined into their target site, replacing the *Gr* coding sequence with *LEXA* or *GAL4* and producing six new knock-in/null alleles: $Gr5a^{LEXA}$, $Gr64a^{GAL4}$, $Gr64b^{LEXA}$, $Gr64c^{LEXA}$, $Gr64e^{LEXA}$ and $Gr64f^{LEXA}$ (for details, see Experimental Procedures and Supplemental Material). When these alleles are combined in a fly with specific reporter genes containing transcription factor binding sites for *GAL4* or *LEXA* (*UAS-RFP* or *lexAop-GFP*), they should replicate endogenous *Gr* gene activity. Indeed, all new knock-in alleles revealed expression either only in GRNs or in

GRNs and additional chemosensory cells of the olfactory system or nutrient sensing brain neurons. A detailed expression analysis of these alleles is described in a separate study [116].

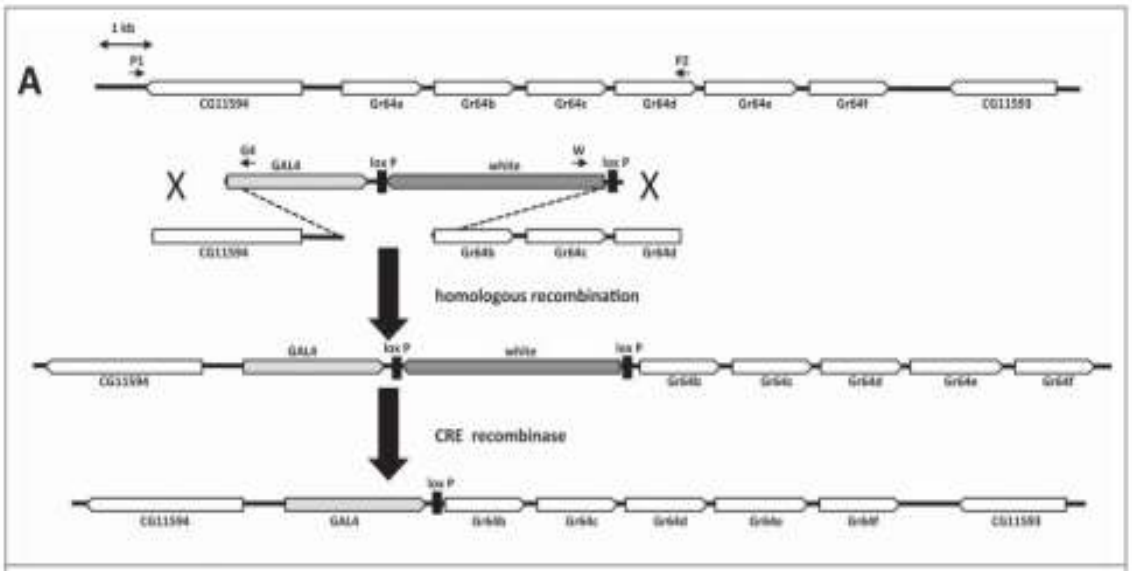

Figure 15 GAL4/LEXA knock-in strategy for sugar Gr genes using homologous recombination

A) Genomic region of the *Gr64* locus and the targeting construct for $Gr64a^{GAL4}$ are shown in the two diagrams at the top. Homologous recombination replaces *Gr64a* with *GAL4* and the w^{+} minigene, which is removed via CRE mediated recombination (bottom diagrams). Positions of primer used for PCR analysis are indicated by short arrows.

B) PCR analysis of genomic DNA isolated from successfully targeted homozygous $Gr64a^{GAL4}$ and $Gr64f^{LEXA}$ lines (1) and respective donor lines (2). Location of primes P1, P9 and P2, P10 (see A) anneal to genomic DNA upstream and downstream of, but not within, the donor construct; Expected DNA fragment sizes for the 5' and 3' products are 4.2 kb and 5.9 kb for $Gr64a^{GAL4}$ and 5.7 kb and 4.6 kb for $Gr64f^{LEXA}$. Primers G4, LA and w are specific for GAL4, LEXA and *white* gene.

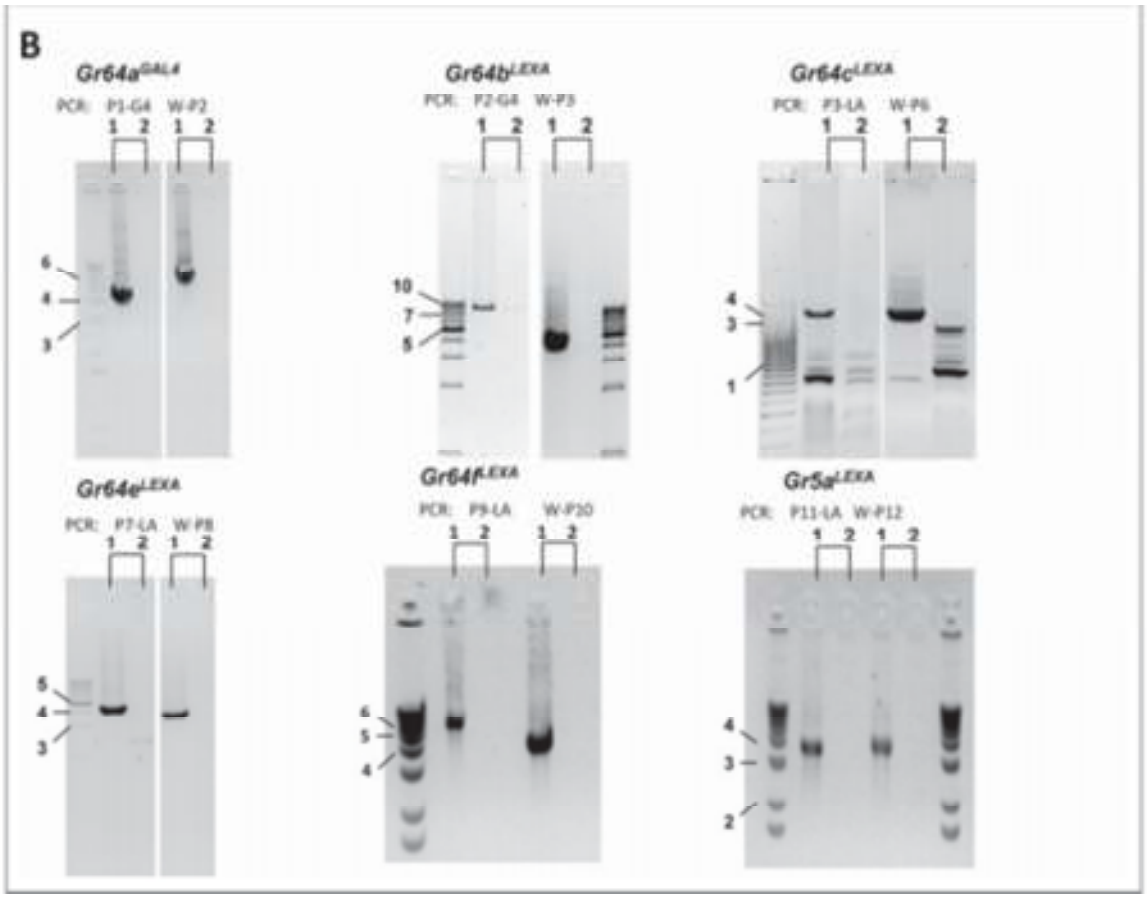

Figure 15 Continued

Effects of individual knock-in mutations on cellular response

Utility of the single gene mutations in assessing the effect on gustatory receptor neuron responses was tested for the *Gr64a*^{GAL4} mutation. *Gr64a* was chosen because it has been proposed to be essential for proper sensing of many sugars, including fructose, maltose, maltotriose, stachyose, raffinose, and others [117, 176] by labellar taste neurons. Yet, lack of expression of *Gr64a*^{GAL4} in labellar neurons, as well as absence of a PER

phenotype [116] is not consistent with a major role for this *Gr* gene in sugar sensing. Thus, we determined the cellular responses of homozygous and heterozygous $Gr64a^{GAL4}$ mutant flies in tarsal neurons, where $Gr64a^{GAL4}$ is expressed, using Ca^{2+} imaging (Figure 16). We focused these imaging experiments on GRNs of the 5b sensilla, as opposed to the 5v sensilla (albeit both produced similar responses; data not shown), because the neurons of the latter also express the *Gr43* fructose receptor [116], which alone is sufficient to mediate response to sucrose and fructose [120]. Heterozygous control flies showed robust neuronal responses to all sugars tested (Figure 2B). Consistent with the relatively mild behavioral deficits of $Gr64a^{GAL4}$ mutant flies [116], GRNs of $Gr64a^{GAL4}$ mutant flies produced robust responses upon stimulation with most sugars, and reduction, but not a complete loss, of Ca^{2+} responses, to maltose and maltotriose only (Figure 16B). Thus, the precise gene knock-in mutations are likely to provide a more accurate assessment for the contribution of individual sugar *Gr* genes than gene deletions used in previous studies, many of which also included regulatory sequences [117, 176].

Generating a sugar-blind Drosophila strain

Null alleles (i.e. lack of function alleles), such as $Gr64a^{GAL4}$, are useful when determining the contribution of a single gene to a particular taste trait. However, for delineating sufficiency (i.e. which genes together may encode for functional sugar receptors) null alleles are of limited value. Sufficiency is best assessed with the help of heterologous expression systems, which, unfortunately, have been met with little success

in the context of insect taste receptors. An alternative approach to heterologous expression systems is the generation of an "empty neuron" system, whereby deletions/mutations are introduced in every *Gr* gene expressed in a particular neuronal subtype. Such an "empty neuron" system has been powerfully employed in the *Drosophila* olfactory system, where it was used to unambiguously identify the ligands for numerous olfactory receptors [20]. While single GRNs express many more *Gr* genes than olfactory neurons express *Or* genes, we sought to test whether neurons of flies lacking all eight sugar *Gr* genes could be used as a "sugar *Gr* deficient neuron system". We therefore generated a strain in which all eight sugar *Gr* genes carried null alleles (octuple mutant). Variations of this octuple mutant strain, also referred to as "sugar-blind" strain (or sugar $Gr^{-/-}$), were also equipped with a GRN specific GAL4 driver and a transgene for either the calcium indicator GCaMP6.0 (octuple mutant DRIVER strain) or one or more sugar *Gr* transgenes (octuple mutant REPORTER strains; Table 1). When octuple mutant flies from these strains are crossed, the effects on the cellular and behavioral responses of single or pair wise combinations of sugar *Gr* genes in otherwise sugar blind flies can be quantitatively assessed using Ca^{2+} imaging and the proboscis extension reflex (PER) assay, respectively. To verify suitability of the *sugar* $Gr^{-/-}$ strain, we first examined the sugar-induced neuronal responses in two types of GRNs, one expressing the non-canonical fructose receptor Gr43a (associated with the 5v sensilla) and one lacking expression of that gene (5b sensilla), of octuple mutant flies (as well as heterozygous control flies) [116]. No neural activity was observed in 5b associated sweet GRNs in homozygous flies upon stimulation with any sugar solution tested, whereas

control flies responded robustly to all sugars (Figure 17A). In contrast, the 5v-associated GRN was activated when stimulated with fructose and sucrose, to a level approximating that of heterozygous control flies. These observations are consistent with our previous analysis of the Gr43a nutrient sensor, which showed that this receptor functions independently of the sugar Gr proteins in hemolymph fructose sensing [120]. Moreover, they suggest that the "sugar *Gr* deficient neuron system" is adequate to determine the response profile upon re-introduction of sugar *Gr* genes.

