Analysis of the *Drosophila* Sugar Receptor Genes

by

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University Program in Genetics and Genomics

Duke University

Date: ___December 1, 2009___

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Dan Tracey

Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the University Program in Genetics and Genomics in the Graduate School of Duke University

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ABSTRACT

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Abstract

Gustation, also known as taste perception, is critical for the survival of most animal species. The fruit fly *Drosophila melanogaster* employs 68 different gustatory receptors (GRs) for the detection of sugars, bitter or toxic compounds, and pheromones. However, with a few notable exceptions, the functions of most GRs involved in feeding are unknown. Our research has focused on a cluster of highly-related *Drosophila Gr* genes, known as the *Gr64* family, that have been shown to be critical for the perception of multiple sugars. Furthermore, we have demonstrated that another gene related to the *Gr64* genes, *Gr61a*, is a sugar receptor that is narrowly tuned to a subset of pyranose sugars and may (along with the *Gr64* genes) be indispensable for early fly development.

As a complementary approach to our behavioral analysis, we have examined the expression pattern of the *Drosophila* sugar receptors using knock-in driver alleles created by homologous recombination. As expected, most of these drivers have shown strong expression in various taste tissues. Intriguingly, some of these knock-in drivers are also expressed in the maxillary palp and antenna, tissues previously thought to be involved only in olfaction. These expression patterns raise interesting questions about the true range of function of these chemosensory receptors and whether or not they might be involved in olfaction as well as in gustation.
Dedication

To my family, who probably don’t get thanked enough.
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1. Chapter 1: Introduction

Careful, accurate observation of the environment is critical to the survival of all living organisms, from the simplest microbes to the most complex plants and animals. Different organisms have adopted a variety of strategies for accomplishing this goal. In the case of higher eukaryotes, such as insects and vertebrates, intricate networks composed of specialized neurons have evolved to detect and integrate diverse types of sensory stimuli. These sensory neurons mediate detection of light, temperature, volatile and non-volatile chemicals, sound, etc. Optimal performance from all sensory systems, as well as efficient and accurate integration of this information in higher regions of the central nervous system, provide an organism with a distinct advantage compared to its competitors.

Chemosensation, the detection of biologically relevant chemicals, is particularly vital to the health and survival of most living things. It is utilized by a wide variety of organisms to find mates, avoid predators, communicate with other members of the same species, and to discriminate between potential food sources and dangerous substances like poisons and noxious compounds. Because of its role in a variety of biological and ecological processes, chemosensation has received attention from researchers concerned with pest control and public health. Applied research into chemosensation is also of great interest to perfumers and food companies who are interested in making their products more attractive to consumers. Finally, many researchers study chemosensation out of a basic interest in how animals sense and understand the world around them.
The process of chemosensation in mammals and insects, while dissimilar in many important ways, also shares some interesting properties. Perhaps the most intriguing of these shared properties is the fact that many mammalian (Nelson et al. 2001; Zhao et al. 2003) and insect (Larsson et al. 2004; Benton et al. 2006) chemosensory receptors function as dimers or multimers of different protein subunits. It is also known that insects and mammals share a preference for sweet and salty substances (Falk and Atidia 1975), and an aversion for bitter substances (Thorne et al. 2004; Wang et al. 2004). Moreover, the organization of the insect and mammalian olfactory centers in the brain are also organized in a strikingly similar way (Ressler et al. 1994; Vassar et al. 1994; Mombaerts et al. 1996; Gao et al. 2000). That insect and mammalian chemosensation share so many different properties suggests that shared ecological pressures have pushed these organisms towards convergent evolution of their chemosensory systems. It also suggests that knowledge obtained from one system may be useful in understanding the other. This chapter will describe what is currently known about chemosensation in both types of animals and set the stage for the studies of insect taste perception that will be described in later chapters.

1.1 Two chemosensory pathways

Chemosensation can be divided into two general categories: olfaction and gustation. Olfaction (sense of smell) is the detection of volatile chemicals present in the air, while gustation (taste) is the detection of non-volatile chemicals in foods, in liquids and on the bodies of other animals. The distinction between these two categories of
chemosensation is not always clear. For example, carbon dioxide is detected by a variety of organisms as a gas in the air, but it can also be detected as “carbonation” when it is dissolved in water. However, chemicals that exist primarily in a volatile form and chemicals that exist primarily in a nonvolatile form tend to be broadly distinguishable.

Most animals have evolved separate sets of sensory organs devoted to detecting olfactory and gustatory stimuli, respectively. This separation holds true in both the peripheral nervous system (PNS) as well as in the central nervous system (CNS), with taste and olfactory sensory cells transmitting their signals to separate parts of the CNS. One example of this type of organization can be found in the mammalian chemosensory system, where the cranial nerves that transmit information from the taste cells to the brain project their axons to the nucleus of the solitary tract (Hamilton and Norgren 1984), while olfactory sensory neurons project to the main olfactory bulb (Clark 1951). Even within the olfactory system, there is a functional subdivision, with sensory neurons that express different olfactory receptors projecting their axons to separate, stereotyped structures within the olfactory bulb (referred to as glomeruli) (Ressler et al. 1994; Vassar et al. 1994; Mombaerts et al. 1996).

1.2 The fruit fly chemosensory system

*Drosophila* chemosensory neurons can be found in a variety of fly organs and tissues (Figure 1). As in other organisms, *Drosophila* chemosensation can be divided into olfactory and gustatory pathways.
1.2.1 *Drosophila* olfactory system

The antenna, the main *Drosophila* olfactory organ (Figure 1), consists of three segments. The third, most distal segment contains sensory sensilla that have long been known (based on electrophysiological evidence) to be involved in the detection of odorants (Ayer and Carlson 1992). These sensilla are divided into three classes—
basiconic, tricoid, and coeloconic—based on their size, shape, and spatial positioning on the third antennal segment (Venkatesh and Singh 1984). Electrophysiological analysis has demonstrated that, even within the same class, different sensilla exhibit specific response patterns when stimulated with different odorants (Clyne et al. 1997; de Bruyne et al. 2001) due to the different complements of olfactory receptor proteins expressed in the neurons within each sensilla. Unlike the third antennal segment, the two most proximal segments of the antenna are dedicated to non-olfactory functions such as hygrosensation (the detection of humidity) and sound perception.

In addition to the third antennal segment, flies possess an accessory olfactory organ on the proboscis called the maxillary palp (Figure 1). This organ is covered with basiconic sensilla that are known to respond to olfactory stimuli (Ayer and Carlson 1992). Interestingly, in some insect species the maxillary palp is used as a taste organ rather than an olfactory organ (Blaney and Duckett 1975).

1.2.2 Drosophila gustatory system

The main organ of the Drosophila melanogaster gustatory system is a sensory structure on the tip of the proboscis known as the labellum (Figure 1). It consists of a pair of mirror-symmetrical surfaces known as labial palps that are covered with 33-34 chemosensory sensilla (Figure 2A) (Nayak and Singh 1983). In addition to the external chemosensory sensilla of the labellum, there are less well-characterized sensory structures inside the proboscis known as taste pegs, as well as internal sensory organs such as the dorsal and ventral cibarial sensory organs and the labral sensory organ (Figure 1). Finally, there are taste sensilla located on the legs, as well as poorly characterized
chemosensory sensilla along the anterior wing margins and on the tips of the abdomens of female flies (Figure 1) (Falk et al. 1976; Nayak and Singh 1983; Stocker 1994; Shanbhag et al. 2001; Amrein and Thorne 2005).

The taste sensilla of the labial palp are usually classified into three groups—long (L-type), intermediate (I-type), and short (S-type)—based on their relative length (Figure 2A) (Hiroi et al. 2002). Out of the approximately 33 sensilla of the labial palp, roughly one-third of the total number of sensilla fall into each class (that is, there are about 10 sensilla per class) (Falk et al. 1976; Nayak and Singh 1983; Ray et al. 1993). Each class of labellar taste sensilla has a characteristic number of gustatory receptor neuron (GRNs), with the L and S-type sensilla containing four GRNs and the I-type sensilla containing just two (Hiroi et al. 2004). In addition to GRNs, each taste sensillum contains a mechanosensory neuron and several support cells (Falk et al. 1976; Nayak and Singh 1983; Ray et al. 1993).
Figure 2: Each chemosensory sensillum contains GRNs involved in sensing different types of ligands.

(A) The labial palp contains three types of taste sensilla: S (short), I (intermediate), and L (long). S and L-type sensilla contain four gustatory receptor neurons, while I-type sensilla contain only two GRNs. Image is a reprint from Thorne et al. (2004). (B) Each sensillum shown represents a hypothetical S or L-type sensillum containing a bitter, sugar, low-salt, and water-sensing neuron. Most Gr genes investigated so far seem to be involved in bitter sensation, with different bitter neurons containing different combinations of Grs. However, Gr5a, a trehalose receptor gene, has been shown to be expressed in a distinct set of neurons involved in sugar sensation. The Grs expressed in water and low-salt neurons, if any, are unknown. Each gustatory sensillum also contains a mechanosensory neuron and several support cells (not shown).

1.3 Electrophysiology of taste neurons

An important concern for many neuroscientists is how to determine when a neuron has responded to a stimulus. The most straightforward way of doing this is to directly record its electrical activity. When neurons respond to stimuli, they experience a change in the electrical potential of their membranes (membrane potential); this change in membrane potential is often referred to as an action potential. These action potentials can be observed as spikes in the membrane potential of the neurons, and are therefore also referred to as “spikes.” An increase in the number of spikes produced by a neuron over a given time interval is usually interpreted as a sign that the neuron has been activated by a stimulus.

Collectively, the field of study concerned with the measuring the electrical properties of neurons (or any other type of cell) is referred to as electrophysiology. In insects, there are two common ways that electrophysiological responses are measured in
taste neurons: the tip-recording method and the side-wall method (Figure 3). The tip-recording method, the favored technique in *Drosophila* taste research, involves placing a glass capillary tube on the tip of a taste sensillum (Hodgson et al. 1955). This capillary tube is filled with a special solution of electrolytes that allows changes in electrical activity to be transmitted to a recording electrode, while also permitting taste ligands to be introduced to the sensillum. The second recording method, the side-wall method, uses similar equipment as the tip-recording method, except that the recording electrode is pierced through the cuticle at the base of the sensillum (Morita and Yamashita 1959), while ligands are delivered to the tip of the sensillum by a separate glass capillary. This allows the electrical activity of the GRN cell bodies to be recorded, whereas the tip-recording method records the membrane potential in the dendrites of the gustatory neurons. The side-wall technique also has the advantage of allowing both hydrophobic and hydrophilic ligands to be delivered to the dendrite tip, whereas the tip-recording technique is limited to ligands that can be dissolved in aqueous solution. In both methods, an additional reference electrode is also placed into the body of the fly being tested, and the activity recorded by this electrode is subtracted from the activity recorded by the electrode attached to the sensillum. This allows spurious electrical activity unrelated to the ligand response to be eliminated from the analysis.

Since both recording techniques simultaneously measure the responses of all of the taste neurons in a sensillum, it is not always possible to determine which neuron is responding to a given stimulus. It has been observed, however, that different types of GRNs produce electrical spikes of different amplitude when they are activated, which
often allows the responding neuron to be identified among the total electrical signal coming from the sensillum (Rodrigues and Siddiqi 1981; Singh 1997; Meunier et al. 2003; Hiroi et al. 2004). For example, in the L and S-type sensilla that contain four GRNs, researchers have observed a water-sensing neuron (W cell), a low-salt-sensing neuron (L1 cell), a high-salt-sensing neuron that also detects bitter and aversive stimuli (L2 cell), and a sugar-sensing neuron that also detects other attractive stimuli (Figure 2B) (Rodrigues and Siddiqi 1981; Singh 1997; Meunier et al. 2003; Hiroi et al. 2004).

Figure 3: Electrophysiological recording techniques for Drosophila taste sensilla.

(A) Diagram of a typical S or L-type taste sensilla containing four GRNs (L2, L1, S, and W). GRN cell bodies are located near the base of the sensillum, while GRN dendrites extend out to the tip of the sensillum. The mechanosensory neuron and
support cells for the sensillum are not shown. (B-C) Two methods for detecting electrophysiological responses in taste sensilla. In the tip-recording method (B), the recording electrode is placed inside the glass capillary tube that is used to deliver ligands to the tip of the sensillum (Hodgson et al. 1955). In the side-wall technique (C), the recording electrode is separated from the capillary tube and is instead placed inside the cuticle, near the cell bodies of the GRNs (Morita and Yamashita 1959). In both techniques, a separate “indifferent” electrode (not shown) is placed elsewhere in the fly’s body to account for changes in electrical potential unrelated to the ligand response. (D) Sample recordings of action potentials recorded from sugar neurons using the tip-recording method. Image in Panel D is a reprint from Dahanukar et al (2007).

1.4 Chemosensory receptors

1.4.1 Mammalian chemosensory receptors

In order for a sensory neuron to perform its dedicated function, the cell must express one or more membrane-embedded proteins that detect the neuron’s ligand and then cause the neuron to generate a series of action potentials. In the mammalian chemosensory system, this function is performed almost entirely by G-protein-coupled-receptors (GPCRs), a class of receptors that function by producing intracellular signaling cascades involving another class of proteins called G-proteins. Mammalian olfactory neurons (Buck and Axel 1991) and vomeronasal sensory neurons (Dulac and Axel 1995; Matsunami and Buck 1997) are known to use GPCRs as their chemoreceptors. Those mammalian taste neurons involved in the sensation of sweet and umami (savory) compounds (Chaudhari et al. 2000; Max et al. 2001; Montmayeur et al. 2001; Nelson et al. 2001; Li et al. 2002; Nelson et al. 2002; Damak et al. 2003; Zhao et al. 2003; Xu et al. 2004), as well those that detect bitter stimuli (Adler et al. 2000; Chandrashekar et al. 2000; Matsunami et al. 2000), are also known to use members of the GPCR family.
However, neurons that sense salty and sour stimuli seem to use ion channels as receptors (Lyall et al. 2004; Huang et al. 2006; Ishimaru et al. 2006; LopezJimenez et al. 2006; Shigemura et al. 2008), indicating that mammalian chemosensory receptors are not exclusively GPCRs.

1.4.2 Drosophila chemosensory receptors

The first family of chemoreceptors to be discovered in Drosophila were the olfactory receptor (OR) proteins. They were revealed by the efforts of several groups using a combination of topology prediction algorithms and reiterative BLAST (Clyne et al. 1999; Gao and Chess 1999; Vosshall et al. 1999; Kim et al. 2000; Vosshall et al. 2000; Kim and Carlson 2002). Currently, there are 62 predicted olfactory receptor proteins (Robertson et al. 2003), with a relatively low amount of sequence conservation (on average, about 20%) across the entire family of proteins (Clyne et al. 1999). Most of the OR proteins have been deorphanized (that is, their ligands have been identified) by this point (Hallem et al. 2004; Goldman et al. 2005; Hallem and Carlson 2006). The “empty-neuron” expression system, which allows olfactory receptors to be deorphanized by expressing them in neurons that have had their native ORs genetically removed, has proven to be a particularly powerful system for characterizing these receptors (Dobritsa et al. 2003). Interestingly, it has been observed that olfactory sensory neurons (OSNs) that express the same unique OR converge to a common glomerulus in the Drosophila antennal lobe (Gao et al. 2000), which is strikingly similar to the organization of olfactory neuron projections in the mammalian olfactory bulb (Ressler et al. 1994; Vassar et al. 1994; Mombaerts et al. 1996).
Another group of predicted chemosensory receptors, the gustatory receptor (GR) proteins, were revealed shortly after the discovery of the OR proteins (Clyne et al. 2000; Dunipace et al. 2001; Scott et al. 2001; Kim and Carlson 2002). There are currently 68 predicted gustatory receptor proteins. One of the more interesting observations about these receptors is that phylogenetic analysis suggests that the OR proteins are actually an offshoot of the GR superfamily, rather than a completely independent group of proteins (Robertson et al. 2003). This suggestion of a common origin for the OR and GR families raises the possibility that there may be some overlap in the types of ligands detected by the two groups of receptors, with gustatory receptors detecting volatile ligands and olfactory receptors detecting non-volatile ligands.

