GUSTATORY PERCEPTION OF SUGARS IN *DROSOPHILA MELANOGASTER* LARVAE

An Honors Fellow Thesis

by

Alex Cole Broussard

Submitted to Honors and Undergraduate Research
Texas A&M University
in partial fulfillment of the requirements for the designation as

HONORS UNDERGRADUATE RESEARCH FELLOW

May 2012

Major: Biochemistry
Genetics

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Approved by:

Research Advisor: Hubert Amrein
Associate Director, Honors and Undergraduate Research: Duncan MacKenzie

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ABSTRACT

Gustatory Perception of Sugars in Drosophila Melanogaster Larvae. (May 2012)

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Drosophila melanogaster larvae have historically been targeted as favorable models for the study of chemosensation and chemosensory learning due to the relative simplicity of their neuroanatomy. Sugars are traditionally used as a 'reward' in learning assays, though the neurons and genes involved in larval sugar sensation are not fully known. Analysis of transgenic *D. melanogaster* larvae coexpressing *GAL4* promoter constructs with *UAS-GFP* confirmed the expression of one putative sugar gustatory receptor (*psGr*) gene in the larval stage of the life cycle, along with a novel sugar receptor, *Gr43a,* which is not a member of the traditional *psGr* gene family. *Gr43a* was found to be expressed in six neurons; two in the dorsal pharyngeal sense organ, and four further down the oesophagus, and *Gr5a* shows expression in 18 bilaterally symmetric neurons distributed along the length of the larva (segmental nerves.) Analysis of the *Gr64a, Gr64f,* and *Gr61a* genes has yielded no evidence of expression. Larval two-choice assays (LTCA) revealed a decreased preference for the sugar fructose in *ΔGr43a* larvae, indicating that it functions as a high-affinity fructose receptor, as it does in the adult. Experiments with

multiple-knockout strains indicate one or more genes in the *Gr64a-f* cluster or *Gr61a* also play a minor role in the perception of fructose. These experiments demonstrate the utility of the LTCA in deorphanizing chemoreceptors.

DEDICATION

This undergraduate thesis is dedicated to Mr. Chadwick Huckabee. In high school, I had the pleasure of studying under Mr. Huckabee in the subjects of chemistry, aquatic science, and physics, and he also coached me for UIL science. Mr. Huckabee modeled his classes after undergraduate courses, teaching general concepts in class but stressing the importance of independent study to master the material. It is not an exaggeration to say that as I entered my freshman year of college, I had gleaned nearly all of my knowledge in the areas of chemistry, physics, and biology from Mr. Huckabee's teaching. He prepared me for a university-level education, and I credit him with much of my academic success. Thank you for teaching me redox reactions, harmonic motion, phylogeny, how to identify shorebirds of the gulf coast…. Thank you for putting up with all my pranks and shenanigans over the course of four years.

ACKNOWLEDGMENTS

Special thanks to Dushyant Mishra, with whom I have collaborated on virtually every aspect of this project. Without your technical expertise and knowledge of larval physiology, I would have found it difficult to meet my deadlines.

Thanks also to Dr. Amrein, who had the courage to hire a hapless freshman in the winter of 2009. Your guidance has been invaluable.

I'd like to express my gratitude to the entire staff of the Amrein laboratory for procedural assistance and entertaining flyroom banter.

Finally, thanks to my friends and family for their continued support. Without your words of encouragement and occasional distractions from my writing, I could not have completed this thesis with my sanity intact.

NOMENCLATURE

LTCA	Larval two-choice assay
Gr	Gustatory receptor
psGr	Putative sugar gustatory receptor
DO	Dorsal organ
TO	Terminal organ
VO	Ventral organ
DPS	Dorsal pharyngeal sense organ
VPS	Ventral pharyngeal sense organ
PPS	Posterior pharyngeal sense organ

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CHAPTER I

INTRODUCTION

Sensory neurons are the means through which organisms acquire information about their external environment, information required to make decisions in processes from mating to feeding [1]. Understanding the genetic and molecular basis for perception, and how perception affects higher-order processes such as learning, is a field that has been extensively explored using the model organism *Drosophila Melanogaster* [2]. Despite this, the study of gustation has trailed behind the study of other chemosensory processes such as olfaction [3].

Gustation in larval *Drosophila*

Chemosensation occurs in the larval head via six major sensory organs (figure 1), three of which are external: the dorsal organ (DO), terminal organ (TO), and ventral organ (VO). The DO is chiefly an olfactory structure, but the TO and VO primarily serve a gustatory function. Additionally, there are three pharyngeal sense organs: the dorsal, ventral, and posterior sense organs (DPS, VPS, and PPS), which house gustatory sensilla [4]. Nonvolatile tastants are recognized by any of the 68 members of the putative gustatory receptor (*Gr*) gene subfamily (figure 2). Sweet compounds, such as sugars,

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This thesis follows the style of PLoS Biology.

generally induce feeding behavior, while bitter compounds, which are generally non-nutritive and have the potential to be toxic, illicit aversive behavior. This "primitive sense" allows organisms to distinguish between nutritious and unfit food substrates [5].