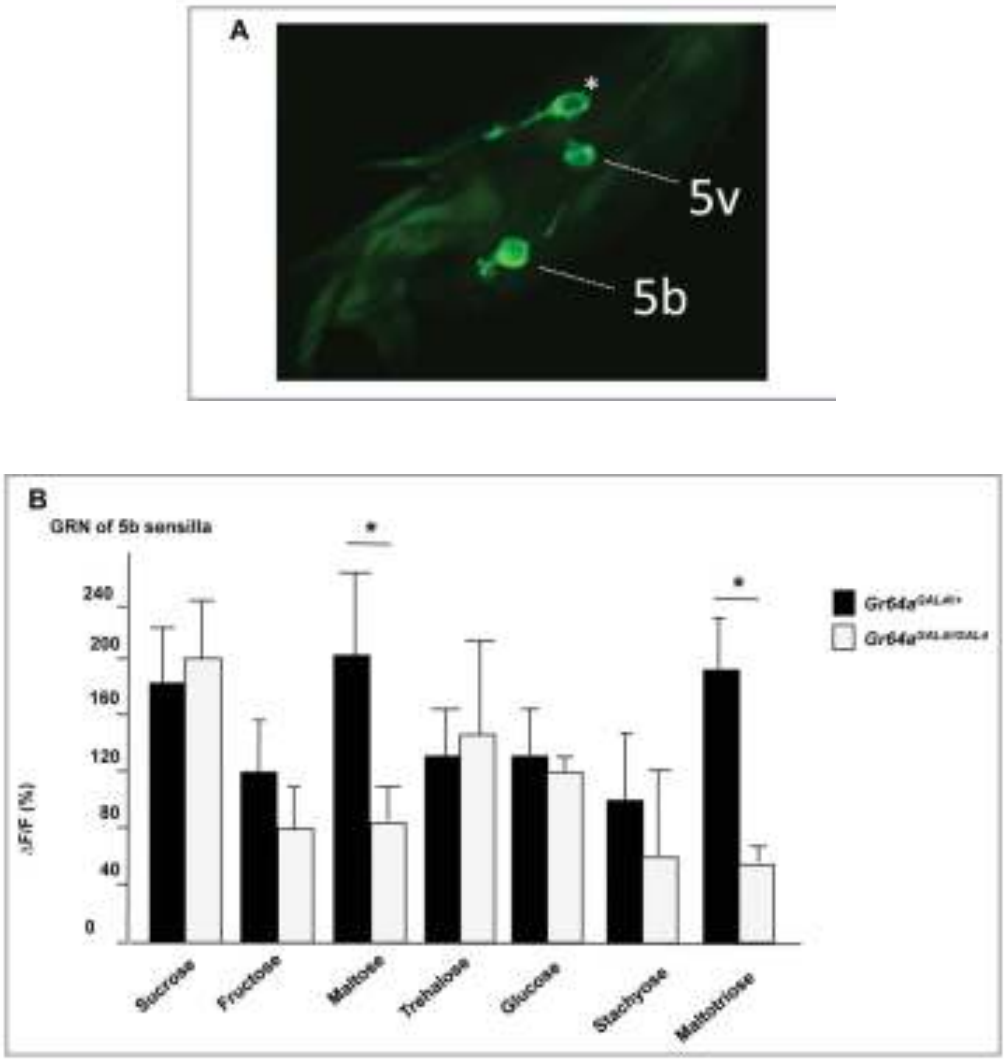

Figure 16 Sweet taste neurons of *Gr64a*^{GAL4} mutant flies respond normally to most sugars.

(A) Antibody staining of tarsi of *Gr64f*-GAL4;UAS-mCD8GFP shows 3 labeled sweet neurons; the two neurons associated with 5b and 5v sensilla were used for Ca^{2+} imaging. The additional, 5s associated sweet neuron is indicated with an asterisk. Note that *Gr64f*-GAL4 and *Gr64a*^{GAL4} are co-expressed in the sweet GNR of these sensilla.16 (B) Ca^{2+}responses of sweet GRNs associated with the 5b sensillum of *Gr64a*^{GAL4}/+ (control) and Gr64a$^{GAL4/GAL4}$ flies. Responses to maltose and maltotriose, but to none of the other sugars, were significantly reduced in homozygous mutants. All sugars were at 100 mM. Student's t-test: * $P < 0.05$; $4<N<8$

Table 1 Strains generated and used in this study.
List of strains used for the examination of phenotypes of (i) mutations in single sugar Gr genes (top six lines) and of transgene rescue in octuple mutant (*Gr5a*^{LEXA}; Δ*Gr61a* Δ*Gr64a-f*) background (bottom four lines). R1 is an X linked genomic construct that contains two essential non-Gr genes missing in the Δ*Gr64a-f* deletion.

Genotype	Description	Remarks
Gr5a^{LEXA};+;+	Gr5a null allele	Coding Region Replaced by LEXA
+;+; *Gr64a*^{GAL4}	Gr64a null allele	Coding Region Replaced by GAL4
+;+; *Gr64b*^{LEXA}	Gr64b null allele	Coding Region Replaced by LEXA
+;+; *Gr64c*^{LEXA}	Gr64c null allele	Coding Region Replaced by LEXA
+;+; *Gr64e*^{LEXA}	Gr64e null allele	Coding Region Replaced by LEXA
+;+; *Gr64f*^{LEXA}	Gr64f null allele	Coding Region Replaced by LEXA
R1,Gr5a^{LexA};+;*Δ61a, Δ64a-f*	All sugar Grs deleted	sugar blind strain
R1,Gr5a^{LexA}; *Gr61a-GAL4;GCamP6m/Cyo; ΔGr61a, ΔGr64a-f*	Driver line for the rescue experiment in octuple mutant background	Yields sugar blind flies suitable for Ca^{2+} imaging when crossed to sugar blind strain
R1,Gr5a^{LEXA}; *UAS-Gr64X/Cyo; ΔGR61a, ΔGR64a-f*	UAS lines used for single rescue experiment in octuple mutant background	Yields flies with single sugar Gr suitable for Ca^{2+} imaging when crossed to sugar blind strain
Gr5a^{LEXA}; *UAS-Gr64X,UAS-Gr64Y/Cyo ; ΔGr61a, ΔGr64a-f/TM6b*	Reporter strains used for double Rescue experiment in octupleMutant background	Yields flies with two sugar Gr suitable for Ca^{2+} imaging when crossed to sugar blind strain

*UAS-Gr64f construct is on the X chromosome (recombined onto *R1, Gr5a*^{LEXA} chromosome) and *UAS-Gr5a* construct is on third chromosome (recombined onto *ΔGr61a, ΔGr64a-f* chromosome).

Sugar receptors are encoded by two or more subunits encoded by sugar Gr genes

It was recently reported that olfactory neurons expressing any single sugar *Gr* gene are activated when bathed in a sugar solutions, and the authors suggested that single Grs function in the absence of other sugar Grs. This observation, however, contradicts evidence from numerous studies which strongly argue that functional sugar receptors are composed of two (or more) different sugar Gr proteins [74, 117, 118, 176]. To address whether or not single Gr proteins can mediate sugar responses, we expressed each of the eight sugar *Gr* genes in sweet GRNs of octuple mutant flies and performed Ca^{2+} imaging experiments on the tarsal 5b sensilla. None of the Gr proteins, when expressed singly,

led to a fluorescence increase after application of eight different sugar solutions. Interestingly, when we measured cellular responses of the 5v associated Gr43a expressing neuron, expression of single sugar *Gr* genes was sufficient for activation following application of some sugars (Table 2). For example, expression of *Gr64a* alone elicited a maltose response in the *Gr43a* neuron, expression of *Gr64b* alone or *Gr64e* alone elicited responses to arabinose, melezitose and glycerol, and expression of *Gr61a* alone elicited a glucose response. The interpretation of these results is that Gr43a can form complexes with sugar Gr proteins to form receptors for selected sugars.

The experiments described thus far are consistent with the hypothesis that Gr proteins do not function as single receptors or homodimers, but are composed of at least two different Gr subunits. To test this idea further, we randomly chose four pairwise *UAS-Gr* combinations, expressed them in octuple mutant flies and monitored activity in the sweet GRN of the 5b sensillum (which does not express *Gr43a*; Figure 18). Indeed, two combinations lead to strong neural responses to a select group of sugars. Specifically, the Gr64a/64e pair induced strong response to maltose and sucrose, but not to glucose, trehalose, fructose, arabinose, melezitose and glycerol. In contrast, the Gr64b/64e pair was able to induce glycerol-specific responses, but did not mediate any responses to the other seven sugars we tested. We note that two other combinations of Gr proteins - Gr64a/Gr64b and Gr64a/Gr64d – failed to convey cellular responses in 5b associated sweet GRNs of octuple mutant flies when tested with any of the eight sugars.

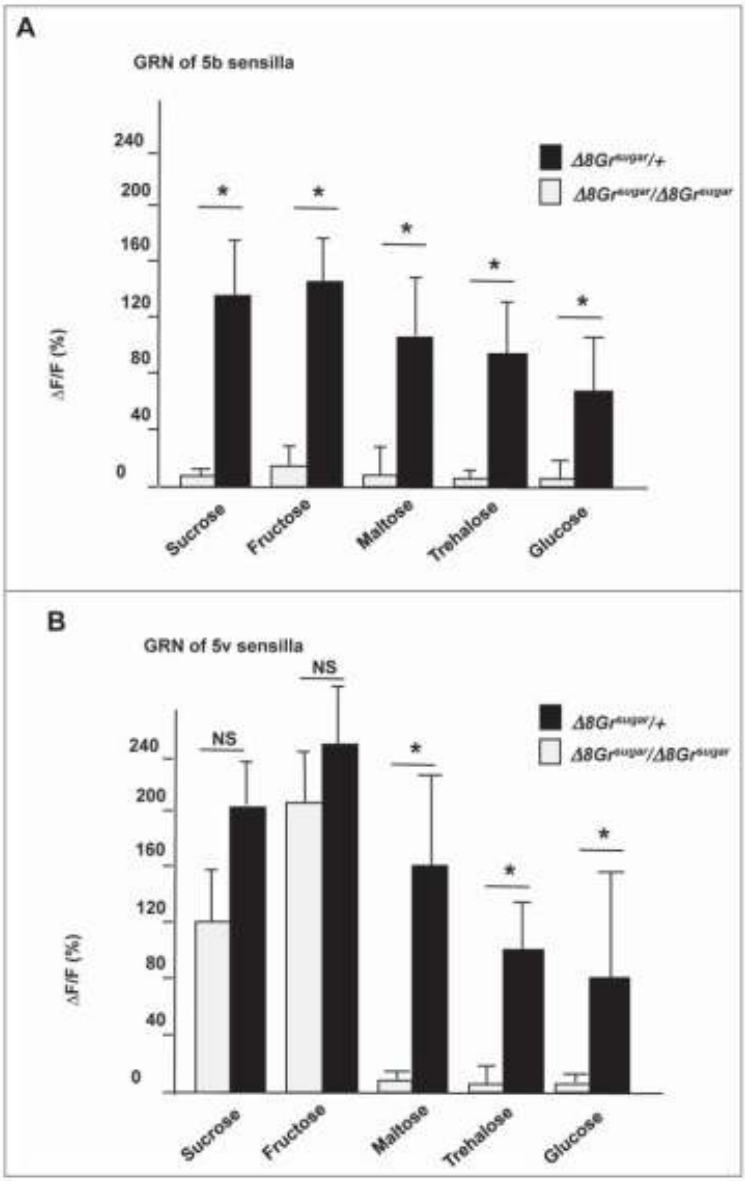

Figure 17 Many sweet taste neurons of octuple mutant flies lack sugar responses
(A) 5b associated sweet GRNs of $\Delta Gr^{sugar}/\Delta Gr^{sugar}$ flies (homozygous mutant for all 8 sugar Gr genes) lack responses to any sugar tested, while imaging of the same neuron of sugar ΔGr^{sugar} /+ flies respond robustly to all sugars tested. (B) 5v associated sweet GRNs, which express the atypical fructose receptor *Gr43a*, of ΔGr^{sugar} /ΔGr^{sugar} flies respond to sucrose and fructose, but not to maltose, trehalose, and glucose. Heterozygous flies show somewhat stronger responses to sucrose and fructose, indicating that receptors for these sugars are formed by sugar Gr proteins. All sugar concentrations were at 100 mM. Student's t-test: NS, Not Significant; * $P < 0.05$; $3<N<7$