Since the discovery of the GR family, a handful of GR proteins have been deorphanized, indicating that GRs are involved in detecting a wide variety of chemicals, including sugars (Dahanukar et al. 2001; Ueno et al. 2001; Chyb et al. 2003; Inomata et al. 2004; Isono et al. 2005; Dahanukar et al. 2007; Jiao et al. 2007; Slone et al. 2007; Jiao et al. 2008), bitter stimuli (Moon et al. 2006; Lee et al. 2009; Moon et al. 2009), and pheromones (Bray and Amrein 2003; Miyamoto and Amrein 2008; Moon et al. 2009). In fact, as might be predicted by the common evolutionary origin of the GR and OR families, some GRs have been implicated in sensing volatile stimuli like CO₂ (Suh et al. 2004; Jones et al. 2007; Kwon et al. 2007). In designing their experiments, researchers studying the GR proteins must always be careful to remember that the GR family is primarily a phylogenetic and not a strictly functional categorization.
In addition to these long established receptor families, recent work has revealed a new class of chemoreceptor proteins, the IR receptors (Benton et al. 2009). The name of this group of receptors is based on the fact that they share loose sequence similarity with ionotropic glutamate receptors. Most of these genes seem to be expressed in a class of olfactory sensory neurons (coeloconic sensilla) that have previously shown almost no expression of olfactory receptor transcripts. Assuming that at least some of the IR proteins are involved in olfaction, the discovery of this receptor family helps to explain the residual olfactory sensitivity to some odorants that has been observed in flies lacking functional ORs (Larsson et al. 2004). IR expression has also been observed in the proboscis, suggesting that this family may have chemosensory functions in gustation as well as in olfaction (Benton et al. 2009). Future work should help elucidate the precise chemosensory contributions of ORs, GRs, IRs, and any other chemoreceptor families that remain to be discovered.

1.5 Drosophila gustatory receptors

Despite the significant amount of work that has already been published on the gustatory receptor proteins, there is still much that is unknown about the majority of these receptors. The following is a brief summary of what is currently known about the Drosophila gustatory receptors.

1.5.1 Role of GRs in taste perception

The first Drosophila Gr to be deorphanized was Gr5a, a receptor gene that was shown to be involved in the detection of the disaccharide trehalose (Dahanukar et al.
An additional cluster of sugar receptor genes, the *Gr64* genes, was revealed through subsequent analysis (Dahanukar et al. 2007; Jiao et al. 2007; Slone et al. 2007; Lee et al. 2009). Separate studies revealed additional receptor genes—*Gr66a* (Moon et al. 2006), *Gr93a* (Lee et al. 2009), and *Gr33a* (Moon et al. 2009)—that encode bitter receptors, while other publications identified pheromone receptor genes (*Gr68a* and *Gr32a*) (Bray and Amrein 2003; Miyamoto and Amrein 2008) and carbon dioxide receptor genes (*Gr21a* and *Gr63a*) (de Bruyne et al. 2003; Jones et al. 2007; Kwon et al. 2007). However, the majority of *Gr* genes remain uncharacterized, so that broad conclusions about this protein family are currently difficult to make.

While the ligands remain unknown for the majority of GR receptors, it is known that a large fraction of transgenic reporters for orphan *Grs* are expressed in a subset of cells that express *Gr66a*. This suggests that many of these receptor genes code for bitter receptor proteins that are coexpressed in the L2 cells of taste sensilla (Thorne et al. 2004). Similarly, a subset of *Gr* genes closely related to *Gr5a* seem to be coexpressed with *Gr5a* in the sugar-responding neurons (Thorne et al. 2004; Dahanukar et al. 2007). Most of these genes have since been shown to encode sugar receptors (Dahanukar et al. 2007; Jiao et al. 2007; Slone et al. 2007; Lee et al. 2009), and additional data proving this point will be described in this dissertation.

In the past, studies of the taste perception of a variety of insect species has suggested that there are at least two different sugar receptors in insect sweet-sensing neurons: one receptor that detects sugars that exist primarily in pyranose ring forms.
(glucose, maltose, trehalose, etc.), and a different receptor that detects sugars that adopt a furanose ring structure (fructose being the most ecologically relevant example of such sugars) (Omand and Dethier 1969; Shimada et al. 1974; Rodrigues and Siddiqi 1981; Shimada and Tanimura 1981; Tanimura and Shimada 1981; Shimada et al. 1985). The results described for the sugar receptor genes identified so far agree with this hypothesis, since deletion of either Gr5a or the entire Gr64 cluster has a severe effect on the detection of pyranose sugars but a relatively weak effect on the detection of fructose (Dahanukar et al. 2007; Jiao et al. 2007; Slone et al. 2007; Lee et al. 2009). Even a deletion for Gr5a and Gr64a-c, which completely eliminates behavioral responses to multiple pyranose sugars, fails to completely eliminate fructose response (Dahanukar et al. 2007; Jiao et al. 2007; Slone et al. 2007; Lee et al. 2009). Since a mutant lacking all eight of the Gr5a-related sugar receptor genes has yet to be described in the literature, it is currently unknown if this residual response to fructose remains in the absence of the canonical sugar receptors, a result that would confirm the existence of additional sugar receptor genes.

### 1.5.2 Non-gustatory roles of GRs

One crucial question that remains to be answered about the GR family is whether or not they can accurately be called “gustatory” receptors. Some of the GR proteins, such as GR5a and GR66a, have been demonstrated to be involved in taste perception. However, as mentioned above, the Gr receptor genes Gr21a and Gr63a have a well-established role in the detection of carbon dioxide gas (de Bruyne et al. 2003; Jones et al. 2007; Kwon et al. 2007). Using the Gal4-UAS system, these genes have been shown to
be expressed in sensilla in the third antennal segment that are known to respond to carbon
dioxide, and, in mutants lacking either gene, these sensilla have a severely reduced
response to CO$_2$ (Jones et al. 2007). There is at least one other gustatory receptor gene,
Gr10a, that has also been shown to be expressed in the antenna, although the ligand for
this receptor remains unknown (Jones et al. 2007). It is also probable that the $Gr$ genes
that are expressed along the fruit fly wing margin are involved in olfaction, since this
appendage is less likely to be brought into contact with food sources than the labellum,
legs, or abdomen.

In addition to GRs expressed in the antenna, there are also $Gr$ genes that have
been shown to be expressed in multidendritic neurons, in the CNS, and in other cells not
associated with the direct detection of chemosensory stimuli (Thorne and Amrein 2008).
Furthermore, at least one report implicates the neurons expressing Gr68a (a receptor
involved in normal male courtship behavior) in the detection of sounds produced by the
movement of other flies (Ejima and Griffith 2008). Thus, even for those receptors with a
proven gustatory function, a role in non-gustatory sensation cannot be ruled out. In
Chapter 4 of this work, evidence will be presented for a novel, non-gustatory function for
some of the *Drosophila* sugar receptors.

**1.6 Introduction to thesis work**

The focus of the work presented in this dissertation is twofold. First, in Chapters
2 and 3, we confirm and dissect the role of a subset of GR proteins in the detection of
sugars and other attractive compounds. The ultimate goal of this line of investigation is
to generate a fly lacking all eight sugar receptor genes, although it now appears that at least some of the sugar receptor genes are essential for embryonic viability. This surprising result is the first evidence that any chemosensory receptor is actually required for the viability of an insect species, although the exact mechanism of the sugar receptors’ involvement in fruit fly development remains a mystery. Second, as discussed in Chapter 4, we study the expression of the sugar receptor genes, and our data both confirms the sugar receptors’ role in sweet taste perception and also reveals a possible role for these receptors in olfactory sensation.
2. Chapter 2: Characterization of a gene cluster encoding sugar receptors in *Drosophila*

2.1 Introduction

In fruit flies and probably most other insects, non-volatile compounds, most notably many food chemicals, are thought to be recognized by seven-transmembrane receptors which are expressed in taste neurons located on the labial palps (the equivalent of the mammalian tongue), the legs and the wings (Stocker 1994; Amrein and Thorne 2005). The *Drosophila* gustatory receptor (*Gr*) gene family is comprised of 68 relatively poorly conserved genes, with amino acid sequence similarity in the range of 8 to 20% between most pairs (Robertson et al. 2003) (Figure 4). *Gr* orthologs are found in other insects, but they are absent in vertebrates, *C. elegans* and more primitive organisms such as yeast or bacteria. The large differences in *Gr* gene number between species—the honeybee has only 12 GRs, while *Drosophila* has almost 70 (Robertson and Wanner 2006)—and their poor conservation suggests that this gene family is subject to rapid adaptation driven by the vastly different ecological niches these insect species occupy.
Figure 4: Sequence conservation of the sugar receptors in *Drosophila melanogaster*.
(A) Alignment of the eight putative sugar receptors. Alignment was constructed with the Multiple Alignment feature of MacVector, using a BLOSUM series matrix and the default parameter settings. (B) Evolutionary relationship between GR proteins. The phylogenetic tree was generated in MEGA4, using the Neighbor-Joining method (Tamura et al. 2007). Bootstrap values are indicated next to the branches. The eight sugar receptors are at the bottom (indicated by a bracket) and have bootstrap support of 100%.

Due to the dispersed location of taste sensilla throughout the body—flies and many other insects harbor taste sensilla not only on the labellum, but also on legs and wings—and the overall low abundance of Gr mRNAs in gustatory receptor neurons (GRNs), expression analyses of Gr genes has been performed mainly with the use of the Gal4/UAS system (Brand and Perrimon 1993; Dunipace et al. 2001; Scott et al. 2001; Hiroi et al. 2002; Thorne et al. 2004; Wang et al. 2004). These studies have led to the identification of two distinct sets of GRNs, which are characterized by the mutually exclusive expression of different Gr genes. The first group is composed of about 22 GRNs – a single taste neuron of each of the 22 I- and S-type sensilla – and expresses Gr66a (Thorne et al. 2004; Wang et al. 2004). However, all of these neurons express additional, but distinct Gr genes, and hence, each neuron is defined by a unique Gr expression code (see Figure 2 in Chapter 1). Functional studies revealed that Gr66a-expressing neurons detect bitter compounds, most notably caffeine, as flies in which these cells are impaired show significantly reduced sensitivity to such chemicals (Thorne et al. 2004; Wang et al. 2004). Thus, it is generally assumed that the Gr genes expressed in these 22 I- and S-type sensilla encode receptors for harmful, noxious and toxic (and to humans, bitter-tasting) compounds. The second group of GRNs is currently represented
by a single Gr gene, Gr5a, and ablation/inactivation of these neurons leads to reduced sensitivity for trehalose and, to a lesser extent, to some other sugars as well (Thorne et al. 2004; Wang et al. 2004). Importantly, Gr5a and Gr66a are expressed in distinct, non-overlapping sets of GRNs, and mediate distinct behavioral responses (feeding and avoidance, respectively) (Thorne et al. 2004; Wang et al. 2004).

The specific molecular roles of all but a few Gr genes are currently unknown. Two of the most well-characterized GRs expressed in gustatory receptor neurons (GRNs) are GR5a and GR66a, which detect the sugar trehalose and the bitter compound caffeine, respectively (Dahanukar et al. 2001; Ueno et al. 2001; Moon et al. 2006). Gr5a is a member of a gene subfamily comprised of seven additional members, Gr61a and Gr64a-f, which evolved through recent gene duplication events (Robertson et al. 2003) and therefore share relatively high similarity to each other (41 to 73 % at the amino acid level; Figure 4). In this chapter, we investigate the role of Gr64a-f and show that they encode receptors for many sugars. Moreover, our data suggests an uncommon mode of co-expression of these genes, being transcribed as multi-cistronic mRNA(s), which would provide an efficient and elegant strategy to express these receptors in the same taste neurons.

2.2 Materials and methods

2.2.1 Deletion of the Gr64 gene cluster

Insertion lines f03449 and d06001 were obtained from the Bloomington and Harvard stock centers, and used to generate the Gr64 deletion using an approach
previously described by Exelixis, Inc (Parks et al. 2004). Briefly, insertion line f03449 was crossed to a line carrying an *hs-FLP* insertion, and the resulting progeny were crossed to the *d06001* insertion line. Then, the *hs-FLP* insertion was activated in the progeny of the second cross by heat-shock, which resulted in deletion of the *Gr64* genes through recombination between the FRT sites contained in the piggyBac insertions. Deletions were detected using a pair of primers that anneal within the piggyBac elements, and then confirmed using a second pair of primers that anneal within the genomic sequences flanking the site of the deletion. Both PCR products were sequenced and it was confirmed that the end points of the deletion coincided with the insertions sites of *f03449* and *d06001*. The primers used for the detection of *Gr64* deletions, and a control DNA (the *alpha-tubulin* gene) were as follows:

- Primer 11—GACGCATGATTATCTTTTACGTGAC
- Primer 12—AATGATTCGCAGTGGAAGGCT
- Primer 13—GGAAAGTGCGCCGGCGGTGGGTGAGGCC
- Primer 14—CTGATCGCAACTAGTTGAGGGGATTCG
- Primer T1—CCTTGTCGCGTGTGAAACACTTCC
- Primer T2—GATAGCCTCGTTGTCGACCATGAA

### 2.2.2 Transgenic Constructs

The genes flanking the *Gr64* cluster that were also deleted during the excision process were rescued using genomic fragments digested out of BAC clone RPCI98-9C2 (Roswell Park Cancer Institute Drosophila BAC Library). A 14.2 kb EcoRI fragment
containing fd64A, CG1134, and CG11594 was cloned into pUAST (Construct R2), and a 15.3 kb NsiI fragment containing CG11593 and CG1135 was cloned into the PstI site of pCaspR4 (Construct R1). In addition, the Gr64abcd_GFP_f rescue construct was derived from the same BAC clone and subcloned into the NotI and Acce65I sites of pUAST. During the cloning process, the coding sequence of Gr64e was replaced with that of egfp (pEGFP-N3; Clontech Inc.) by PCR using the TAKARA LA Taq PCR kit, according to the manufacturer’s protocol. This was accomplished by cloning PCR fragments of the Gr64 cluster upstream and downstream of Gr64e into a vector containing the egfp coding sequence, and then cloning the entire insert into the pUAST vector. The following primers were used to clone the Gr64abcd_GFP_f construct:

Primer Gr64a-f UP 5’—AAAGCGGCCGCGGACCAGCGTCATGAAAGGACC
Primer Gr64a-f UP 3’—ATTACTAGTATCATGTTCCGTACTCGACTGACAACC
Primer EGFP 5’—TGATACTAGTAATGGTGAGCAAGGGCGAGGAG
Primer EGFP 3’—ATTCTGCAGTTACTTGTACAGCTCGTCCATGCC
Primer Gr64a-f DOWN 5’—TTACTGCAGATTTTGTGGAAGTGGCAGGGG
Primer Gr64a-f DOWN 3’—TTAGGTACCATGTAGTTATCGTGTAGCTTCC