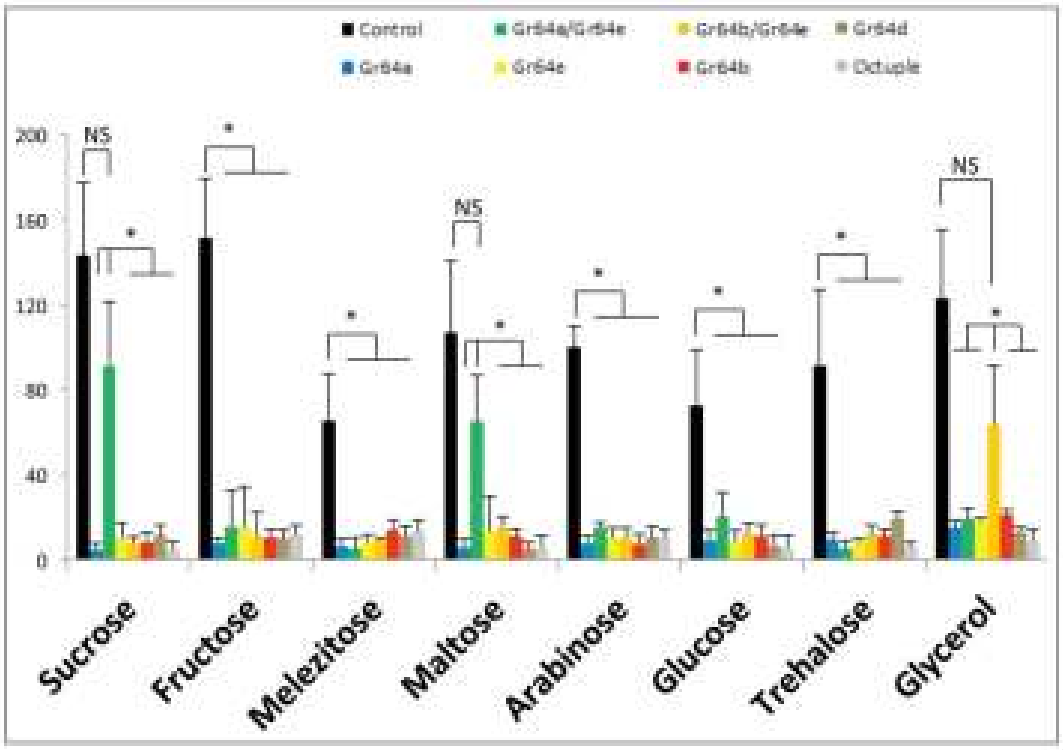

Figure 18 Two sugar Gr proteins are necessary to form functional sugar receptors
5b-associated sweet GRNs of octuple mutant flies (gray) expressing Gr64a (blue), Gr64b (red), Gr64d (tan), or Gr64e (yellow) do not respond to any of the 8 sugars we tested. However, when octuple mutant flies were provided with Gr64a and Gr64e (green), or Gr64b and Gr64e (orange), responses to maltose and sucrose, or glycerol, was recovered. All sugars were at 100mM. Student's t-test: $P < 0.05$; $3<N<7$.

Table 2 Summary of single and double rescue Ca^{2+} imaging experiments. Sweet GRN responses using Ca^{2+} imaging observed in the 5b sensilla (lacking expression of *Gr43a*; red), and in the 5v sensilla (expressing *Gr43a*; blue) of sugar blind $\Delta 8Gr^{sugar}/\Delta 8Gr^{sugar}$) flies expressing a (top) or pairs of select sugar Gr genes are shown. Empty fields indicate no statically significant increase compared sugar blind ($\Delta 8Gr^{sugar}$ $/\Delta 8Gr^{sugar}$) flies; +,++ and +++ indicate statistically significant increase compared to sugar blind flies with cellular response < 33%, 33 to 66% and > 66% compared to control flies ($\Delta 8Gr^{sugar}$ /+), respectively. Note that the GRN of the 5v sensilla responds to sucrose and fructose, due to expression of the *Gr43a* gene in that neuron. Also note that expression of single UAS-Gr transgene in the 5b-associated GRN fails to restore responses to any sugar, while expression in the 5v-assocaited GRN leads to the recovery of some sugar responses. However, expression of pairs of UAS-Gr genes recovers select sugar response in the 5b-associated neuron (red). UAS transgenes were expressed under the control of *Gr61a*-GAL4

SugarTransgene(s	Maltose	Glucose	Trehalose	Fructose	Arabinose	Melezitose	Glycerol	Sucrose
none				+++				+++
UAS-Gr64a	+++			+++				+++
UAS-Gr64b				+++	++	++	+++	+++
UAS-Gr64c				+++				+++
UAS-Gr64d				+++				+++
UAS-Gr64e				+++	++	++		+++
UAS-Gr64f				+++				+++
UAS-Gr61a		++		+++				+++
UAS-Gr5a			+	+++		+		+++
UAS-Gr64a|UAS-Gr64b				+++				+++
UAS-Gr64a|UAS-Gr64d				+++				+++
UAS-Gr64a|UAS-Gr64e	+++			+++				+++ +++
UAS-Gr64b|UAS-Gr64e				+++			++	+++

Conclusion

We have generated a number of precise sugar *Gr* mutations that can be used both as null alleles as well as expression alleles. We also generated a sugar-blind fly strain that lacks measurable sugar responses in sweet taste neurons (except in those expressing Gr43a), and we have explored the utility of the sugar-blind strain to answer some important, pressing questions. Indeed, one of the main findings from our study is that sugar receptors are multimeric complexes composed of two or more subunits, rejecting the suggestion derived from pseudo-heterologous expression studies that single sugar *Gr* genes can mediate sugar responses on their own [20]. Using sugar-blind flies, we have determined the necessary components of two sugar receptors, one tuned to the disaccharides sucrose and maltose and one to the sugar alcohol glycerol. Surprisingly, while the Gr43a fructose receptor functions on its own in the brain and probably other chemosensory organs [120], as well as in heterologous expression systems [195], it is capable of forming additional sugar receptors when combined with other Gr proteins (Table 2). This observation suggests that some Grs, albeit none of the sugar Grs, function as homomultimers, but in combination with other Gr proteins, they can combine to form receptors with novel ligand properties.

Our imaging analysis of *Gr64a* mutant flies, together with behavioral studies of single *Gr64* mutant flies [116], demands re-evaluation of the promoted model of sweet taste, which suggested that two multimeric receptors composed by only three Gr proteins (including Gr64a) function as the major, if not sole, receptors for sweet chemicals. Indeed, the conspicuous expression of *Gr64a* in nutrient sensing neurons in the brain,

along with the absence in labial palp neurons [116], suggests that the main function for this gene is likely the sensing an internal sugar, rather than a dietary one.

Lastly, whether the GRNs in the octuple mutant strains represent a true empty neuron system remains to be determined. It is impossible to rule out that other *Gr* genes are expressed in sweet GRNs; moreover, a number of *Ionotropic chemoreceptor* genes are expressed in the gustatory system [89, 136, 196] and it is not known whether they are expressed in sweet GRNs. Finally, sweet GRNs were recently shown to mediate taste response to fatty acids, suggesting expression of receptors in these neurons that recognize such chemicals [45]**.** Regardless, the complete lack of sugar mediated responses in GRNs that lack expression of Gr43a should make the octuple mutant strain a powerful tool to analyze not only *Drosophila* sugar *Gr* genes, but also putative sugar *Gr* genes from other insect species.

Experimental procedures

Molecular cloning of knock-in constructs

Targeting constructs for ends-out homologous recombination were based on the CMC-loxP-Gal4 [120] and CMC-loxP-LexA::VP16 vectors. CMC-loxP-LexA::VP16 was obtained by first adding loxP sites into the AvrII the BstEII sites of the CMC vector [21]. From the resulting plasmid ("CMC-loxP"), we cloned the LexA::VP16 sequence into the SpeI and AvrII sites yielding the targeting vector CMC-loxP-LexA::VP16. To generate gene-specific targeting constructs, PCR fragments flanking the gene being targeted were cloned into the TOPO-XL vector (Life Technologies) and then subcloned into the upstream and downstream multiple cloning sites of CMC-loxP-Gal4 (*Gr64a*) or CMC-loxP-LexA::VP16 (*Gr5a*, *Gr64b*, *Gr64c*, *Gr64e*, and *Gr64f*).

In most cases, restriction sites were introduced into the primer sequence used to generate the PCR fragments, with the following exceptions: (1) Gr5a 3' flank—Internal SpeI site in the PCR product and a SpeI site in the TOPO vector were used to ligate fragment to NheI site in 3' MCS. (2) Gr64b 3' flank—Internal NheI site in the PCR product was used to ligate fragment to NheI site in 3' MCS. (3) Gr64c 3' flank—Internal NheI site in the PCR product was used to ligate fragment to NheI site in 3' MCS (4) Gr64f 3' flank—Internal NheI site in the PCR product was used to ligate fragment to NheI site in 3' MCS.

Primer Gr5a 5' Flank Sense- CGTACGCCGCAACTGGAAATGGAAATCTGA

Primer Gr5a 5’ Flank Antisense—

ACTAGTTGTGTACAAGCTCTAAATCCTGACTAAACG

Primer Gr5a 3’ Flank Sense—GGTGACCCACCCTTCAATCTTGATTAGACGCAC

Primer Gr5a 3’ Flank Antisense —GCTAGCGTTTTTACGCCTGCTGTCTGCTG

Primer Gr64a 5’ Flank Sense— GGCGCGCCCTGTCGTTGGTTCTCCAGCAGC

Primer Gr64a 5’ Flank Antisense—CGTACGGACGCTGGTCCCTTTTGCACTGAC

Primer Gr64a 3’ Flank Sense—GCGGCCGCTGGACAACAATAGCCACCAACACC

Primer Gr64a 3’ Flank Antisense—GCTAGCCAAGCCGCACTTCCCACATAGG

Primer Gr64b 5’ Flank Sense—

GGCGCGCCGCAAATGGGGGAAGATCATTACTGGG

Primer Gr64b 5’ Flank Antisense—

CGTACGGGCCAAACTAGCACTAACCAAACGAC

Primer Gr64b 3’ Flank Sense—

GCGGCCGCATCCTAGAATTTACTACTCGTATCTCCAATTCAAGAACG

Primer Gr64b 3’ Flank Antisense—

GCTAGCCTCACTTTTCGAACTGGCATCAAAGC

Primer Gr64c 5’ Flank Sense—

GGCGCGCCGTAGCTATATTACTACTGCCCTACGTTCACTG

Primer Gr64c 5’ Flank Antisense—ACTAGTGGCTTGACTGTTGGGTAGCAAATG

Primer Gr64c 3’ Flank Sense—

GCGGCCGCTTCTAGTTTGAAATTTGCATTCTGTCGCACCTTC

Primer Gr64c 3’ Flank Antisense—GCTAGCCTTTTCTTCAGCCGCCTCAACTTG

Primer Gr64e 5’ Flank Sense—

GGCGCGCCGTGAGTTGAGAAATGACTTTACACAGCTTAG

Primer Gr64e 5’ Flank Antisense—

ACTAGTGTTCCGTACTCGACTGACAACCAATC

Primer Gr64e 3’ Flank Sense—

GCGGCCGCATTTTGTGGAAGTGGCAGGGGGTTAAG

Primer Gr64e 3’ Flank Antisense—GCTAGCGATGCGGATGTGTCCCAGTACTTG

Primer Gr64f 5’ Flank Sense—GGCGCGCCGTGGAGTGCAAGCTGGATGCGAAC

Primer Gr64f 5’ Flank Antisense—ACTAGTCCTAGGACCTGCTGGGGTAAACTG

Primer Gr64f 3’ Flank Sense—

GCGGCCGCCCGCTAGAGAGATTCTACGTGTGTCCG

Primer Gr64f 3’ Flank Antisense—GCTAGCCTTATGGCGGACACTGCAATCCTGG

The transgenes were excised and linearized as described by Miyamoto et al. [120] and potential relocation onto the third chromosome for the $Gr64^{GAL4/LEXA}$ constructs and the X for the $Gr5a^{LEXA}$ construct was evaluated based on segregation from respective chromosome balancers. Between 2 and 10 lines with integration on the respective chromosome were generated and genomic DNA of homozygous flies with putatively recombined alleles was isolated. To determine whether the coding sequence of the respective *Gr* genes was precisely replaced with either that of LEXA or GAL4, we performed PCR using a primer within the targeting construct and a primer

complementary to a sequence just upstream of downstream of the targeting construct for each *Gr* gene.

Ca^{2+} Imaging of tarsal taste sensilla

Preparation of forelegs and Ca^{2+} imaging of taste sensilla was performed as described by Miyamoto et al. [192]. Concentration of all sugar was 100 mM.

CHAPTER IV

COMBINATORIAL STRATEGY OF GR PROTEIN ASSEMBLY GENERATES 18 DIFFERENT MULTIMERIC SUGAR TASTE RECEPTORS IN *DROSOPHILA*

Introduction

Taste is the central sensory modality to evaluate food sources for nutritious value and contaminating toxic chemicals, and failing to do so may result in malnutrition, overconsumption or poisoning. With its broad array of genetic, neural imaging and electrophysiological tools and powerful behavioral assays, *Drosophila melanogaster* is an ideal insect model to study gustatory perception and its underlying neurogenetic architecture. Appetitive chemosensory behavior is initiated by volatile cues perceived by olfactory sensilla located on the antenna and maxillary palp, leading the insect to a potential food source. Once in close proximity, flies use an anatomically dispersed gustatory system, organized in discrete, anatomically conserved arrays of taste sensilla (i.e., taste bristles) located at the tip of the proboscis and the most distal segments on the tibiae of all legs to evaluate its content. Additionally, some sensilla are located within the pharynx, at the wing margins and possibly on the female genitalia near the ovipositor [73, 77]. Taste sensilla are composed of up to four gustatory receptor neurons (GRNs) each of which is tuned to one distinct taste modalities, which enables flies to discriminate between toxic and potentially harmful chemicals and nutritious compounds, such as sugars. In addition, a number of support cells in each sensillum provide

secretions essential for functional interactions of taste ligands with taste receptors [108, 153].

The GRNs are located at the base the taste sensilla and they extend a long dendrite that is populated with taste receptors in to the bristle shaft [76]. A terminal bristle pore allows external chemicals to come in contact with the dendritic surface, which is bathed in the taste lymph secreted from some of the support cells. A single mechanosensory neuron thought to sense food texture/density completes the cellular content of each taste sensillum [197-199]. Based on electrophysiological studies on select tarsal taste sensilla, the four GRNs are proposed to mediate different taste modalities, most prominently sweet, bitter/high salt, low salt and water taste, respectively [44]. However, recent behavioral and molecular genetic studies have extended the taste range of *Drosophila* to include fatty acid taste (Ahn et al. in review ;Masek and Keene), sour taste[46, 48] and possibly amino acid taste [47], and hence, the proposed stereotypic organization of taste sensilla might vary among sensilla.

Most GRNs project an axon to the primary taste processing center in the brain, a region referred to as subesophageal zone (SEZ). These projections terminate in different areas of the SEZ, depending of the taste modality they mediate and the taste organ in which respective GRNs are located [86, 153]. Sweet GRNs project mostly in the lateral region of the SEZ, while bitter/high salt neurons project to the medial part of the SEZ. In addition, GRNs located in the pharynx project to more anterior positions in the SEZ, while GRNs from the labellum and leg terminate in a more posterior part of the SEZ compared to those from the pharynx [86]. However, some GRNs, most prominently

many of sensilla located in the tarsi, project to the thoracic ganglion, and not directly to the SEZ in the brain [86, 200].

The *Drosophila* genome contains 68 putative *gustatory receptor* (*Gr*) genes, which encode proteins of about 400 amino acids and are characterized by seven transmembrane spanning regions. Gr proteins were initially thought to be a distinct type of G protein coupled receptors (GPCR) [94, 201]. However, Olfactory receptors (Ors), which are evolutionarily related to Grs [105], are characterized by an inverted membrane topology (intracellular N terminus and extracellular C terminus), and studies from Ors using heterologous cell expression systems suggested that they function as ligand gated ion channels [12, 202]. A similar mode of action was proposed from experiments with Gr43a, showing that this receptor functions as ligand gated ion channel as well [125]. However, some reports also suggested that Grs interact with GPCRs in sensing sugars and bitter molecules [203, 204]. In addition, several components of GPCRs, $G\gamma_1$, $G_s\alpha$ and $G_0\alpha$, were found to be expressed in taste neurons, and mutations in some of these genes was associated with partial loss in sweet taste responses [203, 205-207]. However, no study to date has shown that impairment of G protein function abolishes sugar responses severely, suggesting G protein signaling is not essential in sugar perception [203-207].

The majority of *Gr* genes are expressed in GRNs that respond to bitter chemicals and high salt, and therefore, these *Gr* genes are thought to encode bitter taste receptors. Bitter Gr proteins are characterized by comparably little amino acid conservation, with the exception of a few small subfamilies, comprised of up to four genes arisen through

recent gene duplication events [105]. This diversity of primary amino acid sequence is consistent with the expectation that these receptors have to cover a diverse range of chemical structures [5, 102, 106, 107].

A much smaller class of *Gr* genes is expressed in the GRN that responds to sugars [74]. In most *Drosophila* species whose genome has been sequenced to date, this clade consists of eight highly conserved *Grs* (*Gr5a*, *Gr61a*, *Gr64a*, *Gr64b*, *Gr64c*, *Gr64d*, *Gr64e* and *Gr64f*), and a somewhat distinct 9th gene (*Gr43a*). The eight sugar *Gr* genes arose recently through multiple gene duplication events in the *Drosophila* lineage, but at least one pair of orthologous sugar *Gr* genes can be found across all insect orders analyzed to date [122]. Numerous studies in *Drosophila melanogaster* indicated that several sugar *Gr* genes are expressed in a single GRN of many, but not all, taste sensilla in the labial palps and the legs [107, 116, 176, 182]. The sole exception is *Gr5a*, which is found in up to three GRNs of many, but not all sensilla [100, 116]. Our comprehensive expression analysis that relied on a gene-knock in strategy based on homologous recombination in which two bipartite expression systems were combined (GAL4/UAS and LEXA/lex$_{op}$) revealed that sweet GRNs can be subdivided in at least eight different types, each characterized by a specific sugar *Gr* gene expression code [116]. This observation is consistent with electrophysiological studies that found distinct sugar tuning profiles for taste neurons associated with different labellar taste sense [85] . Moreover, our study also confirmed expression of *Gr5a* in non-sweet GRNs, raising the possibility that this gene has additional roles in taste.

In this paper, we performed a comprehensive Ca^{2+} imaging analysis of the *Drosophila* sweet taste receptor repertoire. Work from numerous laboratories suggested that sugar taste receptors are multimeric complexes composed of two different Gr subunits [116, 118, 176, 208]. Using a fly strain in which most sweet GRNs were rendered sugar-insensitive by virtue of mutations in all eight sugar *Gr* genes (i.e. the octuple mutant strain), we carried out Ca^{2+} imaging experiments on tarsal sweet GRNs that expressed all 28 possible pairwise combinations of sugar *Gr* genes by measuring the responses to eight different sugars. We found that many receptor pairs were able to generate strong responses to a small subset of sugars. Importantly, we found for each sugar at least one receptor pair capable of generating as strong a response as observed in neurons of heterozygous control flies. In addition, we conducted a limited number of experiments in which three sugar *Gr* genes were co-expressed simultaneously, which indicated that for at least some sugars, complexes consisting of more than two *Gr* genes yield novel responses than were not observed by any pairwise combinations from the respective genes. Given that most sweet GRNs express up to six sugar *Gr* genes, our experiments suggest sugar receptors function as multimeric complexes that contain at least three Gr subunits. Lastly, we were able to confer sugar sensitivity to bitter GRNs by expressing pairs of sugar Gr proteins. However, not all combinations successfully conferred sugar sensitivity to bitter GRNs, implying that the presence of bitter GR in these cells likely interferes with the assembly of some sugar receptor complexes.

Results

Heterologous expression systems are ideal to elucidate the requirement of specific receptor components and determine their response profiles. However, such strategies have largely failed for *Gr* genes [118]. Instead, most studies relied on loss of function mutations in varies sugar *Gr* genes, especially P-element insertion mutations into the 5' region of the *Gr64* locus (*Gr64a-f*), and electrophysiological and behavioral analyses of receptive taste sensilla and flies, respectively. The major caveat of these studies is linked to the organization of the *Gr64* locus, in which six of the eight sugar *Gr* genes are tightly clustered and, hence, are not easily amenable to loss of function analysis [74]. Indeed, these studies did not lead to much of a consensus as to their specific roles in sugar taste [118, 176]. While the precise replacement of individual *Gr64* coding sequences by GAL4 or LEXA provided important new information on their specific expression, the homozygous condition of these alleles provided only limited information about their specific roles in sugar taste. Specifically, these alleles fail to clarify questions about the actual composition of specific receptor complexes, or the number of functional receptors that might be formed in sweet taste neurons.

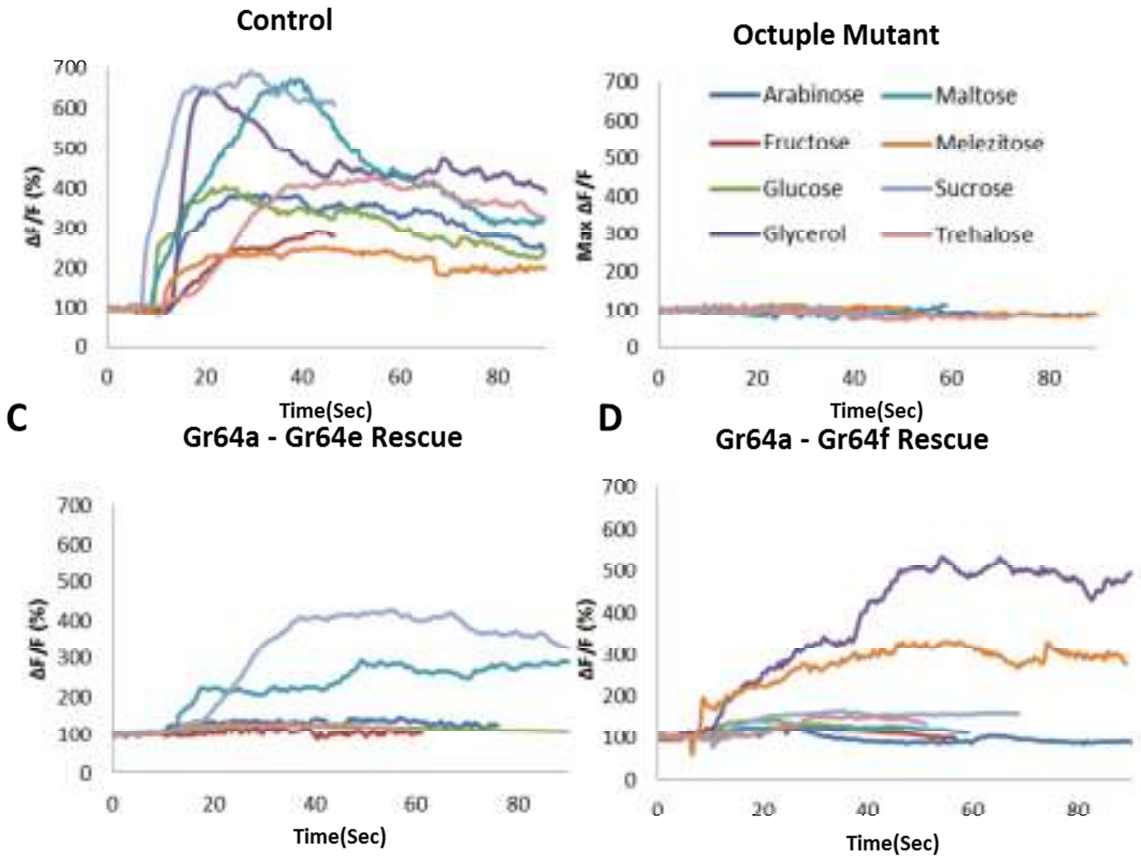

Figure 19 Expression of Gr gene pairs in sweet GRNs reconstitutes sugar responses in octuple mutant flies.

Ca^{2+} traces of the 5b-associated sweet GRN of heterozygous control flies (A), octuple mutant flies (B) and octuple mutant flies expressing Gr64a-Gr64e (C), Gr64a-Gr64f (D) after application of the eight indicated sugar ligands. Different Gr pairs elicit responses to different subsets of sugars. Note that responses to some sugars are stronger than those observed in control neurons (see also text). Generic genotype of flies is *$Gr5a^{LEXA}$/$Gr5a^{LEXA}$ R1; Gr61a-GAL4 UAS-GCaMP6/UAS-GrX UAS-GrY; ΔGr61a ΔGr64a-f/ΔGr61a ΔGr64a-f*, whereby *GrX* and *GrY* are the two Gr coding sequences indicated in each panel.

All sugar Gr proteins participate in sweet taste receptor formation

To address the important questions outlined above, we reversed strategy and used a gain of function approach by exploiting the features of an octuple mutant strain, in which all eight sugar *Gr* genes were eliminated [208]. We earlier showed that taste neurons in this strain lacked any sugar taste response when subjected to Ca^{2+} imaging experiments against a panel of eight sugars [208]. Moreover, expression of single sugar *Gr* genes also failed to restore responses to any of these sugars. These observations strongly argued for multimeric sugar receptors composed of at least two Gr proteins.