For the single gene UAS rescue constructs, the coding sequences for each Gr was cloned either from w1118 or ORE-R cDNA (for Gr64a, Gr64b, Gr64c, and Gr64f) or from a genomic fragment contained in the BAC clone RPCI98-9C2 (for Gr64d and Gr64e). The following primers were used to clone the cDNAs:
Primer Gr64a cds 5’—
TTTTGGTACCCCCACCATGAAAGGACCCAATCTGAATTTCG
Primer Gr64a cds 3’—TTTTTCTAGATCGCGGCACAGTGGCTG
Primer Gr64b cds 5’—TTTTGGTACCCCCACCATGCCGCAGGGCGAGAC
Primer Gr64b cds 3’—TTTTTCTAGACTAATATTTCCCTGCGACTGAGTC
Primer Gr64c cds 5’—
TTTTGGTACCCCCACCATGCAGCAGTCGGGTCAAATAAG
Primer Gr64c cds 3’—TTTTTCTAGACTAAGGAAAATGCTCATGGAG
Primer Gr64d cds 5’—TTTTGGTACCCCCACCATGCTGAGGAGCTTTGTC
Primer Gr64d cds 3’—
TTTTTCTAGACTAAGGAAAATGCTCATGGAG
Primer Gr64e cds 5’—
GGTACCCCCACCATGCGCCAGGACCCTGGGGATCCTG
Primer Gr64e cds 3’—TCTAGATCAGTTTTCGAACAGCCGGGCACCC
Primer Gr64f cds 5’—
TTTTGGTACCCCCACCATGAGATTCTTCCGAAGCTGGAG
Primer Gr64f cds 3’—TTTTTCTAGACTATGAGTAATAGCTGTGATCGCAG

All constructs were injected into $w^{118}$ embryos, and transgenic flies were recovered according to standard procedures.
2.2.3 RT-PCR

PCR from genomic DNA was performed using the TAKARA LA PCR kit. RT-PCR was performed on DNase-treated RNA samples using the One-Step RT-PCR kit (Invitrogen), according to the manufacturer’s protocol. Each RT-PCR product derived from total head RNA was cloned into the TOPO cloning vector and sequenced in order to confirm that the PCR product represented a spliced cDNA product. In all cases, the PCR products were either completely or partially spliced. The relative position of each primer used for RT-PCR is shown in Figure 5C. The nucleotide sequence of each primer is as follows:

Primer 1—CCACACAGACAGTGGCACTTTC
Primer 2—GGAAGCAGGCGGTAGATTTGTG
Primer 3—CGAAAGATTGTCACAGCCTTGAGG
Primer 4—GATAGCGCACAGTCCACGATG
Primer 5—CCCACTGAGTTTTGGTGCGTG
Primer 6—GCGCTGTTTCCCGGATGATATG
Primer 7—GTTGTCTGGACTGATTCTGGTCTGC
Primer 8—GCTTGATGGCTTCCTGGAAAGATC
Primer 9—GATGAGTCCAAGCGACCACTGG
Primer 10—CCTCCTTATCGCTACGAGACAGC
2.2.4 Behavioral Experiments and Statistical Analysis

Proboscis Extension Response (PER) assays were performed using a modified version of a previously described protocol (Wang et al. 2004). Flies were collected on the day of eclosion, and allowed to feed in food vials for 2-6 days. Flies were starved for approximately 26 to 30 hours at 22 to 24°C, and mounted on a glass slide the following afternoon. During the mounting process, flies were immobilized using ice rather than carbon dioxide to avoid behavioral effects due to carbon dioxide exposure. Mounted flies were allowed to recover from the ice treatment for two to four hours in a humidified chamber, and then tested for sensitivity to various tastants using the PER assay.

During the assay, each fly was first administered water and allowed to drink until satiation. Only flies that responded to water were tested further. Each fly was tested with a given tastant by briefly applying the taste solution to the fly’s labellum and legs and recording whether or not the animal extended its proboscis. Each tastant was applied three times per fly, and the flies were given water between each application of a taste solution. Error bars represent +/- SEM, and statistical significance was calculated in Microsoft Excel using Student’s t-test (assuming unequal variance). All experiments were performed blind.

2.3 Results

2.3.1 The genes of the Gr64 cluster are expressed as polycistronic mRNAs

The Gal4/UAS expression system (Brand and Perrimon 1993) has been used very successfully to identify GRNs that express specific Gr genes (Dunipace et al. 2001; Scott
et al. 2001; Hiroi et al. 2002; Thorne et al. 2004; Wang et al. 2004). We generated four Gr64-Gal4 driver constructs and combined these with UAS-nucGFP reporters, but we did not observe expression in the main taste organs with any of these drivers (Natasha Thorne, Jesse Slone and Hubert Amrein; unpublished data), even though RNAs for all six Gr64 genes are detected by RT-PCR (see below). This suggested that crucial transcriptional regulatory elements are located upstream and/or downstream of the cluster and/or within the introns of the Gr64 genes. Further support for an unusual arrangement of regulatory elements of the Gr64 genes is apparent from the dense genomic clustering of the six open reading frames (ORFs). Assuming at least 50 nucleotides of 5’ and 3’ UTR for each gene, the intergenic, non-transcribed regions harboring putative promoters are extremely short (<100 nt) and lack transcription termination signals (AAUAAA), which are present in most Drosophila genes (Table 1). These observations prompted us to test whether the Gr64 genes might be transcribed as a poly-cistronic mRNA. We isolated mRNA from heads and performed RT-PCR analysis across the whole cluster using primer pairs of adjacent genes (Figure 5C and Figure 5D). To discriminate between products from spliced RNA and residual genomic DNA, primers were chosen such that the amplified fragments would represent spliced transcripts that lack at least one intron (Figure 5C). In each case, RT-PCR readily amplified a spliced RNA product composed of cDNAs corresponding to adjacent ORFs separated by the intergenic sequence (Figure 5D). The same result was obtained when RNA isolated from leg tissue was used (data not shown). These results suggest that coding sequences of adjacent Gr64
genes are present on the same mRNA and, by inference, that possibly all six ORFs may be transcribed as a large polycistronic mRNA.

<table>
<thead>
<tr>
<th>Gene Cluster</th>
<th>A to b AAUAAA</th>
<th>b to c AAUAAA</th>
<th>c to d AAUAAA</th>
<th>d to e AAUAAA</th>
<th>e to f AAUAAA</th>
<th>AAUAAA</th>
<th>AAUAAA</th>
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</thead>
<tbody>
<tr>
<td>Gr22</td>
<td>399 None 233 None 255 None -- -- -- -- 107</td>
<td></td>
<td></td>
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<tr>
<td>Gr93</td>
<td>-- -- 230 74 188 123 -- -- -- -- 111</td>
<td></td>
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<td></td>
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<tr>
<td>Gr98</td>
<td>-- -- 554 39 215 None -- -- -- -- 293</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Gr28</td>
<td>838 164 -- -- -- -- -- -- -- 67</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Gr64</td>
<td>200 None 191 None 136 None 183 None 180 None 30</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gr59a,b</td>
<td>477 8 -- -- -- -- -- -- -- 178</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Gr59c,d</td>
<td>-- -- -- -- 207 None -- -- -- -- 9</td>
<td></td>
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<td></td>
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<td></td>
<td></td>
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<tr>
<td>Gr10</td>
<td>309 128 -- -- -- -- -- -- -- 219</td>
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<td></td>
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</tr>
<tr>
<td>Gr36</td>
<td>859 23 273 None 305 None -- -- -- -- 1098</td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

Table 1: Distances between adjacent genes in selected *Gr* gene clusters.

Intergenic distances are indicted and refer to the number of nucleotides from stop codon to start codon. The nucleotide position of the polyadenylation signal (AAUAAA), if present, in the intergenic regions is indicated and represents the number of nucleotides between the stop codon and the first nucleotide of the polyadenylation signal. The last column indicates the position of the AAUAAA sequence after the last gene in each cluster.
Figure 5: Generation of a \textit{Gr64} mutant strain (\textit{AGr64}) and RT-PCR expression analysis of the six \textit{Gr64} genes.
(A) Diagram of the Gr64 gene cluster. The positions of the transposable elements are indicated by triangles. The diagram shows the Gr64 cluster prior to generation of the deletion line. The numbered arrows indicate the positions of the primers used for PCR analysis of the deletion. The black bars represent the rescue constructs for the genes flanking the Gr64 cluster. (B) Molecular analysis of ΔGr64 mutant strain. The diagram shows the structure of the Gr64 deletion (ΔGr64) after trans-recombination. Genomic DNA from w^1118 flies was also analyzed for comparison. Expected band sizes are as follows: 1.1 kb for primers T1 and T2, 1.5 kb for primers 11 and 12, and 6.9 kb for primers 13 and 14. Relevant band sizes from the ladder are marked along the sides of the gel. The 1.5 and 6.9 kb products were cloned and sequenced to further confirm the presence of the deletion. The 1.1 kb product is derived from the tubulin gene and serves as a control for DNA integrity. (C) Exon-intron structure of the Gr64 cluster. Exons are represented by boxes and introns by v-shaped lines. The numbered arrows show the positions of the primers used for RT-PCR analysis. The black bar indicates the rescue construct (UAS-Gr64abcd_GFP_f) in which Gr64e was replaced by egfp (indicated by the dashed line). (D) RT-PCR of total RNA from fly heads indicates the presence of polycistronic transcripts in the Gr64 cluster. RNA was extracted from wild-type ORE-R flies. Each pair of primers spans at least one intron in each of the two genes being investigated. For each pair of primers used in an RT-PCR reaction, a corresponding PCR reaction was performed on genomic DNA to provide a size comparison. RT-PCR products were isolated for each primer pair, cloned and sequenced to confirm integrity of appropriately spliced cDNA products. RT-PCR from leg tissue showed similar results (data not shown). Lanes marked “RT” represent RT-PCR products, while lanes marked “G” represent PCR products from genomic DNA.

With the exception of nematodes, polycistronic transcripts are not thought to be common in higher eukaryotes. In C. elegans, however, a significant number of genes (~15 %) are co-transcribed as operons, and independently trans-spliced to the abundantly expressed spliced leader (SL2) RNA (Blumenthal et al. 2002). However, it has recently become apparent that operon-like gene organizations and polycistronic mRNAs do exist in Drosophila; at least two transcripts initially postulated to be non-coding RNAs were shown to encode multiple, albeit redundant peptides, with functions necessary in early
development (Galindo and Smith 2001; Xu et al. 2008). Examples more similar to the Gr64 genes were described for four pairs of Drosophila Or genes and the Drosophila CheB42a-llz locus (Ben-Shahar et al. 2007; Ray et al. 2007). The basic translation mechanism of poly-cistronic mRNAs of the Or gene pairs is unknown; however the CheB42a-llz bicistronic transcript is subsequently cleaved into two mRNAs that appear to be translated separately (Ben-Shahar et al. 2007). While a polyadenylation signal is present after the upstream gene (CheB42a) in this case, no putative promoter sequences were identified for the intergenic region in the CheB42a-llz locus (Ben-Shahar et al. 2007). A genomic survey by these authors for closely clustered genes lacking promoter sequences in the intergenic region identified almost 1400 Drosophila gene pairs, suggesting that operon-like gene structures may be much more common in eukaryotes than generally assumed (Ben-Shahar et al. 2007). Not surprisingly several Or and Gr gene pairs were found to lack such promoter sequences, including the five downstream genes in the Gr64 gene cluster (see also Table 1).

2.3.2 Loss of the Gr64 genes leads to severe defects in the sugar sensation

2.3.2.1 Deletion of the Gr64 cluster using FRT-based recombination

To gain a basic understanding of sugar perception in Drosophila, we performed a reverse genetic analysis of the six Gr64 genes, which are tightly clustered on the left arm of chromosome 3. We used FRT-mediated trans-recombination (Parks et al. 2004; Thibault et al. 2004) to create a 25 kb deletion of the region containing the Gr64 gene cluster (referred to as ΔGr64), and we confirmed the expected molecular nature of this
deletion using genomic PCR and DNA sequencing from the trans-recombined chromosome (see Materials and methods; Figure 5). In addition to the six Gr64 genes, this trans-recombination event also removed five additional genes on either side of the cluster, resulting in a homozygous lethal mutation, presumably because some of the neighboring genes have essential functions required for viability. We therefore cloned two genomic DNA constructs containing the two genes proximal (R1) or the three genes distal (R2) to the Gr64 locus, respectively, into a transformation vector and generated corresponding transgenic Drosophila lines (see Experimental Procedures; Figure 5). When R1 was crossed into the ΔGr64 mutant strain, viability was completely restored, indicating that at least one of the two proximally located genes provides a life-essential function. Even though unlikely, it is possible that some genes on R1 and/or R2 may have functions related to taste perception. Thus, we used ΔGr64/ΔGr64 flies that carried a copy of each of these rescue constructs for all behavioral experiments (see below).
Figure 6: ΔGr64 mutants are severely deficient in the perception of most sugars.

Proboscis Extension Response (PER) of ΔGr64 mutants and isogenic control flies to 500mM (A) and 100mM (B) sugar solutions. The genotype of ΔGr64 mutants is $R1^+/R2^+; ΔGr64^+/+$. "Probability of Extension" represents the number of times flies from a given strain extended
their proboscis when presented with a tastant, divided by the total number of times that the tastant was presented. For all data shown in Figure 6, each graph is the average of 4-15 experiments +/- SEM (3-11 flies per experiment, 20-105 flies total for each strain and tastant tested). Asterisks indicate a significant difference between the mutant and control strains, as determined by Student’s t-test (* indicates p<0.05, ** indicates p<0.0001). Glycerol was used as 10% or 2% solutions dissolved in water. (C) The Gr5a gene is functional in R1/Y;R2/+;∆Gr64/∆Gr64 flies. Flies heterozygous for ∆Gr64, but containing the same X chromosome (i.e. the same allele of Gr5a) as the homozygous ∆Gr64 flies, show normal and robust response to trehalose at both 100 mM and 500 mM concentrations. (D) PER response of ∆Gr64 mutant and control strains to various bitter tastants in the presence of 500mM fructose. The response to 500mM fructose alone is shown for comparison. There was no significant difference between mutants and controls for any of the bitter solutions by Student’s t-test.

2.3.2.2 ∆Gr64 flies are deficient in the sensation of five different sugars

We first investigated whether the Gr64 genes were required for the detection of six sugars by generating ∆Gr64 homozygous mutant flies that contained one copy of each of the flanking rescue constructs (R1/+;R2/+;∆Gr64/∆Gr64). We determined the behavioral response of these and control flies to sucrose, glucose, trehalose, fructose, arabinose and maltose using the proboscis extension reflex (PER; Figure 6). As controls, we tested flies that were heterozygous for each of the two piggyBac elements used to generate the ∆Gr64 mutation (Figure 6), as well as flies with an intact Gr64 cluster, but containing a copy of R1 and R2 to rule out a dominant phenotype of these transgenes (Figure 7). PER is a robust indicator of a fly’s attraction and motivation to eat a given chemical compound (Dethier 1976). If taste neurons in the labial palps or the forelegs are stimulated with a solution containing sugars, the fly extends its proboscis to attempt feeding. Indeed, we find that both control strains responded with high probability of a
PER, ranging from 42% to 97%, when stimulated with 500 mM solutions of various pyranose sugars (Figure 6A). Even at a five-fold lower concentration (100 mM), both types of control flies responded to all sugars, albeit with a reduced PER (Figure 6 and Figure 7). In contrast, $R1^+/+;R2^+/+;\Delta Gr64/\Delta Gr64$ flies showed a drastic reduction in PER for all sugars at both 500 and 100 mM, except for fructose. In most cases, the reduction was at least 10-fold, while sensitivity for sucrose was reduced only by about three-fold (Figure 6A and Figure 6B). However, PER response to fructose was the same in control flies and $R1^+/+;R2^+/+;\Delta Gr64/\Delta Gr64$ mutant flies at 100mM and reduced by only about 35% at 500mM, suggesting that a high-affinity fructose receptor is present in flies lacking all six $Gr64$ genes.