To obtain a comprehensive view on the potential of dimeric receptor complexes, we expressed all 28 possible pairwise combinations of the eight sugar *Gr* genes (Table 3), along with *UAS-GCaMP6.0*, under the control of the *Gr61a-GAL4* driver in GRNs of octuple mutant flies and performed Ca^{2+} imaging experiments in the sweet GRNs of the three main pairs of taste sensilla on the fifth tarsal segment after application of 100 mM sugar (Figure 19). 100mM was chosen because it is a concentration in the dynamic response range [74]. These experiments revealed two important features: First, all sugars can be effectively recognized by at least one pair of Gr proteins. Specifically, we find that at least one Gr pair, and quite often several, restored Ca^{2+} responses to a given sugar to the same level observed for GRNs of heterozygous mutant (control) flies (Table 3). Moreover, every single Gr protein is represented in these pairs at least once, and one pair (Gr64a/Gr64e) mediated maximal responses to two sugars (sucrose and maltose). Intriguingly, 18 of the 28 receptor pairs mediated responses of 50% or more of the response observed in controls to at least one of the eight sugars, and only five pairs

(Gr64a/Gr64d, Gr64b/Gr64c, Gr64c/Gr64e, Gr64e/Gr61a and Gr64b/Gr5a) failed to produce responses to any sugar of 10% or more of that of control flies. These observations suggest that pairs of Grs, as opposed of any single Gr protein, can form functional receptors to detect all sugar we tested, and that all Gr proteins appear to be required to participate in at least one such functional receptor. The second feature that emerged from this analysis is that the sugar receptors are redundant, i.e. most sugars are detected with high affinity by more than one sugar Gr pair. With the exception of arabinose and maltose, at least two different combinations of Gr proteins elicited Ca^{2+} responses of 80% or more of that observed in GNRs of heterozygous control flies. Arabinose, which is non-nutritious, and maltose are rare sugars in natural food sources, while all other sugars, as well as glycerol, are found abundantly in either fruit or yeast. Thus, the dietary sugars tested in our Ca^{2+} imaging experiments are recognized by at least two different pairs of Gr proteins, providing a safety feature for the detection of dietary carbohydrates.

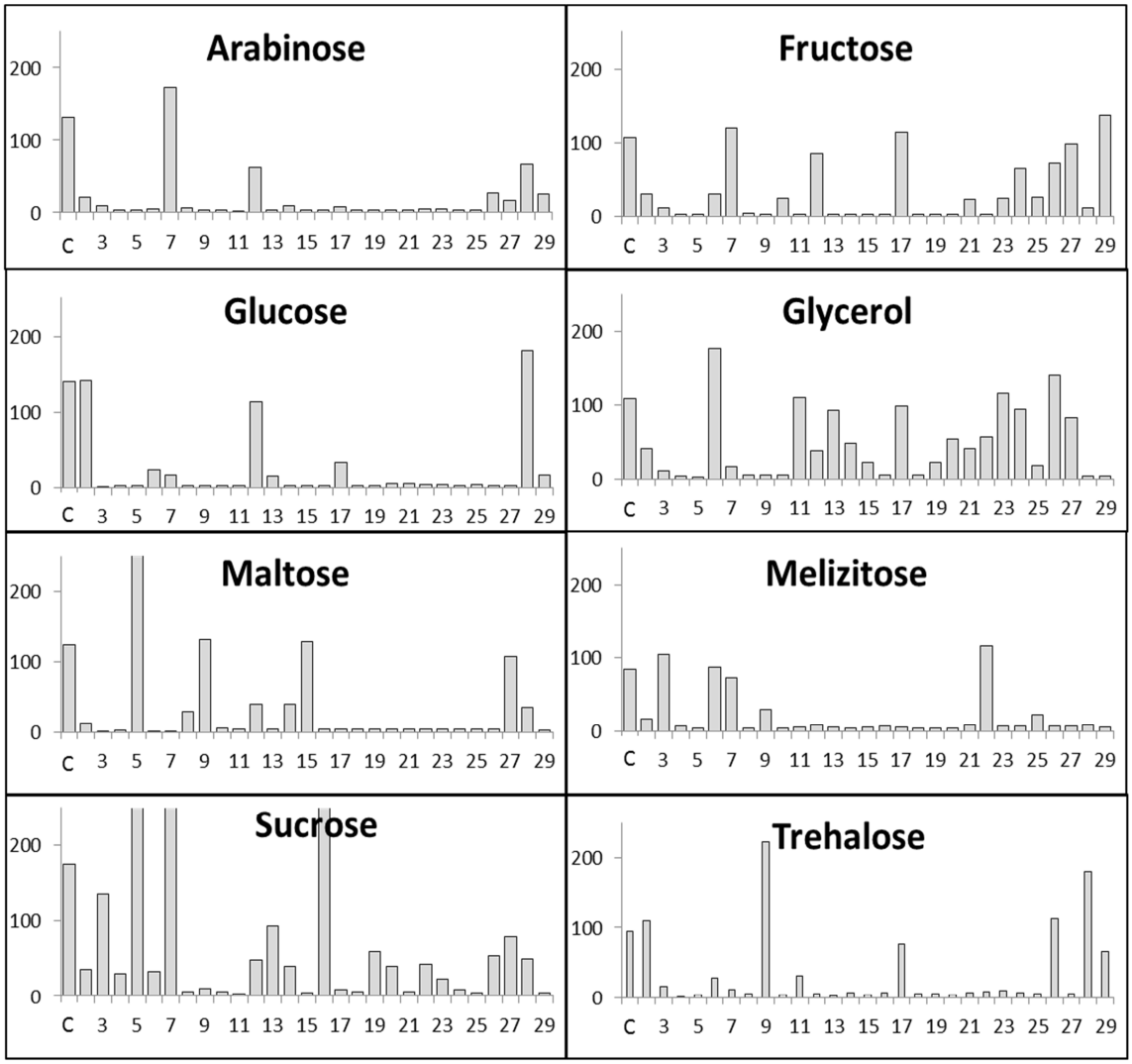

Figure 20 Ca^{2+} responses of sweet GRNs to individual sugars obtained from all 28 possible pairwise combinations of Gr proteins expressed in the empty neuron.

A) Ca2+ response (shown % increase of ΔF/F) in 5b-associated sweet GRNs of octpule mutant flies of the genotypes 1 to 28 (for key, see bottom). Generic genotype is Gr5aLEXA/Gr5aLEXA R1; Gr61a-GAL4 UAS-GCaMP6/UAS-GrX UAS-GrY; ΔGr61a ΔGr64a-f/ΔGr61a ΔGr64a-f, whereby GrX and GrY are two of the eight sugar Gr coding sequences. C indicates response of heterozygous control flies (+/Gr5aLEXA R1; Gr61a-GAL4 UAS-GCaMP6/+; +/ΔGr61a ΔGr64a-f).

B) Grid-display of the data shown in A). X and Y axes show individual sugar Gr genes, and each of the 28 pairs is represented by a column. The pair with the highest response to each sugar is indicated. Color code of activation is shown between the top two grids. Note that for most sugars, only a few pairs (< 4) elicit response comparable to heterozygous controls (> 50%), while a comparably large number of pairs (6 to 8) elicited such responses for fructose and glycerol.

Figure 20 Continued

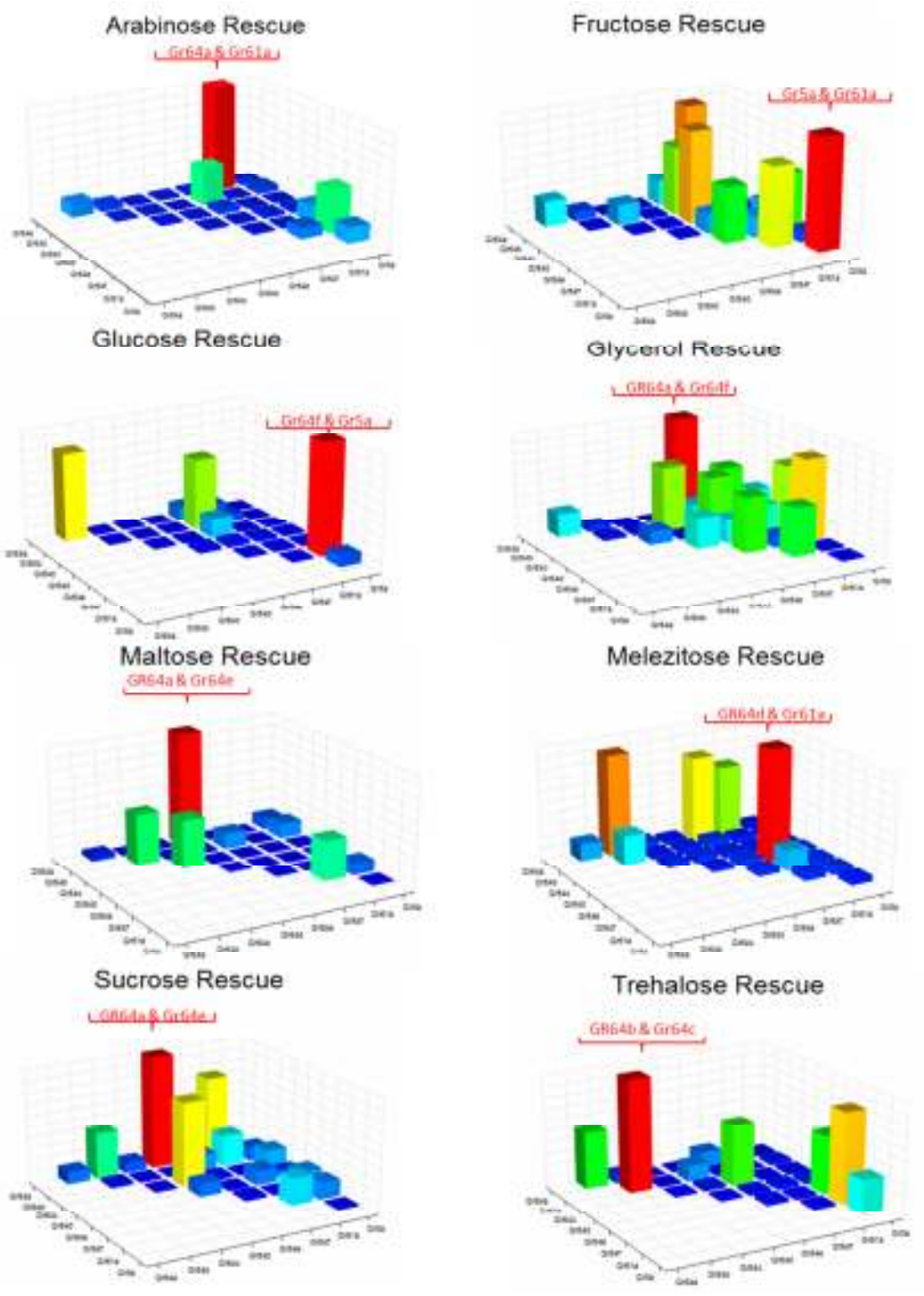

Figure 20 Continued

Table 3 Average ΔF/F values of possible pairwise combinations.
Top left corner of each table shows the sugar label and responses for control flies (Gr5a^{LexA}/+; Gr61a-GAL4:UASGCaMP6.0m /+; 16101/+) Sugars: Ara-Arabinose, Fru-Frucose, Glu-Glycerol, Mal- Maltose, Mel- Melezitose, Suc-Sucrose, Tre-Trehalose.

Fru 107 / Ara 131	64a	64b	64c	64d	64e	64f	61a	5a
64a		22					173	
64b	30					63		
64c								
64d		24						
64e								27
64f	31	85	115	23	66			67
61a	120				25	99		25
5a				25	73	11	136	

Gly 109 / Glu 140	64a	64b	64c	64d	64e	64f	61a	5a
64a		141				23		
64b	42					114		
64c	10					34		
64d			22					
64e		110		54				
64f	177	40	99	43	94			181
61a		93		58	18	84		17
5a		49	23	117	141			

Mel 85 / Mal 123	64a	64b	64c	64d	64e	64f	61a	5a
64a					300			29
64b			132			40		40
64c	105	30		128				
64d								
64e								
64f	87						108	35
61a	73			117	22			
5a								

Tre 95 / Suc 175	64a	64b	64c	64d	64e	64f	61a	5a
64a		36	136	30	342	32	250	
64b	110					47	93	40
64c	16	223			257	10		59
64d					39		43	23
64e		31						54
64f	29		122				79	50
61a								
5a					113	180	66	

Tripartite sugar receptors

Previous studies [74, 117, 118, 176], and the data presented here, clearly indicate that sugar receptors are multimeric complexes that are composed of at least two GR proteins. However, whether these receptors are dimers or multimers cannot be determined in the absence of biochemical studies or more elaborate genetic studies. Interestingly, genetic evidence suggests that at least some Gr-based bitter receptors are multimers composed of three subunits [109, 110, 209-211]. In addition, olfactory receptors (Ors), which are structurally related to the Grs, have been proposed to function as tetrameric complexes consisting of two Orco subunits and two subunits of the same ligand-binding, specific Or protein[212]. Given that most GRNs express four or more different sugar *Gr* genes [116], we explored the possibility whether expression of three sugar *Gr* genes (i.e. *ABC*) altered the sugar response profile, compared to expression of the three respective *Gr* pairs alone (*AB*, *AC* and *BC*). We tested this hypothesis by pairing *Gr64f* and *Gr5a* with four of the six remaining sugar *Gr* genes, creating four tripartite combinations: *Gr5a/Gr64a/Gr64f*, *Gr5a/Gr64b/Gr64f*, *Gr5a/Gr64d/Gr64f* and *Gr5a/Gr64a/Gr61f* (Figure 23). To quantify the response profiles of these tripartite receptors, we directly compared responses obtained from each sugar of GRNs expressing the three receptors to the responses of the most potent sugar *Gr* pair. The profile most consistent with dimeric receptors (or multimeric receptors composed of two different subunits) was obtained with the triple combination Gr5a, Gr64a and Gr64f. Here, the response to individual sugars of the triple combination was similar to that obtained with the best Gr pair. In contrast, the three other combinations elicited a strong response to at least one novel

sugar that was not a suitable ligand for any of the three respective sugar Gr pairs (Figure 23). This strongly suggests that in these three cases, the combination of three Gr proteins leads to a receptor containing at least one of these subunits, and that this receptor is likely to occur in a neuron expressing multiple Grs, at the expense of complexes only containing two different subunits.

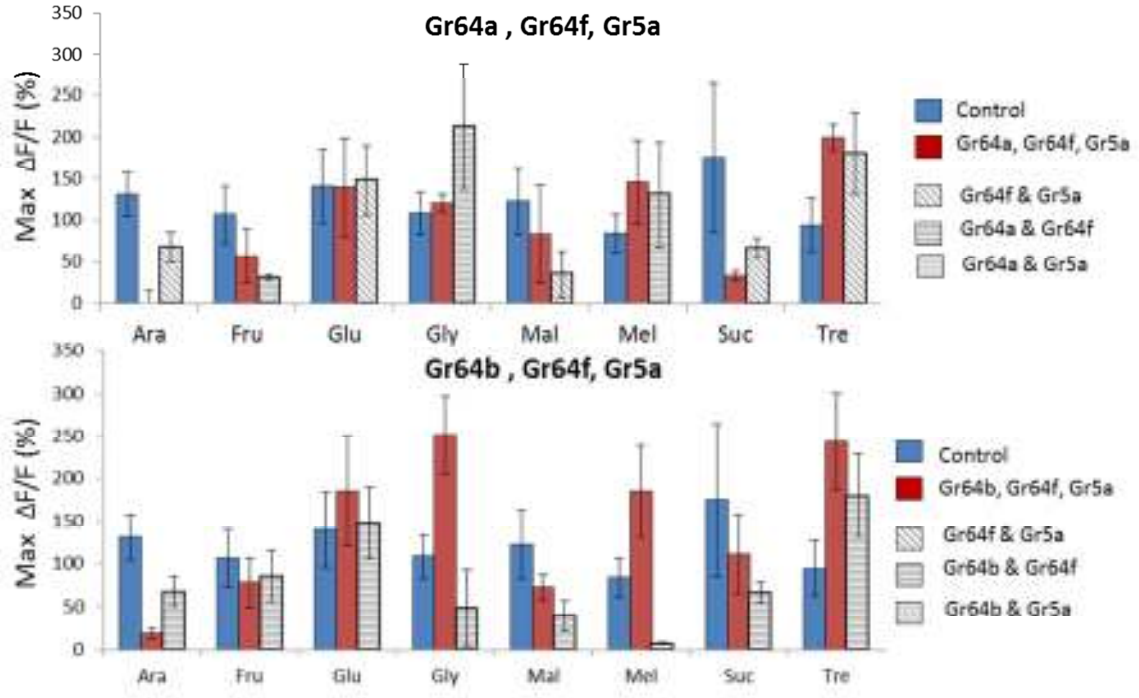

Figure 21Tripartite sugar receptors.
Selected sugar Grs (combination of Gr5a, Gr64f and a third gene is from Gr61 a-e and 61a) were rescued in the 5b associated sensilla in triple combinations. A) Gr64a, Gr64f and Gr5a. B) Gr64b, Gr64f and Gr5a. C) Gr64d, Gr64f and Gr5a. D) Gr61a, Gr64f and Gr5a. Representative fluorescence traces (bottom) and corresponding Ca2+ responses (top). The driver used for the experiments were Gr61a-Gal4. All sugar concentrations were at 100mM.
Genotype: UAS-Gr64F: Gr5aLexA; Gr61a-GAL4:UASGCaMP6.0m / UAS-Gr (A-E and 61a) ; 16101:UAS-Gr5a /16101 .

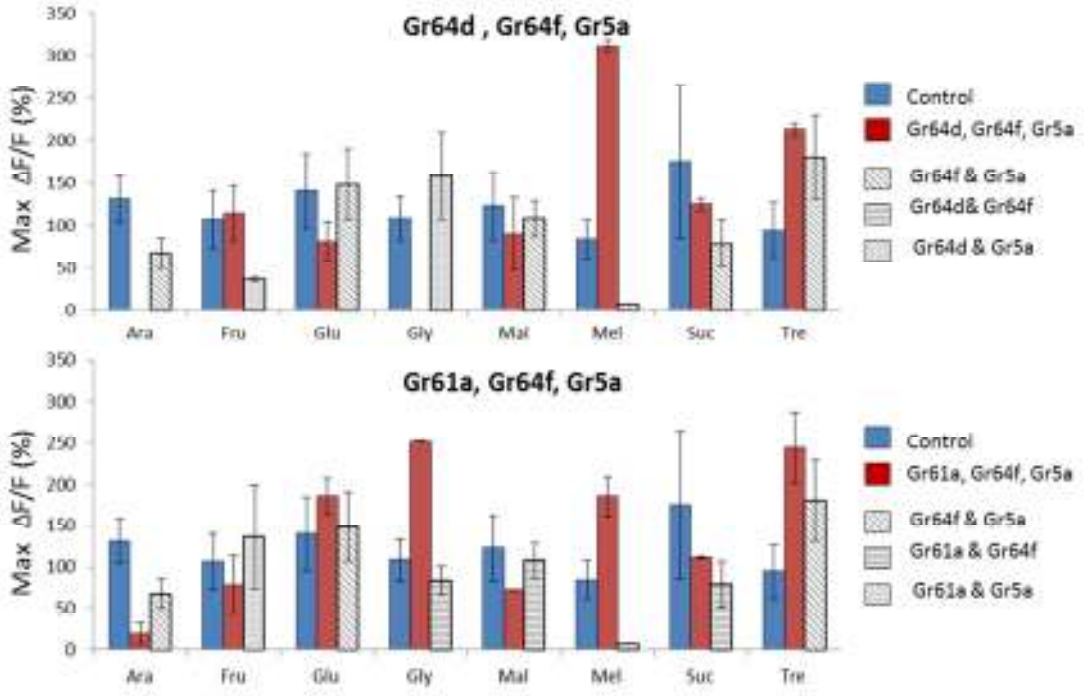

Figure 21 Continued

Reconstitution of sugar receptors in bitter GRNs

A major challenge in identifying ligands of insect taste receptors is the lack of an effective heterologous expression system. Potential alternatives, such as functional expression of bitter *Gr* genes in sweet GRNs, or sugar *Gr* genes in bitter GRNs have been largely although not entirely unsuccessful[118] . Having identified specific combinations of sugar Gr proteins that restored responses to specific sugars in GRNs of octuple mutant flies, we tested whether such Gr protein pairs can confer responses to bitter GRNs when expressed under control of the bitter GRN specific driver *Gr33a-*

GAL4. We selected five pairs of Gr proteins that restored Ca^{2+} responses to glucose (Gr64a/Gr64b and Gr61a/5a), maltose and sucrose (Gr64a/Gr64e), glycerol (Gr64e/Gr5a) and melezitose (Gr64A/Gr64C), as well as three pairs that failed to restore any sugar responses (Gr64a/Gr5a and Gr64b/Gr61a and Gr64d/Gr5a) in sweet taste GRNs of octuple mutant flies (Figure 4). Indeed, four of the five identified sugar Gr pairs (Gr64a/Gr64b, Gr64a/Gr64e, Gr64e/Gr5a and Gr61a/Gr5a) elicited specific responses reminiscent of their profile in sweet GRNs of octuple mutant flies, while one pair failed to respond to the sugar identified in sweet GRNs (Gr64a/Gr64c to melezitose), or any other sugar (Figure 24). Intriguingly, bitter GRNs expressing Gr61a/Gr5a, which in sweet GRNs elicits fairly specific responses to fructose (Figure 22), also responded to glucose with similar intensity (Figure 24). Two of the three pairs (Gr64b/Gr61a and Gr64D/Gr5a) that elicited only weak, if any, Ca^{2+} responses to any sugar in sweet GRNs (Figure 22A,B) also failed to elicit responses to any sugars in bitter GRNs, but one of these pairs (Gr64a/Gr5a) elicited strong Ca^{2+} responses to maltose in bitter GRNs (Figure 3b). Together, these observations indicate that sugar receptors can function effectively in bitter GRNs, but that under the complex representation of many other (bitter) Gr proteins, some of these sugar receptors are not efficiently formed. Most surprisingly, the gain of responses to some sugar in bitter GRNs suggests that Gr proteins might participate in the formation of hybrid receptors that can respond to sugars.

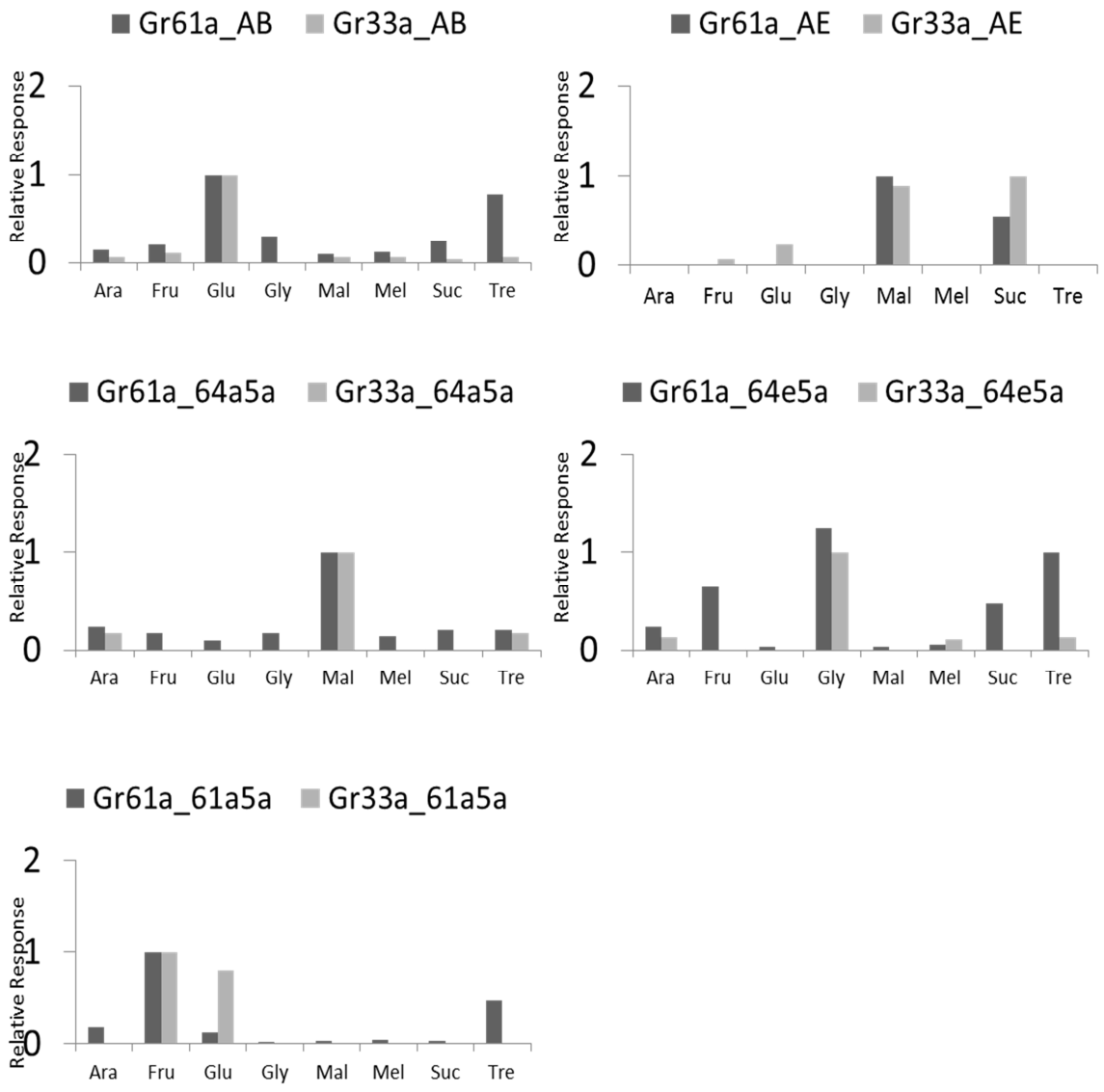

Figure 22 Selected sugar Gr pairs were rescued in the bitter ($Gr33a^{Gal4}$).
Normalized Ca^{2+} responses of the 5b associated bitter GRNs upon stimulation by indicated ligands. Compared to normalized response pattern of sweet GRNs.1 mM denatonium was used as a positive ligand control for bitter response. Genotype: Bitter neuron control: *$Gr33a^{GAL4}$ UAS-GCaMP6m/+,* Sugar *Gr* rescue *: $Gr33a^{GAL4}$ UAS-GCaMP6m/UAS-GrX-Y.*