It was recently shown that flies exhibit a behavioral feeding response to glycerol, a linear triol, and indeed, glycerol was shown to stimulate sugar-sensitive neurons (Koseki et al. 2004). We wondered whether glycerol detection is also mediated by some of the GR64 receptors and therefore examined the PER response in control and $\Delta Gr64$ mutant flies. Indeed we observed almost a 6 and 16-fold reduction of PER to 2% and 10% glycerol, respectively, in mutants when compared to controls.
Figure 7: Flies with $R1$ and $R2$ transgenes show normal PER response.

The graph shows PER responses for four different sugars of flies containing the two transgenes $R1$ and $R2$ and a wild-type copy of the $Gr64$ gene ($R1/++;R2/++;\Delta\Delta\Delta\Delta\text{Gr64}+/+$), in comparison to control flies (see Figure 6), as well as homozygous $\Delta\text{Gr64}$ mutants. $R1/++;R2/++;\Delta\text{Gr64}+/+$ flies show normal response to these sugars, compared to the highly reduced or lost response in mutants. At 500 mM, the response of $R1/++;R2/++;\Delta\text{Gr64}+/+$ flies is the same as that observed in the control strain, while at 100 mM, the response appears slightly higher. For all data shown in Figure 7, each graph is the average of 4-15 experiments +/- SEM (3-11 flies per experiment, 20-105 flies total for each strain and tastant tested). Asterisks indicate a significant difference between the mutant and control strains, as determined by Student’s t-test (* indicates $p<0.05$, ** indicates $p<0.0001$).

2.3.2.2.1 $\Delta\text{Gr64}$ flies are deficient in the sensation of trehalose, similar to $Gr5a$ mutants

The loss of behavioral response to trehalose in $\Delta\text{Gr64}$ mutant flies is surprising, since these flies presumably contain a wild-type $Gr5a$ gene, which encodes a receptor for this sugar (Dahanukar et al. 2001; Ueno et al. 2001). Therefore, the perception of trehalose appears to require at least two $Gr$ genes, $Gr5a$ and one or more members of the
Gr64 gene cluster, suggesting that insect sugar receptors might function as dimers or multimers. To rule out the possibility that the loss of trehalose responses in these flies is caused by a defective Gr5a allele, we tested the PER response of flies heterozygous for ∆Gr64, but containing the same X chromosome (i.e. the same allele of Gr5a) as the homozygous ∆Gr64 flies (Figure 6C). Indeed, these flies showed a robust response to trehalose, indicating that a second receptor gene in the Gr64 cluster is necessary for the detection of this sugar. Thus, trehalose, and possibly sugars in general, would appear to require multiple GR proteins for its proper detection by the taste system. One possible mechanistic explanation for this observation is that sugars are detected by multimeric receptors composed of two or more GRs. There are precedents for insect chemoreceptors as multisubunit transmembrane receptors in the olfactory system: In most olfactory sensory neurons (OSNs), receptors for volatile chemicals appear to function as dimers consisting of the widely expressed OR83b protein and the single OR expressed in a given OSN (Larsson et al. 2004; Benton et al. 2006). Furthermore, a distinct subset of CO2-sensitive OSNs expresses the two gustatory receptors Gr63a and Gr21a, both of which are required for sensing this gas, a stress pheromone in flies (Suh et al. 2004; Jones et al. 2007; Kwon et al. 2007).

2.3.2.3 ∆Gr64 flies are still sensitive to a variety of bitter-tasting compounds

To assess whether lack of the Gr64 gene affects the behavioral responses to other chemicals, we tested PER response to four chemically diverse, bitter-tasting compounds. Such compounds, which are known to inhibit feeding, reduce PER responses if they are
mixed with sugar solutions (Thorne et al. 2004; Wang et al. 2004). Therefore, we tested
PER responses to 500 mM fructose solutions that included caffeine, denatonium
benzoate, berberine or quinine (Figure 6D). Both strains showed a similar decrease in
PER response when stimulated with these solutions, suggesting that the Gr64 genes are
not required for the detection (and avoidance) of bitter compounds. Taken together, our
data suggest that the six Gr64 genes are necessary specifically for the detection of most
sugars.

2.3.2.4 The sugar-sensing defects of ∆Gr64 flies can be restored by a genomic rescue
construct

To conclusively prove that the Gr64 genes indeed encode sugar receptors, we
performed transgene rescue experiments. We cloned a genomic fragment containing five
of the six Gr64 genes into the UAS reporter (UAS-Gr64abcd_GFP_f; with Gr64e
replaced by GFP) and generated two types of R1/+;R2/+;∆Gr64/∆Gr64 flies, the first
containing the UAS-Gr64abcd_GFP_f rescue construct (see Figure 5C), and the second
containing the same rescue construct as well as a Gr5a-Gal4 driver. The Gr5a-Gal4
driver is expressed in sugar-sensitive neurons of both the labellum and the legs (Thorne et
al. 2004) and should confer such expression on the rescue construct. At both 100mM and
500mM concentrations, the UAS-Gr64abcd_GFP_f transgene rescued the PER response
to similar levels as observed in the control strain (Figure 8). Surprisingly, this rescue was
not enhanced by the Gal4 driver, indicating that regulatory elements within the cluster
confer sufficient expression onto the Gr64 genes. This expression was confirmed using
RT-PCR analysis, which showed that regardless of whether the Gr5a-Gal4 was present
or not, the $Gr64$ transcripts were readily amplified (Figure 9). We note that a second $Gr64abcd_GFP_f$ reporter integrated in a different genomic location provided only partial rescue of sugar sensitivity (data not shown).
Figure 8: Rescue of ΔGr64 mutant phenotype.

PER response of ΔGr64 mutants (R1/+; R2/+; ΔGr64/ΔGr64) carrying one copy of the UAS-Gr64abcd_GFP_f reporter, with or without Gr5a-Gal4 driver. Sugars were tested at 500mM (A) and 100mM (B) concentration. PER response of flies with the rescue construct is similar to that of the control flies, regardless of whether or not the Gr5a-Gal4 driver is present. Each graph is the average of 5-10 experiments +/- SEM (3-6 flies per experiment, 20-58 flies total for each strain and tastant tested). Asterisks indicate a significant difference between the mutant and rescue strains, as determined by Student’s t-test (* indicates p<0.05, ** indicates p<0.001, *** indicates p<0.0001).
Figure 9: Expression of the Gr64 genes in flies containing the Gr64 rescue construct.

Flies containing the UAS-Gr64abcd_GFP_f transgene express the Gr64 genes, both in the absence (lanes 1) and presence (lanes 2) of the Gr5a-Gal4 driver. In lanes 3, RNA from homozygous ∆Gr64 mutants flies (R1/+;R2/+;∆GR64/∆GR64) was loaded. RT-PCR analysis of RNA isolated from heads and legs (not shown) was carried out for the first four genes. Integrity of cDNA was confirmed using primers against the tubulin gene. The same primers were used as in Figure 5D (1-6, T1 and T2).

2.3.2.5 Single-gene rescue experiments

In order to more narrowly identify the Gr64 genes that are involved in the detection of particular sugars, we generated R1/+; ∆Gr64/∆Gr64 flies carrying the Gr5a-Gal4 driver and UAS rescue constructs for each individual Gr64 gene. A similar transgenic rescue approach has been utilized previously with a subset of the genes in the Gr64 cluster (Jiao et al. 2008), but no comprehensive attempt has been made to rescue all of the genes in the cluster. Using UAS transgenes containing cDNA or genomic fragments for each of the predicted ORFs in the cluster, it was found that Gr64f gave the
strongest rescue when expressed in a $\Delta Gr64$ background (Figure 10). In fact, rescue of $Gr64f$ alone seemed to provide modest rescue for several sugars, including trehalose, although the statistical significance of each rescue phenotype is not very high when compared to $\Delta Gr64$ flies. This rescue of trehalose perception by expression of $Gr64f$ cDNA in the $\Delta Gr64$ background agrees with previous research from another group, who utilized a different behavioral assay (Jiao et al. 2008). Thus, it appears that GR64f is the major trehalose receptor subunit among the GR64 proteins and can, by itself, rescue a significant portion of the $\Delta Gr64$ phenotype. However, as will be shown in Chapter 4, loss of the $Gr64f$ gene alone does not result in severe taste deficits for most sugars, including trehalose, indicating that there is probably some level of redundancy in the ligand sensitivity of the GR64 proteins. The idea of partial redundancy among the GR64 receptors is further supported by the observation that the addition of GR64a, GR64d, GR64e, and GR64f all seem to increase sucrose response in the $\Delta Gr64$ background, although only GR64e and GR64f increase the response to a statistically significant level (Figure 10).
Figure 10: Taste sensitivity of flies carrying single-gene UAS-Gr64 rescue constructs in Agr64 background.

A Gr5a-Gal4 driver was used to drive expression of single-gene UAS-Gr64 rescue constructs in sugar-sensing neurons of otherwise Agr64 mutant flies. Flies carrying the UAS-Gr64f rescue construct show the strongest rescue of sugar sensitivity in comparison to Agr64 mutant controls. Graphs represent the probability of proboscis extension. Sugars were tested at 100mM concentration. Each graph is the average of 5-11 experiments +/- SEM. Asterisks indicate a significant difference between the Agr64 mutant and rescue flies, as determined by Student’s t-test (* indicates p<0.05).

2.4 Discussion

In this chapter, we have shown that the members of the GR64 protein family are indispensable for the sensation of pyranose sugars. However, they do not appear to be necessary for the sensation of fructose, a result that is consistent with previous work showing that pyranose sugars and fructose are detected by different receptor sites in insects (Omand and Dethier 1969; Shimada et al. 1974; Rodrigues and Siddiqi 1981; Shimada and Tanimura 1981; Tanimura and Shimada 1981; Shimada et al. 1985).
Homozygous ∆Gr64 mutant flies show as robust a response to 100 mM fructose as control flies, indicating that there is at least one functional fructose receptor that does not contain any of the GR64 proteins. A receptor for this sugar might therefore be comprised of a heterodimer between GR61a and GR5a or a homodimer of either one of these two proteins. This hypothesis will be investigated in the future. But other compositions are possible as well, such as heterodimers involving one of these subunits along with another GR protein. Any such dimer may also serve as a low-affinity receptor for non-fructose sugars and therefore be responsible for the residual PER responses to glucose, trehalose, maltose and arabinose in homozygous ∆Gr64 mutant flies (Figure 6).

Sugars are essential dietary compounds for many insects, including most Drosophila species. While a single trehalose receptor has been identified previously (Dahanukar et al. 2001; Ueno et al. 2001), the molecular genetic basis for the perception of sugars in general was unknown. Here we showed that the six Gr64 genes encode receptors for the detection of most sugars: sucrose, glucose, maltose, trehalose, and arabinose. Our data also suggests that the Drosophila taste receptors, similar to insect olfactory (Larsson et al. 2004; Benton et al. 2006) and CO₂ receptors (Jones et al. 2007; Kwon et al. 2007) and mammalian sweet taste receptors (Nelson et al. 2001; Zhao et al. 2003), function as dimers (or possibly multimers), since detection of trehalose requires, in addition to GR5a, at least one of the six receptors encoded by the Gr64 genes (most likely GR64F). However, in contrast to mammals, which use T1R2/T1R3 heterodimers for the detection of all sugars (Nelson et al. 2001; Zhao et al. 2003), Drosophila appears to use distinct combinations of GRs for the detection of different sugars. As GRs have
been difficult to express in cell or heterologous systems, the availability of a mutant lacking the six Gr64 genes should help elucidate the molecular nature of dimeric (or multimeric) sugar receptors in insects. Experiments described in this chapter have already demonstrated that expressing Gr64f in a ∆Gr64 background can rescue a significant portion of the sugar response deficit of ∆Gr64 mutants. In the future, rescue experiments will be conducted using pairwise combinations of the UAS-Gr64f transgene with the UAS transgenes for the other Gr64 family members. The ultimate goal will be to find the minimal complement of GR64 proteins that will restore full sugar sensitivity in the ∆Gr64 background.

RT-PCR analysis of Gr64 transcripts suggests that the six Gr64 genes are transcribed as one or more polycistronic mRNAs, a rare mode of gene expression in eukaryotes other than C. elegans. Two other groups have also investigated the polycistronic expression of the Gr64 genes and have been able to isolate full-length bicistronic cDNAs from this cluster, as well as cDNAs containing sequences from up to three Gr64 genes (Dahanukar et al. 2007; Jiao et al. 2008). Thus, in spite of the fact that a transcript containing all six ORFs has yet to be isolated, the presence of polycistronic transcripts within this cluster is by now well-established.

Of the few known examples of operon-like genes in Drosophila, the CheB42a-llz locus is the best-characterized case of a polycistronic mRNA. The CheB42a-llz RNA, which encodes two proteins, is subsequently cleaved into two transcripts, which appear to be translated independently by a cap-independent process (Ben-Shahar et al. 2007). Elucidating how the Gr64 transcripts are processed post-transcriptionally and by which
mechanism they are translated will be a challenging undertaking, especially if such processing only takes place in the correct cellular context (i.e. taste neurons).

Furthermore, we have obtained expression data (described in Chapter 4) that suggests that the first and the last gene in the cluster may not be expressed in the same number of cells in the labellum. However, assuming that at least some of the genes in the cluster are coexpressed, polycistronic, operon-like transcription of the \textit{Gr64} genes would provide an elegant solution for their coordinated expression in the same subset of sugar-responsive taste neurons.

\textbf{Note:} The material presented in this chapter is adapted from the following paper, as allowed by the journal in which it was originally published:

3. Chapter 3: *Gr61a* is a receptor for a subset of sugars

3.1 Introduction

Given the residual response to fructose that remains in all *Drosophila* sugar receptor gene mutants obtained thus far (Dahanukar et al. 2007; Slone et al. 2007; Jiao et al. 2008), it is likely that there are low-affinity receptors for this ecologically important sugar that are not members of the putative sugar receptor gene family (*Gr5a*, *Gr61a*, and *Gr64a-f*). Alternatively, *Gr61a* could itself be the elusive fructose receptor gene, since it has received only cursory attention in the literature up to this point (Dahanukar et al. 2007) and much remains unknown about this gene. The most conclusive way to investigate both possibilities would be to generate flies lacking all eight of the canonical sugar receptors and then to test their response to fructose. Such a “sugar-receptorless” fly might also be useful as an empty-neuron system for deorphanzing gustatory receptors, similar to the one used to deorphanz the *Drosophila* OR proteins (Dobritsa et al. 2003). This chapter will focus on our investigation of the function of *Gr61a*, and our initial efforts to generate flies that lack all of the sugar receptors.

3.2 Materials and Methods

3.2.1 Behavioral Experiments and Statistical Analysis

Proboscis Extension Response (PER) assays were performed as described in Chapter 2, using a modified version of a previous protocol (Wang et al. 2004). Error bars represent +/- SEM, and statistical significance was calculated in Microsoft Excel using Student’s t-test (assuming unequal variance). All experiments were performed blind.
3.2.2 Transgenic Constructs

A Gr61a genomic rescue construct was generated from w$^{1118}$ genomic DNA using the TAKARA LA PCR kit, according to the manufacturer’s protocol. The PCR product was first cloned using the TOPO-XL cloning kit (Invitrogen), sequenced, and then cloned into the NotI and Acc65I sites of the vector pCaSpR4. The primers used to make the rescue construct are as follows:

Primer 61A-1—ATTAGCGGCCGCGATATTTTGGCCAGCCTTGCTTG
Primer 61A-2—ATTAGGTACCGCCATTTCTGTTCGAAGCGACTT

This construct was injected into w$^{1118}$ embryos, and transgenic flies were recovered according to standard procedures.

3.2.3 Analysis of Embryonic Lethality

To determine the lethal phase of ΔGr61a,ΔGr64 double mutant embryos, R1/R1;ΔGr61a,ΔGr64/TM3Ser,GFP females were crossed to R1/Y;ΔGr61a,ΔGr64/TM3Ser,GFP males and then allowed to lay eggs on apple juice plates overnight. The number of GFP-positive and GFP-negative embryos and larvae resulting from this cross were counted over the following 2-3 days. This was necessary due to the persistence of maternally-contributed GFP in the embryo, which can be observed for up to 24 hours after egg-laying, even in embryos that ultimately showed a GFP-negative phenotype (unpublished observations).
In an attempt to rescue the inability of late-stage $\Delta Gr61a, \Delta Gr64$ homozygous embryos to hatch, the progeny of $R1/R1; \Delta Gr61a, \Delta Gr64/ TM3Ser, GFP$ adults were dechorionated at approximately 15-20 hours after being laid. Dechorionation was accomplished by soaking the embryos in a 50% solution of water and household bleach (6.15% sodium hypochlorite) for two minutes, followed by thorough rinsing with distilled water. Dechorionated embryos were then placed on apple juice plates supplemented with yeast, and the number of GFP-positive and GFP-negative larvae that hatched from this group of embryos was counted over the course of two days. GFP-negative larvae were never observed, out of a total of approximately 192 larvae screened.

3.3 Results

3.3.1 Loss of Gr61a reduces the response to glucose and arabinose

Seven of the eight sugar receptor family genes have been previously characterized (Dahanukar et al. 2001; Ueno et al. 2001; Chyb et al. 2003; Inomata et al. 2004; Isono et al. 2005; Dahanukar et al. 2007; Jiao et al. 2007; Slone et al. 2007; Jiao et al. 2008). However, the last remaining member of the gene family, $Gr61a$, has yet to be associated with any ligand, sugar or otherwise. To determine whether or not $Gr61a$ is involved in the perception of sweet stimuli, we investigated the consequences of $Gr61a$ mutant allele on sugar-sensing behavior. This allele, which removes part of the predicted $Gr61a$ coding sequence, has previously been reported to have no electrophysiological defect in sugar perception, and no behavioral analysis was carried out with $Gr61a$ mutant flies (Dahanukar et al. 2007). When we performed PER analysis on these mutants, however,
we observed a significant defect in their responses to D-(-)-arabinose, glucose, and sucrose compared to \( w^{1118} \) background control flies (Figure 11), although their responses to maltose, trehalose, fructose, and glycerol were unaffected. Furthermore, when we placed a Gr61a genomic rescue construct into the \( \Delta \)Gr61a mutant background, we observed a significant restoration of the glucose response of the mutant flies (although the sucrose and arabinose responses were not increased by a significant amount) (Figure 12). These results, which disagree with the conclusions of the Carlson group, suggest that \( Gr61a \) is indeed a sugar receptor that is involved in the detection of a subset of pyranose sugars.

Figure 11: \( \Delta \)Gr61a mutants are deficient in the sensation of some sugars.

\( \Delta \)Gr61a mutant flies show wild-type responses to maltose and glycerol, but severely reduced responses to glucose and arabinose. The \( \Delta \)Gr61a mutant strain was previously generated by another group (Dahanukar et al. 2007). Graphs represent the probability of proboscis extension. Sugars were tested at 100mM concentration. Each graph is the average of 6-10 experiments +/- SEM. Asterisks indicate a significant difference between the mutant and rescue flies, as determined by Student’s t-test (* indicates p<0.05, ** indicates p<0.001, *** indicates p<0.0001).
Figure 12: Rescue of \( \Delta Gr61a \) mutant phenotype.

Sugar response of \( w^{11}18 \) flies, \( \Delta Gr61a \) mutants, and \( \Delta Gr61a \) mutants carrying one copy of a \( Gr61a \) genomic rescue construct. The \( Gr61a \) genomic rescue construct significantly increases the response of mutant flies to glucose, but not to sucrose and arabinose. Each graph is the average of 5-10 experiments +/- SEM. Asterisks indicate a significant difference between the indicated strain and \( \Delta Gr61a \) homozygotes, as determined by Student’s t-test (* indicates \( p<0.05 \), *** indicates \( p<0.0001 \)).

3.3.2 Combinations of \( Gr5a, Gr61a \) and \( Gr64 \) mutants

3.3.2.1 Mutants lacking \( Gr61a \) and the \( Gr64 \) genes are embryonic lethal

In order to investigate the phenotype of flies lacking all but the GR5a sugar receptor, we recombined the \( \Delta Gr61a \) deletion with the \( \Delta Gr64 \) deletion (both of which are on the 3\(^{rd}\) chromosome) to create a \( \Delta Gr61a, \Delta Gr64 \) double mutant chromosome.

Surprisingly, we found that the \( \Delta Gr61a, \Delta Gr64 \) mutant chromosome is homozygous lethal, even in the presence of the R1 transgene that normally rescues the lethality of the \( \Delta Gr64 \) deletion. This lethality occurs sometime prior to the early larval stages, since we
never observe homozygous mutant larvae (represented by GFP-negative animals) when the double mutant chromosome is placed over a GFP-marked balancer (see crossing scheme in Figure 13A and data in Table 2). GFP-negative embryos are observed, however, comprising approximately 19.0% of the embryos laid by R1/R1;ΔGr61a, ΔGr64/TM3Ser,GFP parents (79 out of 416 total embryos observed, Table 2). This is statistically different (chi-square test, P-value < 0.0001) from the expected ratio of 1:3 GFP-negative to GFP-positive embryos that we would expect to see for this cross (assuming that the non-GFP embryos represent the ΔGr61a, ΔGr64 homozygote class). The magnitude of the difference is relatively small, however, and may represent technical difficulties in identifying GFP-negative embryos.

Most of the non-GFP embryos that could be unambiguously identified showed mouth hooks and other signs of advanced development, suggesting that the embryos die during embryogenesis. One possible interpretation for this phenotype is that the embryos are able to reach the 1st instar stage of larval development, but are too weak to break through the chorion and eventually starve to death within their eggshell. This explanation is supported by the fact that GFP-negative larvae could occasionally be obtained by dechorionating and carefully dissecting the vitelline membrane from the embryos. These progeny showed extremely weak locomotion and died without progressing out of the 1st instar stage of larval development. Significantly, homozygous ΔGr61a,ΔGr64 mutants carrying the UAS-Gr64abcd_GFP_f construct rescue this lethality (see Figure 13B), implying that the sugar receptor deletions are indeed responsible for the lethality of the
double mutant embryos. However, this result is somewhat contradicted by the fact that a genomic rescue construct for \textit{Gr61a} fails to rescue the lethality of the double mutant (data not shown).

Figure 13: Crossing schemes used in the analysis of \(\Delta 61a, \Delta 64\) lethality.

(A) Cross to determine stage of lethality. Lack of GFP indicates individuals that are homozygous for the double mutant chromosome. Non-GFP embryos can be observed among the progeny of this cross, but non-GFP larvae are never observed.

(B) The \textit{UAS-Gr64abcd_gfp_f} transgene can rescue the lethality of the double mutant chromosome.
<table>
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<tr>
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<th># GFP-Plus Animals</th>
<th># GFP-Plus Animals</th>
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<tbody>
<tr>
<td>Embryos</td>
<td>337</td>
<td>79</td>
</tr>
<tr>
<td>Larvae</td>
<td>192</td>
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Table 2: GFP-positive and negative progeny from cross in Figure 13A.

This table shows the number of progeny that were observed for each class of offspring. The number of GFP-positive larvae was estimated by subtracting the number of embryos that failed to hatch from the total number of embryos laid, since all larvae that hatched were observed to be GFP-positive.

3.3.2.2 Phenotypes of other double mutant combinations

The embryonic lethality of the ∆Gr61a, ∆Gr64 double mutant chromosome makes the generation of mutants lacking all eight sugar receptor genes difficult for the time being. However, it should still be possible to examine the phenotype of other sugar receptor mutant combinations. For example, we have obtained Gr5a,Gr61a double mutant flies and shown that they possess no additional loss of sugar sensitivity relative to Gr5a and Gr61a single mutants (Figure 14). Not surprisingly, the Gr5a,Gr61a double
mutants are viable, since they lack only two sugar receptor genes, versus the seven genes that were eliminated in the inviable \( \Delta Gr61a, \Delta Gr64 \) double mutant animals. Most importantly, the \( Gr5a, Gr61a \) double mutants do not show a severe loss in fructose sensitivity, providing additional evidence that there may be a fructose receptor outside of the \( GR5a, GR61a, GR64a-f \) family of proteins. Future examination of \( Gr5a, Gr64 \) double mutants will provide further clarification on this point.

**Figure 14:** Taste response of \( Gr5a, Gr61a \) double mutants.

No additional sugar-sensation deficits are created by combining a \( Gr5a \) and \( Gr61a \) mutation beyond those exhibited by each mutation on its own. Graphs represent the probability of proboscis extension. Sugars were tested at 100mM concentration. Each graph is the average of 6 experiments +/- SEM. Asterisks indicate a significant difference between the mutant and \( w^{1118} \) flies, as determined by Student’s t-test (* indicates \( p<0.05 \), ** indicates \( p<0.001 \)). The phenotype of \( Gr5a, Gr61a \) double mutants is \( Gr5a^{LexA::VP16}; \Delta Gr61a \). Refer to Figure 11 in this chapter and Figure 21 in Chapter 4 for the phenotypes of the \( Gr61a \) and \( Gr5a \) single mutants, respectively.
3.4 Discussion

3.4.1 Detection of sugars is mediated by a subfamily of GR proteins

Homologs of the *Drosophila* sugar receptor genes are present in all currently sequenced insect species (Kent and Robertson 2009) and can even be found in the water flea *Daphnia pulex* (Penalva-Arana et al. 2009). In fact, in *Daphnia*, the only GRs that have any identifiable homology to *Drosophila* GRs are the predicted sugar receptors DpuGR55 and DpuGR56 (Penalva-Arana et al. 2009). While it has not been demonstrated experimentally that the homologs of the *Drosophila melanogaster* sugar receptors in other insect species are also sugar receptors, the fact that homologs of these proteins are found throughout the arthropod phylum strongly suggests that they perform an important and conserved function. Based on the importance of sugars in the diet of many animal species, it is tempting to speculate that sugar perception is the function of these proteins in all arthropods. Given the variety of genetic tools available in the fruit fly model system and the absence of such tools in other insect species, *Drosophila* should provide an excellent platform in the future for determining the function of these putative sugar receptor genes.

3.4.2 A role for sugar receptors in embryonic development

By attempting to create mutants that lack all eight of the predicted sugar receptor genes, we sought to determine whether or not there are additional, low-affinity taste receptors for fructose that exist outside of this gene family. Although we were unable to generate mutants lacking all eight receptors, were able to reveal an indispensable and
unexpected role for the Drosophila sugar receptors in embryonic and/or very early larval development. Specifically, our observations suggest that sugar receptors may be necessary for late-stage embryos to survive and progress to later stages of development. Although we still cannot say with certainty what the potential mechanism of this lethality might be, it is possible that at least some of the sugar receptors are required for the monitoring and/or regulation of internal energy homeostasis, similar to the insulin and insulin-like receptors in most animal species. There are reports indicating that T1R2 and T1R3, the protein subunits of the mammalian sugar receptor found in the taste buds, are also expressed in the gut and are involved in the regulation of metabolism and energy homeostasis through regulation of glucagon-like-peptide 1 levels in the bloodstream (Jang et al. 2007; Kokrashvili et al. 2009). Perhaps Drosophila sugar receptors perform a similar role in energy regulation and homeostasis in fruit flies. One way to investigate this would be to measure glucose or trehalose levels in embryos lacking the Gr61a and Gr64a-f sugar receptor genes, and this will be one approach pursued in future studies. Such analysis may also need to be extended to other sugar receptor genes, such as Gr28b.c, which has been shown to be expressed in the developing embryo using both in situ hybridization and Gal4 drivers (Thorne and Amrein 2008).

We have demonstrated that the UAS-Gr64abcd_GFP_f transgene is capable of rescuing the lethality of the ∆Gr61a,∆Gr64 double mutant chromosome. However, the Gr64abcd_GFP_f rescue construct is located on the 3rd chromosome and was recombined onto the double mutant chromosome in order to create the rescue line. This leaves open
the possibility that the rescue of the lethality is due to the removal of a second-site mutation during the process of recombining the $Gr64abcd_GFP_f$ rescue onto the double-mutant chromosome. The fact that a $Gr61a$ genomic rescue transgene located on the 2nd chromosome fails to rescue the recessive lethality of the $\Delta Gr61a, \Delta Gr64$ chromosome adds some support to this interpretation, although it is also possible that expression from the $Gr61a$ transgene does not fully recapitulate wild-type mRNA levels.

If the sugar receptors in other insects also turn out to be critical for viability, this fact could be used to develop new methods of pest control. One obvious way to do so would be to develop antagonists targeted specifically against insect sugar receptors that would drastically reduce the number of insect larvae produced by a pest species while having little or no effect on crops, livestock, or humans. One concern with this approach would be the potential for such an antagonist to have serious effects on non-targeted insect species, given the presence of sugar receptor homologs across the insect class. Thus, studies determining the functional similarity of the sugar receptors from different species of insects will be vital in pursuing such an approach in the future.

### 3.4.3 Future directions in the study of *Drosophila* sugar receptors

The sugar-sensation defects of the $\Delta Gr64$ and $\Delta Gr61a$ mutants described in Chapters 2 and 3 conclusively show that the receptors encoded by these genes are involved in sugar perception. One of the more interesting questions that remains to be answered about the eight sugar receptor proteins is which sugars are detected by specific members of this GR family? We have already answered this question in the case of
Gr61a, which seems to have a specific and important role in the sensation of the monosaccharides glucose and arabinose, but no role in the sensation of the disaccharides maltose or trehalose. In Chapter 4, the phenotypes of additional single gene mutants will be described.

The inviability of ∆Gr61a,∆Gr64 double mutants, while interesting, creates a temporary setback in the long-term goal of generating flies lacking all eight sugar receptor genes. The possibility that the lethality of the ∆Gr61a,∆Gr64 double mutant is due to an additional, unidentified mutation on the ∆Gr61a,∆Gr64 chromosome is being actively investigated. If it is confirmed that the sugar receptor mutations themselves are responsible for the lethality, there are still several ways that ∆Gr5a;∆Gr61a,∆Gr64 triple mutants could be made. One solution would be to use a mosaic analysis system like MARCM (Lee and Luo 2001) to generate tissue-specific, triple mutant sensilla within an otherwise wild-type animal, and then to conduct electrophysiological and behavioral analyses on the mutant sensilla. Another option would be to use homologous recombination to create a version of the Gr61a gene flanked by loxP recombination sites. This would allow the Gr61a gene to be removed in a taste-tissue-specific manner by expressing UAS-Cre with a Gr5a-Gal4 or Gr64f-Gal4 driver. Whatever the solution, it will be critical to circumvent the lethality of the ∆Gr61a,∆Gr64 double mutant before some of the more interesting questions about the Drosophila sugar receptor gene family can be addressed.
4. Chapter 4: Analysis of the *Drosophila* sugar receptors using homologous recombination

4.1 Introduction

4.1.1 Limitations of standard molecular tools in the study of *Gr* genes

A vital part of the characterization of a gene is determining when, where, and how strongly that gene’s mRNA and protein products are expressed in the living organism. Expression data can be a valuable tool for confirming or disproving the function of a gene of interest. Expression analysis can also yield tools, such as Gal4-promoter fusion transgenes, that can be useful in the future genetic manipulation of cells expressing a gene of interest.