Discussion

Heterologous expression systems have been successful in identifying ligands for mammalian taste receptors. However, numerous attempts to employ such strategies for insect taste receptors have failed, and to date, only a single *Drosophila Gr* gene has been successfully expressed in heterologous cells [125]. This rare success is likely due to the fact that this receptor, Gr43a, functions without the contribution of any other Gr protein, mainly as a nutrient sensor for hemolymph fructose in a few neurons in the brain [120]. In contrast, numerous genetic and electrophysiological studies have indicated that bitter and sugar receptors are multimeric complexes, composed of two or more subunits [109, 211]. In this paper, we have taken advantage of an octuple mutant, "sugar blind" *Drosophila* strain that lacks all eight classical sugar receptor, which are the only *Gr* genes known to be express in most but not all sweet GRNs (*Gr43a* is expressed in the sweet GRN of the 5v sensillum) [120]. Thus, this strain provides an experimental system that is comparable to the "empty neuron system" that was instrumental in deorphanizing olfactory receptors. We note that in contrast to these olfactory neurons, which do not express any other olfactory receptors, the sweet GRNs in the taste system do express members of the IR protein family [49, 213]. As there is no evidence that members of these two protein families interact with each other, our data interpretation were made under the assumption that IRs do not interfere with Grs in the formation of mutlimeric Gr receptor complexes.

A single dimeric G-protein coupled receptor complex mediates the sweet taste of sugar, sweet tasting proteins and artificial sweeteners in sweet taste cells in humans,

mice and many other mammals [58, 65, 214]. In contrast, many insects appear to employ up to a dozen or more putative sugar Gr proteins, and the in the fruitfly, it has become clear that all eight putative sugar *Gr* genes contribute to sweet taste [74, 116, 208]. Our investigations presented here revealed some new and conclusive insights into the nature of multimeric *Drosophila* sugar receptors and the sweet GRNs in which they are expressed. When reintroduced as pairs or triplets, we find that Gr proteins can assemble into many specific receptor complexes, each tuned to generally a small subset of or a single sugar. Importantly, each Gr protein is part of at least one specific receptor complex. Lastly, bitter and sweet GRNs are employ the same signaling mechanism, as we have successfully expressed sugar Gr combinations in bitter GRNs and making them responsive to sugars.

A large number of sugar receptor functions in sweet GRNs

18 out of 28 different pairwise *Gr* combinations expressed in the empty neuron reconstituted functional receptors, eliciting responses equal or higher than GRNs of heterozygous controls to one or two sugars. We note that each Gr proteins contributes to at least three functional bipartite sugar receptor complexes when expressed with a partner in the empty neuron, and several subunits can pair with five or six Grs (Figure 25). Specifically, Gr61a, Gr64a and Gr64f are the most versatile, forming functional complexes with 5, 4 and 6 different other subunits, respectively, and mediating responses to seven or all eight of the sugar we tested. Henceforth, we refer to these as major subunits. However, even the five remaining (i.e. minor) subunit, play crucial roles

in the recognition of specific sugars: for example the Gr64e-Gr64a pair was the most effective complex for the detection of maltose and sucrose, while the Gr64c-Gr64b combination mediated the strongest response to trehalose (Figure 22). We emphasize that even though these experiments show that pairwise combinations can form functional sugar receptors in the empty neuron, they do not prove their existence in a wild type GRNs, which express anywhere from four to eight sugar Grs. Indeed, our experiments whereby three subunits are co-expressed simultaneously argue for more complex receptors that might contain at least three different Gr proteins (Figure 25, see below).

The sugar receptors in wild type sweet GRNs

Reconstitution of Gr pairs in octuple mutant sweet GRNs led in numerous cases to higher responses to a specific sugar when compared to control GRNs, having an all eight sugar *Gr* genes. This observation cannot be interpreted that the particular bipartite combination is a more potent, higher affinity sugar receptor than the one present in wild type sweet GRNs for that particular sugar. For example, there are large quantitative differences in the two situations: In the empty neuron, only one possible pair can be formed, while in a neuron expressing four Grs, six different pairs can be formed, and neurons expressing all 8 can form 28 different pairs. Thus, in the two latter context, different Gr proteins compete with each other for other Gr partners, while in the empty neuron system with only two *Gr* genes provided as transgenes, that competition has been voided. In addition, the level of *Gr* gene expression in the empty neuron is driven via the

GAL4 system, which is known to provide higher levels of expression than endogenous *Gr* promoters.

The work presented here proves that functional sugar receptors are composed of multiple Gr proteins. While the majority of Gr pairs can restore responses to at least one sugar, our data strongly argues that sugar receptors are complexes of more than two different Gr subunits. Comparing the response profile of empty neurons expressing three Gr genes (ABC) vs empty neurons expressing the three possible pairs of the same group of genes (AB, AC and BC, respectively), we find that in three out of four triple combinations, GRNs expressing three Gr proteins simultaneously not only exhibit stronger responses to sugars than that observed by any of the Gr pairs, but in addition also responded to a sugar that failed to activate neurons expressing any of three respective Gr pairs. This observation argues strongly that complexes containing all three Grs are formed at the expense of complexes containing Gr pairs. Whether the broader tuning profile of GRNs expressing three Grs is indeed due to the presence of the tripartite complexes alone, however, cannot be assessed directly, as it is possible that such neurons still express discrete bipartite receptor complexes, which could be responsible for responses to some of the sugars. Nevertheless, the observation that in three of four cases, we find new sugar responses in tripartite configuration implies that sugar receptors contain three Gr proteins in wild type GRNs. Based on these data and on previous functional studies on *Drosophila* bitter receptors [109, 211] and Or-based olfactory receptors, reported to form ligand-gated ion channels proposed to contain four (or eight) Gr subunits, we propose that sugar receptor are multimeric channels most

likely to contain four subunits. Because three major Gr proteins – Gr61a, Gr64a and Gr64f –participate more prominently in sugar sensing, these Gr proteins might collectively have a role similar as that of the obligate Orco subunits in olfactory receptors, while the remaining two subunits in a complex might be represented by any two other Gr subunits. This model makes an important prediction, which is that a fly mutant for *Gr61a*, *Gr64a* and *Gr64f* is expected to have a more severe phenotype than any other triple mutant. In taste neurons of such flies, we expect that the only remaining (and minor) Gr proteins are not present at high enough level to efficiently reconstitute functional receptors, which they can do when expressed at higher levels through the GAL4/UAS system.

Interactions of bitter and sugar Gr subunits

Until now, sugar *Gr* genes have not been functionally expressed in bitter GRNs, and conversely, only in a couple of cases were bitter *Gr* genes expressed successfully in sweet GRNs. Several reasons could account for these difficulties, including the multimeric nature of these complexes and the immensely complex endogenous repertoire present in these cells (especially in bitter GRNs, many of which express even more *Gr* genes than sweet GRNs; [107]. The latter of these possibilities raises the prospect of formation of non-functional "hybrid" complexes between members of the two Gr types that might compete in the formation of functional sugar and bitter GR complexes respectively. Our analysis supports this possibility: Gr64a/Gr64c elicited strong responses to melizitose in sweet GRNs, but failed to elicit any significant

response in bitter GRNs, and the other four pairs that elicit responses in sweet GRNs to specific sugars were generally less potent in bitter GRNs, and often elicited responses to only a subset to the sugars identified as ligand in the sweet GRN. Interestingly, one pair, Gr61a/Gr5a, which elicits no response to maltose did so when expressed in the bitter GRNs, suggesting that a "hybrid" receptor was not only formed but able to functionally respond to this sugar. Taken together, these experiments show that interactions between the two Gr types are quite likely to occur when given the opportunity by forcing their co-expression in the same neuron.

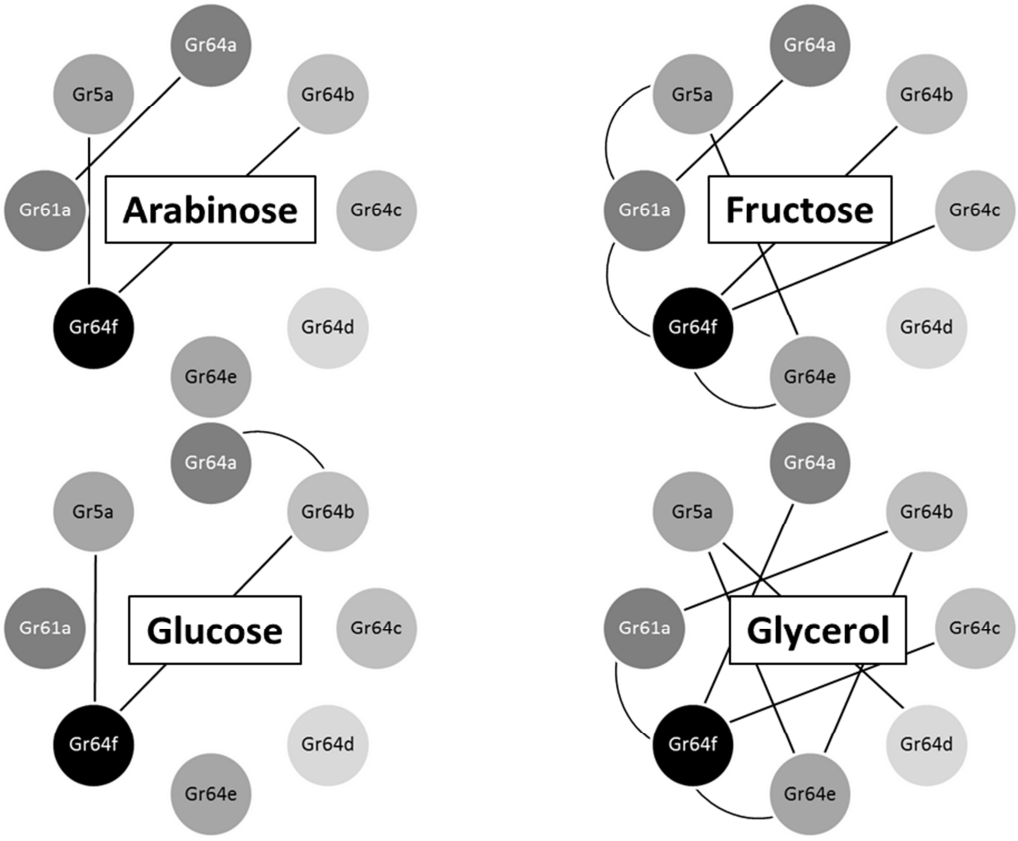

Figure 23 Prevalence of different Gr subunits in functional bipartite sugar receptors.

For each sugar, paring to form functional receptor in the empty neuron are indicated by connecting lines. Gr subunits that can participate in receptors for 3 sugars are shown in

light gray, for 4 sugars medium gray, for 5 sugars in dark gray, and for six sugars in black. Only pairs that elicit >50% of control heterozygous GRNs are shown.