The expression pattern of the *Drosophila* gustatory receptor genes has been studied for much of the last decade. However, detecting *Gr* transcripts using standard techniques like *in situ* hybridization has proven difficult, suggesting that these transcripts are expressed at a very low copy number (Clyne et al. 2000). Although *in situ* analysis been used to successfully detect the transcripts for some of these genes (Dahanukar et al. 2007; Lee et al. 2009; Moon et al. 2009), many *Grs* that can be detected using Gal4 drivers have not been detected using *in situ* hybridization (Scott et al. 2001). Thus, *in situ* analysis alone may not be sufficient to obtain a comprehensive view of *Gr* gene expression. RT-PCR analysis is more sensitive and therefore more successful at detecting low-copy-number transcripts (Dahanukar et al. 2007; Slone et al. 2007; Jiao et
al. 2008). However, it provides no information about which specific cells express a given gene.

In light of the limitations of the techniques described above, transgenic promoter-Gal4 drivers, in combination with UAS-GFP or UAS-lacZ reporters, have been the most effective tools for the study of gustatory receptor expression. The signal amplification inherent in the Gal4/UAS system make it a logical tool for analyzing the expression of genes that are expressed at low levels, such as the Gr genes. In fact, the expression of a significant number of Gr genes has been characterized using this system (Dunipace et al. 2001; Scott et al. 2001; Hiroi et al. 2002; Thorne et al. 2004; Dahanukar et al. 2007). Also, unlike other tools used in expression analysis, Gal4 drivers can be combined with different UAS transgenes to express a variety of proteins in a particular set of cells.

Unfortunately, there are a few caveats to using the Gal4/UAS reporter system to study the Gr genes. In some cases, expression data obtained from such reporters conflicts with other information that is known about the gustatory receptor genes. One such example has already been described in Chapter 2 regarding the Gr64 cluster, where the first five genes in the cluster (Gr64a-e) have not been detected in the labellum using the Gal4/UAS system, but are detected in that tissue by RT-PCR (Jiao et al. 2008) and are known to be crucial for sugar perception. One possible explanation for these conflicting observations is that there are regulatory elements that normally control the expression of these genes but which are not contained within the short genomic fragments that have been used to make the promoters for the Gal4 drivers. Since enhancer elements in
Drosophila have been observed up to 80 kb away from the genes they regulates (Jack et al. 1991), the only way to completely circumvent this problem would be to place the driver into the exact locus occupied by the gene being investigated. This can be accomplished in Drosophila using a technique known as homologous recombination.

4.1.2 Homologous recombination as a tool for studying the sugar receptor genes

Ends-out homologous recombination is a powerful tool for genetic manipulation in Drosophila and other model systems like yeast and mice. This tool allows researchers to take advantage of the biological phenomenon of homologous recombination to precisely replace a gene of interest with a deletion of the gene, an altered version of the same gene, or a reporter. Alterations that add new DNA sequences into the genome rather than removing DNA sequences are often referred to as knock-ins. By generating knock-ins that replace several Drosophila sugar receptor genes with sequences encoding Gal4 and LexA::VP16, we have generated driver alleles that reveal expression patterns that suggest novel functions for the members of this gene family. At the same time, these knock-in alleles represent single-gene null mutations that can be used to more precisely dissect the function of the genes in the Gr64 cluster.

4.2 Materials and Methods

4.2.1 Transgenic Constructs

Targeting constructs for homologous recombination were generated using the Takara LA Taq PCR system, following the manufacturer’s protocol. The CMC105
plasmid (a gift from Gary Struhl’s lab) was used as the basis for making our targeting constructs. As an initial step, we cloned two copies of the loxP sequence into the CMC105 plasmid: one into the AvrII site and another into the BstEII site, both in the same orientation. From the resulting plasmid, we made two additional constructs: one with the Gal4 sequence cloned into the AvrII site (CMC-loxP-Gal4), and another with the LexA::VP16 sequence cloned into the SpeI and AvrII sites (CMC-loxP-LexA::VP16). For each gene-specific targeting construct, genomic fragments flanking the predicted coding sequences of the gene being targeted were generated by PCR. These PCR products were cloned into TOPO vectors, sequenced, and then cloned into the upstream and downstream multiple cloning sites of CMC-loxP-Gal4 or CMC-loxP-LexA::VP16 plasmids. For cloning the Gr5a 3’ flank, an internal SpeI site in the PCR product was used in lieu of the NheI site created by the primer “Gr5a 3’ Flank Downstream.” Likewise, an internal NheI site in the Gr64f 3’ flank PCR product was used in lieu of the NheI site created by the primer “Gr64f 3’ Flank Downstream.” The primers used to generate the flanking genomic DNA fragments are as follows:

Primer Gr5a 5’ Flank Upstream—
CGTACGCGCAACTGGAAATGGAAATCTGA

Primer Gr5a 5’ Flank Downstream—
ACTAGTTGTGTACAAGCTCTAAATCCTGACTAAACG

Primer Gr5a 3’ Flank Upstream—
GGTGACCCACCCCTTCAATTTGATTAGACGCAC
Flies containing the *lexAop-rCD2::GFP* reporter were previously generated by Tzumin Lee’s group (Lai and Lee 2006). For our *lexAop-nucGFP* reporter transgene, a
1.1 kb fragment containing GFP fused to a C-terminal nuclear localization signal (cloned from the vector MA1300, a gift from Dr. Maki Asano) was cloned into the Acc65I and XbaI sites of the vector pLOT (also a gift from Tzumin Lee) (Lai and Lee 2006).

All constructs were injected into w1118 embryos, and transgenic flies were recovered according to standard procedures.

4.2.2 Homologous Recombination

Knock-in alleles for Gr5a, Gr64a, and Gr64f were generated using an ends-out homologous recombination protocol adapted from a previously described protocol (Rong and Golic 2000). Briefly, flies carrying donor transgenes for the gene-targeting constructs were crossed to flies carrying hs-FLP and hs-I-SceI transgenes. Three to four days after the crosses were set up, bottles carrying the progeny were heat-shocked for one hour in a 37°C water bath. When the progeny of these crosses eclosed, white-eyed or variegated virgin females were removed from the bottle and crossed to w1118 males. The offspring of this second cross were then screened for knock-in alleles by looking for red-eyed progeny (indicating the presence of the mini-white marker from the targeting construct), and these red-eyed progeny were crossed to double balancer flies to map the chromosome containing the candidate knock-in allele. Each knock-in allele that mapped to the correct chromosome was ultimately confirmed using PCR. In the case of Gr64aGal4, the floxed mini-white marker was removed from the knock-in allele using an hs-Cre transgene. For molecular confirmation of the knock-in alleles, the following primers were used to perform PCR on genomic DNA from knock-in candidate lines.
(“Primer Gr5a 3’ KO DOWN” is the same as “Primer Gr5a 3’ Flank Downstream” described above, and “Primer Gr64f 3’ KO DOWN” is the same as “Primer Gr64f 3’ Flank Downstream”):

Primer Gr5a 5’ KO UP—CGCATTTAACGTTGTTTCCGGTG
Primer Gr5a 3’ KO DOWN—GCTAGCGTTTTTACGCCTGCTGTCTG
Primer Gr64a 5’ KO UP—GATCACCTTGTACCTGTGTGCG
Primer Gr64a 3’ KO DOWN—GCTTGATGGCTTCCTGGAAAGATC
Primer Gr64f 5’ KO UP—
TTTGGGTACCCCACCATGCAGCAGTCGGGTCAAAAAG
Primer Gr64f 3’ KO DOWN—
GCTAGCCCTTATGGCGGACACTGCAATCCTGG
Primer Gal4 AR Extend—CTTGATTCCACTTCTGTCAGATGT
Primer LexA seq 321 REV—GACCTTCAATATGCTGTTGCGC
Primer CMC 3’ Left 1—TGACGCTACGTAACGCTACAAACGG

4.2.3 Immunohistochemistry

Brain tissue from adult flies that were at least 3 days old were dissected, fixed in 4% paraformaldehyde, and stained according to a previously published protocol (Thorne and Amrein 2008). In summary, whole-mount brains were stained with rabbit anti-GFP (Molecular Probes, Catalog# A6455) at 1:100 dilution and mouse anti-nc82 (a non-commercial antibody from Reinhard Stocker, used as a counterstain against neuropil) at
1:10 dilution. Staining was performed overnight, at room temperature, in PBS with 5% heat-inactivated goat serum and 0.1% Triton-X (blocking solution). The following day, tissues were washed several times and then stained again overnight, at room temperature, with secondary antibodies (all at 1:100 dilutions) in blocking solution. The secondary antibodies that were used were Goat-anti-mouse Cy3 (Jackson ImmunoResearch Labs, Catalog# 115-166-072) and Goat-anti-rabbit Alexa488 (Molecular Probes, Catalog# A11070). Following staining with the secondary antibodies, brains were mounted on glass slides in Vectashield (Vector Laboratories, Inc) and sealed with coverslips. Spacers were placed on both sides of the samples before the coverslip was placed on top to avoid compression of the brain tissue and, specifically, of the antennal lobes. Other tissues that were stained, such as the labellum, were stained using a similar procedure, except that the spacers were not used, and mouse anti-elav-9F8A9 (DSHB, Iowa City, IA) was used (at 1:10 dilution) instead of mouse anti-nc82. In most cases, however, GFP expression in peripheral chemosensory tissues was recorded by directly imaging fluorescence from freshly dissected tissue (referred to as “live-GFP” in the text).

**4.2.4 Behavioral Experiments and Statistical Analysis**

Proboscis Extension Response (PER) assays were performed as described in Chapter 2, using a modified version of a previous protocol (Wang et al. 2004). Error bars represent +/- SEM, and statistical significance was calculated in Microsoft Excel using Student’s t-test (assuming unequal variance). All experiments were performed blind.
Trap assays to detect a role for the sugar receptors in olfaction were performed in chambers made from 250 mL Pyrex beakers that contained traps constructed from 1.7 mL Eppendorf tubes. Four traps were placed into each chamber, two containing water alone and two containing the sugar solution that was to be tested. Each trap also contained a piece of steel mesh that was designed to physically separate the flies from the water or sugar solution, while still allowing them to smell volatile compounds released by the solution. Flies were starved overnight, and then immobilized on ice before being placed in the chamber, which was then placed in the dark. The number of flies in each trap was counted after 3 hours and also after 18-24 hours (“overnight”). The total number of flies that went into the water traps and into the sugar traps was counted for each set of experiments, and statistical significance was calculated using a chi-square test.

4.2.5 RT-PCR Analysis

RT-PCR was performed on DNase-treated RNA samples using the One-Step RT-PCR kit (Invitrogen), as described in Chapter 2, except that dissected maxillary palps were used as the tissue source instead of whole heads. Each putative RT-PCR product was directly sequenced to determine whether it represented the expected cDNA product or genomic contamination. Since the Gr5a and Gr64f cDNA products had gaps that corresponded to predicted intronic sequences, they were assumed to be derived from spliced mRNA. The nucleotide sequence of each primer used for RT-PCR analysis is as follows (primers T1 and T2, which are specific to the alpha-tubulin gene, were used as controls):
Gr5a RT 5’—CAACTGGCTGGCTTGCTGGGCAAGAT
Gr5a RT 3’—TCTAGACTAATCAAGATTGAAGGTGAGCAGTC
Gr64f RT 5’—GTTTCGCCAGCTCAACGATG
Gr64f RT 3’—TTTTTCTAGACTATGAGTAATAGCTCTGATCGCAG
Gr66a RT 5’—GTAAAGGCCTCATCCACCAG
Gr66a RT 3’—GCGGCCGCGATGGACAACATGGCGCAG
Primer T1—CCTTGTCGCGTGTGAAACACTTCC
Primer T2—GATAGCCTCGTGTGAAACACTTCC

4.3 Results

4.3.1 Knock-in driver alleles for three sugar receptor genes

In order to obtain accurate reporters for the expression of the sugar receptors, we generated transgenic lines carrying targeting constructs for three of the sugar receptor genes: *Gr5a*, *Gr64a*, and *Gr64f*. Using these targeting constructs, we created knock-in Gal4 and LexA::VP16 drivers for these genes. LexA::VP16 is a transcriptional activator that binds to a different activator sequence than Gal4, and has recently been developed as an alternative to the Gal4/UAS system (Lai and Lee 2006). By using two different driver/reporter systems to make the sugar receptor knock-ins, we will ultimately be able to perform double-staining experiments to determine the degree of overlap in the expression of these genes.
To confirm the molecular nature of the knock-in alleles, we used PCR reactions that would only produce products if the intended knock-in allele had been created (Figure 15). PCR was also conducted on animals homozygous for the knock-in alleles to confirm that the gene sequences targeted for deletion by the knock-in drivers were no longer present in the genome (data not shown). These knock-in alleles, besides providing drivers, also represent null alleles for the genes that were targeted. Thus, these alleles provide additional tools for investigating the ligand specificities of the sugar receptors.

![Figure 15: Molecular Confirmation of Sugar Receptor Knock-ins.](image)

Confirmation of the *Gr5a*<sub>LexA::VP16</sub> (left column), *Gr64a*<sub>Gal4</sub> (middle column), and *Gr64f*<sub>LexA::VP16</sub> (right column) knock-ins by PCR. The diagram at top shows the structure of the wild-type locus, the targeting construct used to generate the knock-
in, and the knock-in allele. The primers used to confirm the knock-ins are indicated by arrows. The gels show PCR-amplification products from genomic DNA derived from knock-in alleles (1) or w1118 (2). The mini-white gene has been removed from \textit{Gr64a}^{Gal4} using \textit{loxP} sites that flank the marker.

### 4.3.2 Expression of the \textit{Gr5a}^{LexA::VP16} knock-in

Since \textit{Gr64a} and \textit{Gr64f} are members of a gene cluster that has the potential for complex, interdependent, and polycistronic regulation (see Chapter 2), we decided to begin our analysis with the \textit{Gr5a} knock-in. The data obtained from this knock-in would then provide a point of comparison when we analyzed the \textit{Gr64a} and \textit{Gr64f} knock-ins.

As expected from previous Gal4 expression data, the \textit{Gr5a}^{LexA::VP16} knock-in driver shows expression in the legs and labellum when crossed to \textit{lexAop-rCD2::GFP} and \textit{lexAop-nucGFP} reporters (Figure 16B,C,E). An interesting observation yielded by analysis of the \textit{Gr5a}^{LexA::VP16} driver is that it labels approximately 26 neurons in each labial palp when it is combined with \textit{lexAop-rCD2::GFP}, but almost twice as many neurons per labial palp (approximately 59) when it is combined with \textit{lexAop-nucGFP} (based on an average of cell counts from 8-14 labial palps for each genotype). Previous \textit{Gr5a-Gal4} drivers have also shown expression in a similar number of neurons (~55-71) (Thorne et al. 2004) as those seen when \textit{Gr5a}^{LexA::VP16} is combined with \textit{lexAop-nucGFP}. However, there is only predicted to be one sugar-sensing neuron per sensillum in the labial palp, which only adds up to about 33-34 neurons per palp. Thus, the number observed when \textit{Gr5a}^{LexA::VP16} is combined with \textit{lexAop-rCD2::GFP} is more in line with what one would expect if \textit{Gr5a} is a sugar receptor that is only expressed in S cells. These
differences are likely due to the stronger fluorescence exhibited by the lexAop-nucGFP reporter, which would allow it to pick up more Gr5a-expressing cells than lexAop-rCD2::GFP. Assuming that this interpretation is correct, it would mean that Gr5a is expressed in an additional type of taste neuron besides the sugar-responding S cell.

In the future, it will be interesting to determine what other kind of neuron might be expressing GR5a in gustatory sensilla. One plausible hypothesis is that GR5a and perhaps other sugar receptors are expressed in water-sensing cells, since it has been noted that the firing rate of the W cell in response to an aqueous solution is suppressed by the addition of sucrose to the solution (Rodrigues and Siddiqi 1981). Alternatively, GR5a could have a role in the low-salt (L1) neuron, although there is no data indicating that GR5a has a role in salt perception.
Figure 16: Expression of the $\text{Gr64f}^{\text{LexA::VP16}}$ and $\text{Gr5a}^{\text{LexA::VP16}}$ knock-in drivers in various chemosensory tissues.

Live GFP epifluorescence is shown for the $\text{Gr5a}^{\text{LexA::VP16}}$ and $\text{Gr64f}^{\text{LexA::VP16}}$ drivers in the labellum (A-C), the legs (D-E), the antenna (F-G), and the maxillary palp (H-I). The reporter used in panels A-B and D-I is $\text{lexAop-rCD2::GFP}$, while the reporter used in panel C is $\text{lexAop-nucGFP}$. $\text{Gr5a}^{\text{LexA::VP16}}$ labels approximately 30 neurons per labial palp when the $\text{lexAop-rCD2::GFP}$ reporter is used (B), in contrast to the approximately 55-71 cells labeled by different $\text{Gr5a-Gal4}$ drivers (Thorne et al. 2004). However, the number of cells marked by $\text{Gr5a}^{\text{LexA::VP16}}$ when $\text{lexAop-nucGFP}$ is used is closer to 60 (C). Expression of $\text{Gr5a}$ and $\text{Gr64f}$ in the maxillary palp is confirmed by RT-PCR (J). Primers specific to $\text{Gr66a}$ are used as negative controls to rule out RNA contamination from the labellum. Primers against the alpha-tubulin gene are used to ensure integrity of the RNA sample.

4.3.3 Expression of the $\text{Gr64a}^{\text{Gal4}}$ driver

We next decided to investigate the $\text{Gr64a}^{\text{Gal4}}$ knock-in. Consistent with $\text{Gr64a}$ being a sugar receptor gene, the $\text{Gr64a}^{\text{Gal4}}$ driver shows expression in taste neurons on the forelegs, midlegs, and hindlegs of male and female fruit flies (Figure 17C). The $\text{Gr64a}^{\text{Gal4}}$ allele also shows expression in the pharynx, although the precise organs expressing the reporter have not been identified (Figure 17D). Previous $\text{Gr64a-Gal4}$ transgenes have been shown to drive expression in one of the pharyngeal organs from this region, the VCSO (Natasha Thorne, unpublished data), and it is likely that the $\text{Gr64a}^{\text{Gal4}}$ driver is recapitulating this expression. As expected, the $\text{Gr64a}^{\text{Gal4}}$ driver shows no expression in the antenna, maxillary palp, or wing margins—tissues with no demonstrated involvement in sugar perception.

The $\text{Gr64a}^{\text{Gal4}}$ driver also shows expression in a single neuron in the labial palp (Figure 17A-B). This expression suggests that $\text{Gr64a}$ serves a relatively minor role in
taste perception in the labellum, since it is known that there are multiple sensilla that respond to sweet stimuli in the labial palp (Hiroi et al. 2002). This expression pattern also runs counter to the hypothesis that all of the Gr64 genes are expressed on a polycistronic transcript in most of the sensilla of the labial palp, since it appears that Gr64a is only expressed in a single GRN, while a previously-generated Gr64f-Gal4 driver (Dahanukar et al. 2007) and our own Gr64f LexA::VP16 knock-in (see below) both show expression in approximately 20-21 GRNs (cells counts of live-GFP from 4-7 labial palps). However, this does not preclude the possibility that subsets of the Gr64 genes are expressed as polycistronic transcripts. Alternatively, it is possible that the Gr64a Gal4 driver does not capture the full Gr64a expression pattern. The most likely explanation for such a result would be the loss of an enhancer element within a Gr64a intron during the creation of the knock-in allele. The generation of a knock-in reporter that deletes none of the Gr64a gene sequence (for example, a myc-tag fused to the GR64a coding sequence) would be one way of testing this hypothesis.
Figure 17: \(Gr64a^{Gal4}\) shows expression in several taste tissues.

The \(Gr64a^{Gal4}\) allele labels a single GRN in the labellum (A-B) and multiple cells in the forelegs (C) and pharynx (D). The tissues in panels A and B were stained with anti-GFP and anti-elav (as a pan-neuronal marker). The images in panels C and D show GFP epifluorescence from freshly dissected tissue (“live GFP”). A UAS-nucGFP transgene was used as the reporter.

4.3.4 Expression of the \(Gr64f^{LexA::VP16}\) driver

As expected for a sugar receptor gene, the \(Gr64f^{LexA::VP16}\) knock-in driver shows expression in the legs and labellum when crossed to a \(lexAop-rCD2::GFP\) reporter (Figure 16A and D). The \(Gr64f^{LexA::VP16}\) driver shows expression in about 20 GRNs per labial palp (average of cell counts from 7 labial palps) when crossed to the \(lexAop-rCD2::GFP\) reporter. This may underestimate the number of cells that express the \(Gr64f\)
driver, since we have yet to cross this driver to the *lexAop-nucGFP* reporter, the stronger of the two *lexAop* reporters.

### 4.3.5 Expression of sugar receptors in the olfactory system

In addition to their expression in the taste system, the *Gr5a*\textsuperscript{LexA::VP16} and *Gr64f*\textsuperscript{LexA::VP16} knock-ins were found to drive expression of GFP reporters in the olfactory system. Both drivers show expression in the maxillary palp (Figure 16H-I), while *Gr64f*\textsuperscript{LexA::VP16} (Figure 16F) but not *Gr5a*\textsuperscript{LexA::VP16} (Figure 16G) shows expression in the third antennal segment. It should be noted that the *lexop-rCD2::GFP* reporter used in these crosses shows no expression in any chemosensory tissues in the absence of a driver, indicating that this expression is not due to ectopic expression by the reporter itself (data not shown). The presence of Gr5a and Gr64f mRNAs in the maxillary palp is confirmed by RT-PCR (Figure 16J).

Since neurons expressing a given OR project their axons to a single, stereotyped glomerulus in each half of the antennal lobe, staining of axon projections in the antennal lobe should provide clues about the identity of the OSNs that express the sugar receptor genes. To gain a more detailed understanding of which olfactory neurons express *Gr5a* and *Gr64f*, brains were dissected from adult flies carrying copies of the *LexA::VP16* knock-in alleles and a copy of the *lexop-rCD2::GFP* reporter. Staining of these brains indicates that *Gr64f*\textsuperscript{LexA::VP16} neurons project to multiple glomeruli in the medial part of the antennal lobe (Figure 18A), while *Gr5a*\textsuperscript{LexA::VP16} neurons project to a single glomerulus in a ventral part of the antennal lobe (Figure 18B).
To help determine which glomerulus receives axons projections from the Gr5a-expressing OSNs, crosses were performed to generate Gr5a<sup>LexA::VP16</sup>; UAS-mCD8::GFP/+; Or-Gal4/lexAop-rCD2::GFP flies. In these crosses, Or-Gal4 drivers representing four of the six types of maxillary palp OSNs (Or33c-Gal4, Or42a-Gal4, Or46a-Gal4, and Or71a-Gal4) were used as tools to map the glomerulus innervated by Gr5a<sup>LexA::VP16</sup> neurons (since Gr64f<sup>LexA::VP16</sup> is expressed in multiple glomeruli, it was not amenable to this particular approach because both reporters use GFP). However, none of the neurons expressing these drivers appear to project to the same glomerulus as Gr5a<sup>LexA::VP16</sup>, since two glomeruli are stained when these Gal4 drivers are combined with Gr5a<sup>LexA::VP16</sup>, while each driver alone only stains a single glomerulus (Figure 19A-B). This leaves the remaining two maxillary palp Or genes (Or85d and Or59c) as possible candidates for coexpression with the Gr5a<sup>LexA::VP16</sup> driver. The Or85d neurons (which project to the VA4 glomerulus) are the most likely to be expressing Gr5a<sup>LexA::VP16</sup>, since Or59c neurons project to a medial glomerulus that is quite distant from the apparent ventral position occupied by the Gr5a<sup>LexA::VP16</sup> glomerulus. A repetition of the approach described above, but with UAS-mCD8::GFP replaced by UAS-mRFP (to allow the expression of the Gal4 and LexA::VP16 drivers to be differentiated), should allow this tentative classification to be confirmed in the future. A similar approach should identify which Or genes the Gr64f<sup>LexA::VP16</sup> driver is coexpressed with in olfactory neurons of the maxillary palp and antenna.
Figure 18: Antennal projections of the *Gr5a* and *Gr64f*-expressing olfactory sensory neurons.

*Gr64f* neurons from the maxillary palp and antenna appear to project primarily to a cluster of medial glomeruli in the antennal lobe (A). Weaker staining is also sometimes observed in a pair of dorsal glomeruli (A). The *Gr5a* neurons in the maxillary palp appear to project to a particular glomerulus in the antennal lobe (B), and this projection pattern disappears when both maxillary palps are removed (data not shown). For both panels A and B, brains were stained with anti-GFP and anti-nc82 (as a neuropil marker).
Figure 19: Mapping the Gr5a-expressing glomerulus.

(A) Staining of dissected brains from adult flies carrying $\text{Gr5a}^{\text{LexA::VP16}}$ in combination with $\text{Or-Gal4}$ drivers that are expressed in the maxillary palp. $\text{UAS-mCD8::GFP}$ and $\text{lexop-rCD2::GFP}$ transgenes were used as reporters for the expression of the Gal4 and LexA::VP16 drivers, respectively. (B) Staining of brains carrying the $\text{Or-Gal4}$ drivers and $\text{UAS-mCD8::GFP}$ reporter in the absence of the $\text{Gr5a}^{\text{LexA::VP16}}$ allele. (C) Depth coding of GFP expression from brains shown in panel A. Pixels sharing the same color are located at the same depth. Glomeruli that overlap on the X-Y axis can be more readily distinguished with the addition of depth-coded information. For all panels, brains were stained with anti-GFP and anti-nc82 (as a neuropil marker).
4.3.6 Behavioral Analysis of the sugar receptor knock-ins

4.3.6.1 PER response of $Gr64a^{Gal4}$ knock-in flies

Since the $Gr64a^{Gal4}$ knock-in allele is also a genetic null for the $Gr64a$ gene, flies homozygous for this knock-in allele were tested for taste sensitivity using the PER assay. Similar to $\Delta Gr61a$ mutants, animals homozygous for the $Gr64a^{Gal4}$ knock-in show severe reductions in the sensation of glucose and arabinose (Figure 20A), a result that is consistent with the observation the GR61a and GR64a proteins are most closely related to each other among the sugar receptor proteins (Figure 4B). Unlike $\Delta Gr61a$, however, the $Gr64a^{Gal4}$ allele also causes additional defects in glycerol and sucrose sensation, demonstrating that the two receptors do not share identical ligand specificities.

The glucose and arabinose defects of the $Gr64a^{Gal4}$ mutant cannot be rescued by expressing $Gr64a$ cDNA using the $Gr64a^{Gal4}$ driver (Figure 20B). This suggests either that the $Gr64a^{Gal4}$ driver does not express in the proper number of cells or that the deletion created by the knock-in allele has disrupted the regulation of downstream genes in the $Gr64$ cluster. The former conclusion is somewhat contradicted by the fact that expression of the $Gr64a$ cDNA using a $Gr5a-Gal4$ driver (which expresses in a larger number of sugar-sensing neurons) fails to rescue the glucose and arabinose defects of the $Gr64a^{Gal4}$ knock-in (Figure 20B). Experiments to determine if expression of the genes downstream of $Gr64a$ can rescue the $Gr64a^{Gal4}$ defect have not yet been performed.
Figure 20: \textit{Gr64a}^{Gal4} knock-in flies have a moderate deficiency in sugar perception.

(A) The sugar responses of \textit{Gr64a}^{Gal4} knock-in flies are reduced relative to two control strains (\textit{w1118} and the \textit{M68-1}, the transgenic donor strain used to generate the knock-in), but are still greater than those of \textit{ΔGr64} mutants (\textit{R1/+;R2/++;ΔGr64/ΔGr64}). (B) The sucrose and glycerol-sensing deficits of \textit{Gr64a}^{Gal4} knock-in flies can be rescued in the presence of \textit{UAS-Gr64a} and a \textit{Gr5a-Gal4} transgenes. \textit{UAS-Gr64a} alone is not enough to rescue any of the behavioral deficits, despite the presence of the \textit{Gr64a}^{Gal4} driver allele. For both panels A and B, graphs represent the probability of proboscis extension. Sugars were tested at 100mM concentration. Each graph is the average of 5-6 experiments +/- SEM. Asterisks indicate a significant difference between the indicated strain and \textit{w1118}, as determined by Student’s t-test (* indicates p<0.05, ** indicates p<0.001, *** indicates p<0.0001).
4.3.6.2 PER response of $Gr5a^{LexA::VP16}$ and $Gr64f^{LexA::VP16}$ flies

To confirm the involvement of $Gr5a$ and $Gr64f$ in sweet taste perception, we examined the sugar responses of the $Gr5a^{LexA::VP16}$ and $Gr64f^{LexA::VP16}$ mutants. Not surprisingly, these animals showed taste defects towards pyranose sugars that agree with the prevailing body of research on genes from this family (Figure 21). Specifically, the defects in melezitose and trehalose perception observed for the $Gr5a^{LexA::VP16}$ mutant agree with electrophysiological studies from John Carlson’s group (Dahanukar et al. 2001; Dahanukar et al. 2007) (Figure 21A). However, we do not observe a defect for glucose perception and we suggest that other neurons may express GRs in $Gr5a^{LexA::VP16}/Gr5a^{LexA::VP16}$ mutants that are responsible for proper behavioral response to this sugar. In comparison, the $Gr64f^{LexA::VP16}$ mutants, similar to the $Gr64a^{Gal4}$ and $\Delta 61a$ mutants, show severe defects in glucose and arabinose sensation, a weak defect in sucrose sensation, and no defect in the sensation of fructose, trehalose, maltose, or glycerol (Figure 21B).
**Figure 21:** Sugar responses of $Gr5^a_{LexA::VP16}$ and $Gr64f_{LexA::VP16}$ mutants.

$Gr5^a_{LexA::VP16}$ knock-in flies show reduced sensitivity for trehalose and melezitose (A), while $Gr64f_{LexA::VP16}$ shows reduced sensitivity to glucose and arabinose (B). Both strains show additional significant behavioral deficits, but the magnitude of these reductions in PER response are relatively small (less than 25%). For both panels A and B, graphs represent the probability of proboscis extension. Sugars were tested at 100mM concentration. Each graph is the average of 6-9 experiments +/- SEM. Asterisks indicate a significant difference between the indicated strain and $w^{1118}$, as determined by Student’s t-test (* indicates $p<0.05$, ** indicates $p<0.001$, *** indicates $p<0.0001$).

### 4.4 Discussion

In addition to confirming previous assumptions about the expression of the

*Drosophila* sugar receptor genes in taste tissues, the data shown in this chapter strongly
suggest a novel role for these sugar receptor proteins in the olfactory system. Our results also demonstrate the benefits of utilizing homologous recombination to create genetic alterations that are both knock-in drivers as well as targeted null alleles that can be studied as mutants for phenotypes in animals.