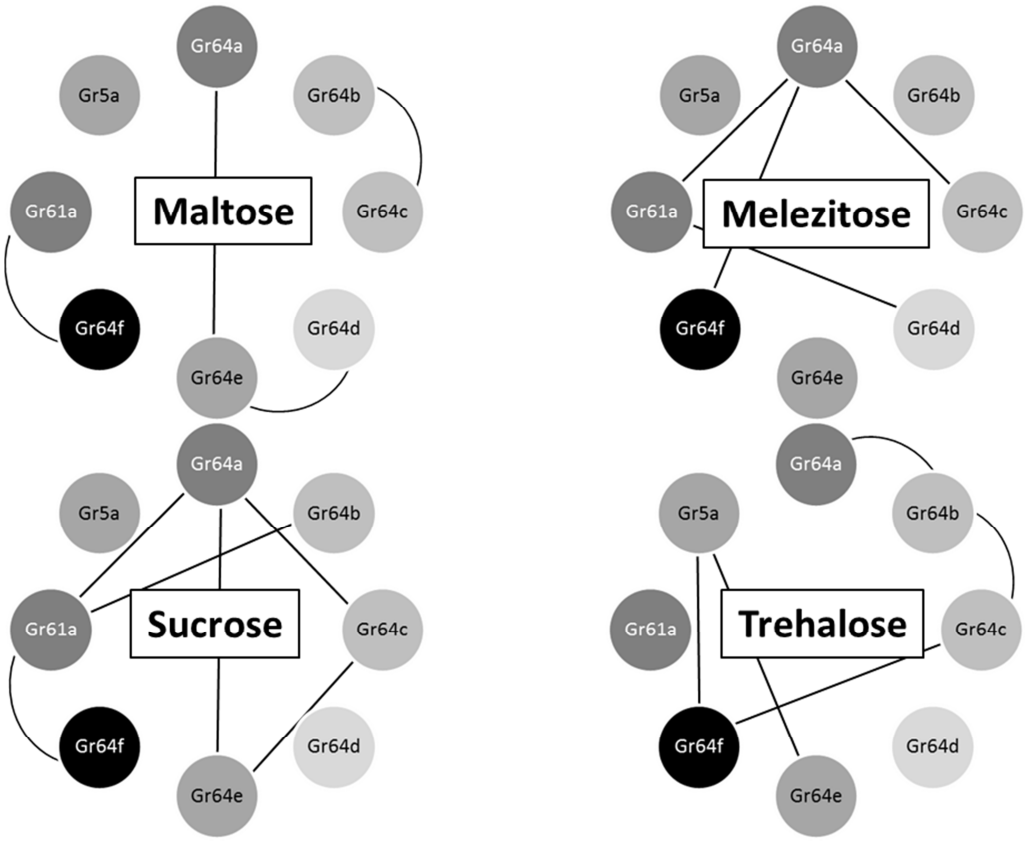

Figure 25 continued

Experimental procedures

Fly stocks

Fly lines were maintained on standard corn meal food under a 12 hours light/dark cycle at 25^{o} C. The heterozygous octuple mutant and w^{1118} strains were used as negative and positive controls for Ca^{2+} imaging. *Gr61a-GAL4* [119] and *Gr64f-GAL4* [176] were used as drivers in Ca^{2+} imaging assays. *UAS-GCaMP6.0m* (BDSC # 42750) used as a Ca^{2+} indicator. Octuple mutant flies were R1 $Gr5a^{LexA}$; ; *ΔGr61a ΔGr64a-f*.
Ca^{2+} imaging data of sweet neuron were obtained from the progeny of flies of the following genotypes: $Gr5a^{LexA}$; UAS-Gr (A-E and 61a)/Cyo; *ΔGr61a ΔGr64a-f* /TM6B and $Gr5a^{LexA}$; Gr61a-GAL4:UASGCaMP6.0m; *ΔGr61a ΔGr64a-f* /TM6B. For rescue lines involving Gr64f, UAS-Gr64F:$Gr5a^{LexA}$; Gr61a-GAL4:UASGCaMP6.0m /Cyo; *ΔGr61a ΔGr64a-f* /TM6B and for rescue lines involving Gr5a, $Gr5a^{LexA}$;+/Cyo; *ΔGr61a ΔGr64a-f*: UAS-Gr5a:/TM6B were used [208].

Chemicals

Crystalline (D) form of sugars (except for Arabinose, for which the (L) form is the more abundant form in nature), were purchased from Sigma-Aldrich, with purity >99%. Denatonium benzoate (Sigma-Aldrich #D5765)) was of >98% purity. Sugars stock solutions (200 mM) were prepared each week in MilliQ water and kept at 4^{o}C.

Calcium imaging

Preparation of forelegs and Ca^{2+} imaging of taste sensilla was performed as described by Miyamoto et al. [119]. Briefly, flies were collected on the day of eclosion and kept on standard food for 2 to 8 days. The foreleg of female flies was cut between the femur and

the tibia with a razor blade. The distal end of the cut leg was dipped in silicone oil (Dow Corning) and placed laterally on double-sided scotch tape that was attached to a glass bottom dish (MatTek Corporation). The tibia and the first three tarsal segments of the leg was placed on the tape while the fourth and fifth tarsal segments were left hanging. The leg on the tape was covered with 1% agarose to keep the preparation still. The whole preparation was then covered with 100 µl of water and immediately used for imaging with a Nikon eclipse Ti inverted microscope. The ligands were applied at 10 seconds after recording started and in a none-responsive sample imaging was terminated at 90 seconds. For responsive sample, imaging was continued for 2 minutes. The imaging frequency was 0.5 seconds. Two to four different ligands were tested in each preparation, before testing with fructose or sucrose as a positive control. Preparations were washed with MilliQ water three times after a ligand was tested. The next test was initiated when the fluorescence level had returned to base level to assert that a lack of response was not due to desensitization. Preparations in which the sweet GRNs of the s - sensilla expressing the fructose receptor Gr43a did not respond to test ligand were discarded from the dataset.

CHAPTER V

SUMMARY AND DISCUSSION

Sweet taste of many insects, including the model system *Drosophila melanogaster*, is characterized by a relatively large repertoire of distinct taste receptor genes, which is a notable and perhaps unexpected deviation from sweet taste of mammals. In most mammals where this taste modality has been investigated, a single dimeric receptor consisting of T1R2 and T1R3 mediates not only sugar taste, but also the sweet perception of artificial sweeteners, as well as sweet tasting proteins [215]. In contrast, the genome of *Drosophila* and many other dipterian insects contain usually six to eight genes that are thought to mediate sweet taste. My dissertation aimed to find the molecular underpinnings of how the eight putative sugar Gr genes participate in and contribute to the sensation of sugars. In the first paper [116], we characterized the expression pattern of seven *Drosophila* sugar *Grs* and classified the respective sweet taste GRNs into eight different types, characterized by the specific expression profile of the sugar *Gr* genes. We also analyzed sweet taste behaviorally of flies carrying mutations for individual sugar *Gr* genes using the proboscis extension reflex assay (PER). Two clear features emerging from the behavioral analysis were the findings of functional redundancy between different genes and the lack of a predominant role for any of the eight sugar *Gr* genes. Our expression analysis also revealed that a few sugar *Grs* are non-canonically expressed in tissues not directly related to taste. *Gr5a*, *Gr64b* and *Gr64f* are expressed in the olfactory organs, whereas *Gr64a* is co- expressed with

Gr43a expressing, nutrient sensing neurons in the brain. Ca^{2+} imaging studies of these neurons suggests a potential role for Gr64a in internal nutrient sensing

In the second paper [208], we established a critical tool for the de-orphanization of the sugar Gr proteins. We generated an octuple mutant strain that lacked all sugar *Gr* genes and found that this strain is essentially sugar-blind when tested using PER. The hallmark of this strain is that most of its sweet GRNs are "empty" of functional Gr proteins (i.e. the empty neuron), and Ca^{2+} imaging confirmed that the empty neurons is no longer activated when exposed to any type of sugar. We then explored its potential as a decoder neuron by expressing single or select pairwise combination of sugar *Gr* genes. Only re-introduction of some (but not all) pairwise combinations, and none of the single Gr genes, restored Ca2+ responses to some sugars. These findings established that the empty neuron can be used a decoder and it also showed that single Gr proteins are not capable to function as sugar receptors. We also corrected a long standing, overreaching role of *Gr64a* in the field. Our functional analysis using calcium imaging and a specific mutation showed that *Gr64a* is not involved in perception of our eight tested sugars, as previously claimed

Lastly, in the third paper, we conducted a comprehensive analysis in which we reintroduced all 28 possible pairwise combinations of sugar *Gr* genes in the "empty neuron, followed by functional Ca^{2+} imaging. We found that 18 of these 29 combinations led to the reconstitution of distinct GRN responses, and we found at least one combination for each of the eight sugars that were used in our initial panel of wild type flies. In addition, we produced a selective set of flies that express three different

sugar *Gr* genes in the "empty neuron". Interestingly, this experiment revealed that for two of the four triple combination, responses to sugars were recovered that were absent with any of the three respective pairwise combinations, suggesting that at least some sugar receptors contain at least three different subunits.

How Gr works –Model

Gr5a mutant flies have been shown to have a trehalose insensitivity phenotype [112, 113]. Later works, showing the involvement of *Gr64* family members for trehalose perception, suggested that sugar *Grs* might work as heteromers [74, 118]. However, there was little evidence for how sugar receptors work. In my studies, I have shown that most sugar *Grs* co-express in an overlapping manner in sweet GRNs. Behavioral experiments with individual sugar *Gr* mutant fly suggested redundant function of these genes. I further demonstrated that at least two sugar *Grs* are sufficient for sugar taste in *Drosophila* and different pairs can be used to recognize a particular sugar. However, additional *Grs* might be involved to form the complete sugar Gr protein.

Future Directions

Our findings suggest the possibility of flies being able to differentiate between various sugars. Further studies are needed to find out to what extent they can do this and whether it affect the fly's behavior. If eight sugar *Grs* are needed to differentiate sugars, insects with fewer sugar *Grs* might not possess this ability, for example, the honeybee. If true, the connection of secondary order neurons to specific sugar sensilla might reveal ways

for flies to differentiate between sugars. Our study focused on the response profile toward eight biologically relevant sugars. However, the number of sugars flies encounter in their natural habitat is broader and therefore further work is needed to understand the complete sugar response repertoire of the fruitfly Surprisingly, flies' response profiles to natural and artificial sweeteners are highly similar to that of humans [1], therefore we could extend our sugar panel to include these sweeteners. Considering fly *Grs* and human taste receptors belong to different receptor families and some of these sweet molecules do not bind to sugar binding pockets [67], it is striking that the detection of sweet molecules developed such similar solutions. Fatty acid preference is also mediated through sugar neurons [45] and it is intriguing to consider whether flies are able to differentiate between sugars and fatty acids using the same neurons. If so, how is sugar and fatty acid perception resolved in the higher order neurons? It is also possible that resolution is happening at the chemosensory neuron level, which would require a revision to the "labeled-line" hypothesis.

Our work has identified some apparent redundancy amongst the *Drosophila* receptors with several pairs recognizing the same sugar. A bioinformatics analysis could be carried out on the external domains of these pairs to study common domains for binding specific sugars.

The empty neuron system has provided a powerful tool to analyze the function and composition of the *Drosophila* sugar receptors. We propose that this system is likely going to be useful in decoding the sugar receptor of other insect species. For example, it will be interesting to see how broadly tuned the only known pair of sugar *Gr* genes of hymenoptera mediate are in this *Drosophila* system. Will such neurons respond to all eight sugars we tested? Or can these Gr proteins function on their own, possibly as homo-multimeric complexes. Likewise, how do the many putative sugar *Gr* genes of mosquitos combine to form sugar receptor complexes? Male mosquitoes rely exclusively on nectar feeding, and are in need of high energy demands to form swarms and mate with females. Thus, learning about their potential feeding preferences could inform us about potential strategies to control their rapid propagation, which is of high relevance in the transmission of mosquito born diseases, such as dengue, malaria, yellow fever and zika.

Our analysis has also revealed broader, but still unknown functions for sugar *Gr* genes in olfaction and internal nutrient sensing. For example, do sugar Gr proteins expressed in olfactory neurons form complexes with Or proteins? If so, what are the ligands that such "hybrid" receptors bind? Likewise, the discovery that the fructose sensing neurons in the brain not only express *Gr43a*, but also *Gr64a*, suggests that these neurons are likely to sense more than just fructose, specifically given the fact that lack of *Gr64a* does not affect their response to this sugar. Our Ca^{2+} imaging experiments revealed two distinct subsets of fructose sensing neurons: high responders and low responders. Further analysis can ascertain whether this dichotomy represents a functional

division. Interestingly, a putative sugar *Gr* gene of *B. mori* has been shown to be co-expressed with the *Gr43a* ortholog in brain neurons of the silkworm, suggesting a conserved role for sugar Gr genes in the internal nutrient sensing [126].

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