4.4.1 Utility of knock-in drivers in the analysis of Gr genes

In this chapter, we have demonstrated that knock-in driver alleles can reveal expression patterns that are not observed using standard promoter-driver fusion constructs. This is not entirely unexpected, given the fact that regulatory sequences can be located at a great distance from a gene’s promoter (Jack et al. 1991), but it does raise questions about the proper approach to analyzing Gr expression in fruit flies. It now seems quite possible that the results obtained from previous Gal4 drivers may—at least in some cases—be incomplete. Even the knock-in drivers described in this work may present an incomplete picture of in vivo expression patterns, since the sequences of the genes that were knocked out (particularly the introns) may themselves contain important regulatory information. Ultimately, the best way to conclusively confirm the data produced by these drivers will be to develop complementary tools (for instance, polyclonal antibodies raised against peptide sequences specific to each sugar receptor) for detecting Gr transcripts and proteins directly. For the time being, however, knock-in driver alleles are probably the best way of obtaining accurate expression data for the gustatory receptor genes, so long as proper care is taken in the design and creation of the driver alleles.
4.4.2 Phenotypes of single-gene sugar receptor mutants

As might be expected, all of the single-gene sugar receptor mutants studied so far \((Gr5a^{LexA::VP16}, Gr64f^{LexA::VP16}, Gr64a^{Gal4} \text{ and } \Delta Gr61a)\) have shown less serious taste defects than the \(\Delta Gr64\) mutant (which removes six sugar receptor genes). Interestingly, the phenotypes of the single-gene mutants seem to fall into two groups. One group—including \(Gr64a^{Gal4}, Gr64f^{LexA::VP16}, \text{ and } \Delta Gr61a\)—shows severe defects in the detection of arabinose and glucose, modest defects in the sensation of sucrose and fructose, and no defects in the detection of maltose and trehalose. The second group, which consists of \(Gr5a^{LexA::VP16}\), shows defects in trehalose and melezitose sensation, but wild-type responses to glucose, arabinose, and all other sugars tested. As additional knock-in alleles are generated in the future, it will be interesting to see if the remaining sugar receptor genes fall into one of these two established groups, or if they fall into novel groups with unique ligand specificities.

As a whole, the findings from the studies of the single-gene sugar receptor mutants are consistent with the expression analyses discussed in this chapter, showing that individual sugar receptor proteins have distinct but partially overlapping functions. This suggests that individual GRs may co-operate and form multimeric complexes that respond to specific sugar compounds, an idea that was first proposed in Chapter 2 based on the overlapping phenotypes of \(\Delta Gr5a\) and \(\Delta Gr64\) mutants. The molecular nature and stoichiometry of these receptor complexes, however, remain a mystery.
4.4.3 A possible role for sugar receptors in olfaction

As described in previous chapters, there is evidence that at least a few gustatory receptors are involved in the detection of volatile chemical signals. The novel argument put forth in this chapter is that the some of the *Drosophila* sugar receptors, which have already been characterized as receptors for non-volatile ligands, may have roles in olfaction as well. LexA::VP16 knock-in drivers for two of these receptors, *Gr5a* and *Gr64f*, show expression in olfactory sensory neurons of the maxillary palp, while the driver for *Gr64f* shows additional expression in OSNs in the antenna. A few potential explanations for this observation are described below.

Perhaps the most likely explanation for sugar receptor expression in the olfactory system is that these particular GRs function together with ORs to form a novel olfactory receptor for volatile ligands. In light of the phylogenetic observation that the ORs are an expansion within the GR family (Robertson et al. 2003), it is not unreasonable to suggest that the two types of chemoreceptors may be able to interact to form a functional receptor complex. One report has even demonstrated that ORs can be functionally expressed in sugar-sensing taste neurons (Hiroi et al. 2008), although it has not been determined if this result is dependent on the presence of the sugar receptor proteins in those neurons.

In light of the close proximity of the maxillary palp to the labellum, it is also possible that the maxillary palp is used to detect sugar molecules dissolved in food that has come into contact with the maxillary palp during the feeding process. One study demonstrates that loss of the maxillary palp reduces the taste response of fruit flies to
Sucrose, suggesting that information from the maxillary palp is integrated into the fly’s taste response (Shiraiwa 2008). Perhaps some of the information that the maxillary palp integrates into the gustatory response is whether or not the maxillary palp has come into contact with sugar molecules. Unfortunately, this hypothesis would not explain the expression of *Gr64f* in the antenna.

Another possible explanation for sugar receptor expression in the olfactory system is that OSNs are using GR5a and GR64f as olfactory receptors to detect extremely low concentrations of sugar molecules that have volatilized into the air. There is some precedence for this in rodents, as at least one report indicates that rats can anticipate the sugar content of a solution in licking assays, presumably by smelling sugar molecules released by the solution (Rhinehart-Doty et al. 1994). This is perhaps the most difficult of the hypotheses to investigate, as sugars are relatively non-volatile chemicals, and the fly is likely to need to be quite close to a food source before it can detect significant concentrations of volatile sugar. Our initial attempts to test this hypothesis found no clear evidence that wild-type flies can smell sugar (Figure 22). This does not rule out a role for sugar receptors in the detection of volatile sugars, but it does suggest that using behavioral assays to detect such a phenomenon may be technically difficult.
Figure 22: Sugar trap assays on ORE-R wild-type flies.

(A) Design of trap assays. Funnels were placed at the top of each trap to allow flies to enter and also to prevent their escape. (B) ORE flies show little preference for sugar-containing traps versus water traps. For each experiment, the number of flies in the sugar and water traps was counted after 3 hours and also after approximately 18-24 hours (“Overnight”). The only significant difference between the number of flies found in the sugar traps versus the water traps was at the 3-hour time point for males tested with glucose (chi-square test, * = p<0.05). This difference did not hold up after running the experiment overnight.

The next logical step in the investigation of the hypotheses described above will be to identify the Ors that are coexpressed with the sugar receptor genes in the maxillary palp and antenna. Once the Ors that are coexpressed with Gr5a and Gr64f have been
determined, investigation of the potential olfactory ligands for these receptor genes can then proceed, most likely using electrophysiological recordings from the sensilla of mutant animals. Since the odorants that activate specific maxillary palp neurons have already been identified (de Bruyne et al. 1999), knowing which OSNs express the sugar-receptor GRs will provide us with a narrow list of likely olfactory ligands for the GRs. Electrophysiological techniques can also be used to record the responses of the Gr5a and Gr64f- expressing OSNs to a variety of sugar molecules. No matter what results are obtained from these experiments, prior assumptions about the function of the sugar receptors and/or the olfactory system are likely to be challenged.

### 4.4.4 Investigation of potential axon-guidance defects in Gr64f mutants

Previous work with antibodies against the *Drosophila* bitter receptors GR33a and GR93a indicates that these proteins localize not only to the dendrites and cell body, but also to the axons (Lee et al. 2009; Moon et al. 2009). This is an unexpected result and suggests that some gustatory receptors might serve an important role in axon function. One particularly intriguing possibility is that GRs, including the sugar receptors, might serve a role in guiding the axons of the peripheral sensory neurons to the proper areas of the CNS. A role for sugar-binding proteins in axon guidance is not without precedence, since at least one report in mice implicates a galactose-binding receptor in neurite outgrowth and proper axon targeting of a subset of olfactory sensory neurons (Puche et al. 1996). Thus, it is reasonable to suspect that sugar receptors might have a role in axon guidance, perhaps by binding to glycolipids or glycoproteins that help identify the proper
targets for the axons of sensory neurons. If true, this might provide an alternative explanation for the behavioral defects of sugar receptor mutants, since their taste neurons might be delivering sensory information to inappropriate parts of the brain.

To test the hypothesis that sugar receptors are involved in axon guidance, we generated flies that were homozygous for both \textit{Gr64f}^{LexA::VP16} and \textit{lexAop-rCD2::GFP}. Since such flies lack a functional copy of \textit{Gr64f}, they would be expected to show aberrant axon targeting or glomerular morphology compared to wild-type flies carrying \textit{Gr64f}^{LexA::VP16} and \textit{lexAop-rCD2::GFP}. Unfortunately, we observed no obvious differences in the location or morphology of \textit{Gr64f}-positive glomeruli in the presence or absence of \textit{Gr64f} (Figure 23). We were also unable to detect obvious defects in targeting of the taste centers in the SOG (Figure 23). However, it remains to be seen if mutants lacking multiple sugar receptors (for example, the entire \textit{Gr64} cluster) will demonstrate axon guidance defects.
Figure 23: Gr64f mutants show no defects in axon targeting of Gr64f-expressing neurons.

Images are from antibody stainings of adults that are heterozygous (A) and homozygous (B) for the Gr64f^{LexA::VP16} knock-in allele. Since the knock-in allele deletes the Gr64f coding sequence, flies homozygous for the Gr64f^{LexA::VP16} allele are mutant for the Gr64f gene. Heterozygous flies carry the knock-in allele over a TM3 balancer that has a functional copy of Gr64f. All flies carry a copy of the lexAop-rCD2::GFP reporter transgene in addition to the knock-in allele. For both panels A and B, brains were stained with anti-GFP and anti-nc82 (as a neuropil marker).
5. Summary

In this work, it was demonstrated that the eight members of the GR5a-related subfamily of *Drosophila* gustatory receptors are necessary for the perception of a number of sugar compounds. Notably, GR61a has been shown to be required for the detection of specific sugars in behavioral assays, in contrast to previously published electrophysiological recordings that failed to detect any differences in firing patterns in taste neurons of wild type and *Gr61a* mutant flies when stimulated with different sugars (Dahanukar et al. 2007). Data has also been presented suggesting that the sugar receptor genes of the *Gr64* cluster are expressed as polycistronic transcripts, and that the proteins encoded by the sugar receptor genes might function as obligate heteromultimers. Furthermore, this work has described preliminary evidence for a role for the *Drosophila* sugar receptors GR5a and GR64f (and possibly others) in olfaction. While the implications of this observation are currently unclear, some intriguing explanations have been offered, along with future experiments that should test these hypotheses and ultimately reveal the specific function that these GRs play in the olfactory system.

5.1 Future directions in insect taste research

A major gap in taste sensory transduction is the lack of information as to which receptor interacts with which ligand, and more knowledge in this area will be necessary in order to make comprehensive conclusions about the specific roles of each receptor. Indeed, it is not clear whether all GRs recognize chemosensory stimuli. Recent expression analyses have revealed that several members of the GR28b subfamily, as well
as Gr43a, are expressed in many other sensory neurons, as well as in central neurons (Thorne and Amrein 2008). These findings suggest that at least some GRs may be involved in other sensory processes such as nociception, proprioception, thermoception, sound perception, etc. It is worth noting that the GRs, not unlike the ORs, are a rapidly evolving gene family, and that the number of genes in each chemosensory family varies substantially in different insect species (Robertson et al. 2003; Robertson and Wanner 2006; Kent et al. 2008; Wanner and Robertson 2008; Smadja et al. 2009). Phylogenetic comparisons will facilitate the identification of GRs involved in common sensory processes (detection of sugars, detection of widespread noxious and toxic compounds etc). Not surprisingly, the GR5a/GR61a/GR64a subfamily is highly conserved among insects, most of which use sugars as a primary source of caloric input. The poor conservation of other GRs, on the other hand, may indicate highly species-specific functions, and it is therefore not surprising that two of the least conserved GRs (GR68a and GR32a) were shown to function as pheromone receptors (Bray and Amrein 2003; Miyamoto and Amrein 2008). In any case, the fruit fly as a model system should serve us well to address the function of GRs from Drosophila as well as other insect species.

The recent identification of the IR proteins is certain to add complexity to chemosensory coding. Members of this gene family were shown to be expressed broadly in olfactory neurons, most of which do not express any ORs (Benton et al. 2009). This suggests that IRs may recognize ligands that are not detected by ORs. Interestingly, some IRs were recently found to be expressed in taste organs (Miyamoto and Amrein,
unpublished results), raising the possibility that IRs may detect soluble ligands, possibly alone, or in combination with GRs.

With respect to the subset of GRs studied in this work, one particularly interesting question is why fruit flies employ eight different receptor proteins to detect sugars, while other insects seem to carry many fewer proteins? For example, the honeybee contains only 12 \textit{Gr} genes, and only two of them appear to be orthologs of the \textit{Gr5a/Gr61a/Gr64a} subfamily (Kent and Robertson 2009). One possibility is that flies can differentiate between different types of sugars, an ability that mammals do not possess because they have only one sugar receptor, made up of two protein subunits (Nelson et al. 2001; Zhao et al. 2003). Experiments indicating that fruit flies and other dipteran insects can discriminate between different types of sugars provide strong evidence for this model (Omand and Dethier 1969; Shimada et al. 1974; Rodrigues and Siddiqi 1981; Shimada and Tanimura 1981; Tanimura and Shimada 1981; Shimada et al. 1985). According to this model, honeybees would be expected to have lower discriminatory ability than fruit flies, based on their possession of only two sugar receptor genes. The development of a reliable cell-based system for deorphanizing gustatory receptors from other insect and arthropod species will be critical in investigating this issue. Such systems have greatly facilitated the elucidation of specific roles of mammalian olfactory and taste receptors (Saito et al. 2004; Ishimaru et al. 2006), and similar systems would streamline the analysis of sugar receptor function in insects. However, GRs and ORs, more than any other class of multitransmembrane proteins, have been notoriously recalcitrant to such analyses, and therefore \textit{in vivo} based assays appear to be the best approach to study not
only the ligand specificity of these receptors (Dobritsa et al. 2003), but also their topology and their potential to interact to form obligate heteromultimers (Benton et al. 2006).

Although it is widely accepted that the GR5a/GR61a/GR64a-f proteins function as sugar receptors, in the absence of data showing physical interaction between receptor and ligand, it cannot be ruled out that they may function downstream of the receptors that detect and bind to sugars. This seems unlikely, given that the GRs are coded by a large family of genes and are predicted to be multiple-transmembrane-domain proteins, both properties that are shared by many families of receptor proteins. Data suggesting that the sugar receptors operate as heteromultimers, described in Chapter 2, are also more consistent with a receptor model than with a signaling component model. Moreover, dendritic expression of two different bitter-sensing GRs, GR33a and GR93a, has been demonstrated using polyclonal antibodies (Lee et al. 2009; Moon et al. 2009), further supporting the argument that members of the GR family operate as chemoreceptors embedded in the dendritic membrane. Thus, despite the lack of direct evidence of ligand binding for any GR, it is widely accepted that these proteins operate directly as chemoreceptors rather than downstream signaling components.

Beyond the studies of the peripheral taste and olfactory systems that have been described in this dissertation, much remains to be learned about the functional organization and logic of the higher brain centers that integrate signals from the peripheral chemosensory system. This is especially true of the taste system, since the SOG (the primary taste processing center) lacks the clear glomerular organization of the
antennal lobe, although broad differences can be observed between the projection patterns of sugar and bitter-sensing neurons (Thorne et al. 2004; Wang et al. 2004). A better understanding of the integration of taste information in the fly gustatory system will advance our understanding not only of the insect brain, but also of the general logic and organization of neurological systems across all animal species.
References


LopezJimenez ND, Cavenagh MM, Sainz E, Cruz-Ithier MA, Battey JF et al. (2006) Two members of the TRPP family of ion channels, Pkd1l3 and Pkd2l1, are co-expressed in a subset of taste receptor cells. J Neurochem 98(1): 68-77.


Biography

Jesse Slone was born in Xenia, Ohio on June 11, 1981. Jesse graduated magna cum laude from The Ohio State University in 2004 with a Bachelor of Science degree in Molecular Genetics. While an undergraduate at Ohio State, he worked in Dr. Helen Chamberlin’s lab on a project designed to develop gene-targeting techniques in C. elegans.

In the course of his graduate studies in Hubert Amrein’s lab at Duke University, Jesse has published one research paper titled “Sugar Receptors in Drosophila.” While at Duke, he has received several fellowships and awards, including the National Science Foundation Graduate Student Research Fellowship, the James B. Duke Fellowship, and the Katherine Goodman Stern Fellowship.

Papers published