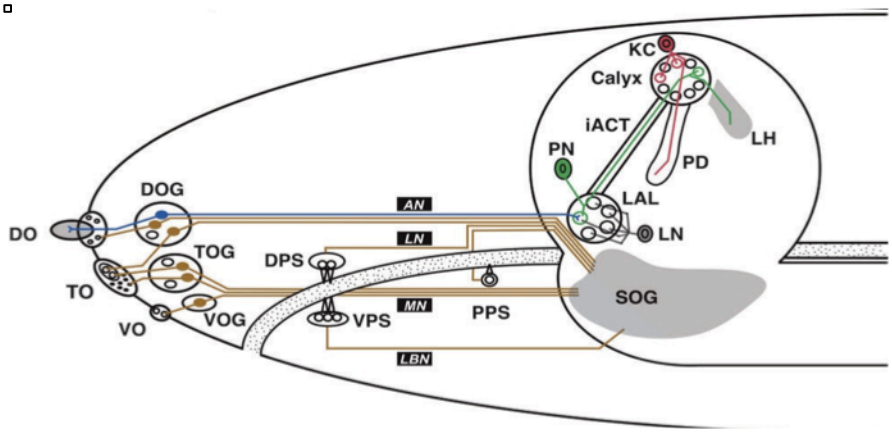

Figure 1: A schematic of the major chemosensory organs in Drosophila larvae. Notice that gustatory neurons project to the suboesophageal ganglion (SOG), known to process gustatory input, while olfactory neurons project to the larval antennal lobe (LAL) [4].

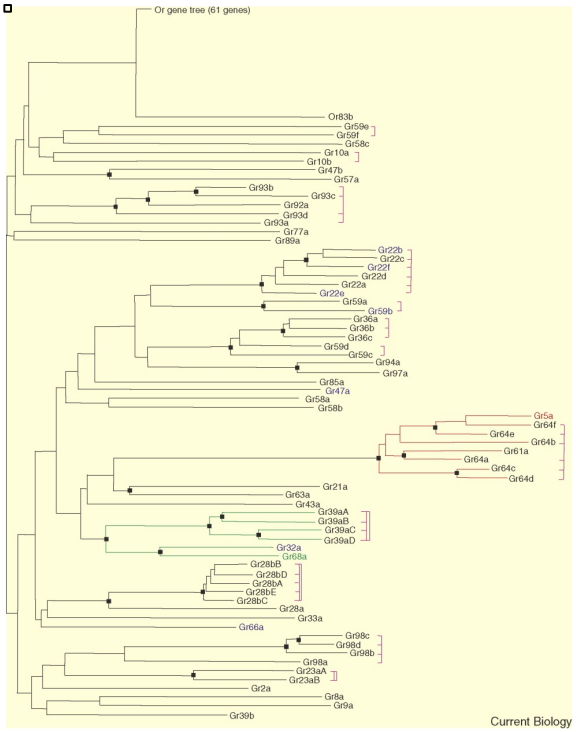

Figure 2: Cladogram of the *Gr* family of genes. The putative sugar *Grs Gr5a*, *Gr64a-f*, and *Gr61a* are displayed in red [5].

Genetic tools in *Drosophila*

With a long history of laboratory use, many genetic tools are available for working with this organism. One useful method of characterizing gene expression is the *UAS/GAL4* system (figure 3). *GAL4*, a yeast transcriptional factor, binds the *UAS* enhancer sequence, (also a yeast transgene) recruiting RNA polymerase and inducing transcription of genes downstream of the *UAS* sequence. Expression of *GAL4* under the control of a selected promoter can be used to restrict *GAL4* transcription to those tissues expressing the gene of interest. Since there are no known *GAL4* target sequences in Drosophila, coexpression of a *UAS* transgene can be used to drive expression of downstream genes in a tissue-specific manner.

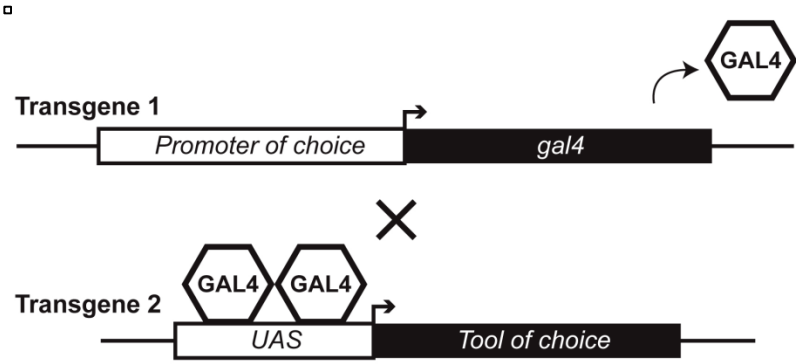

Figure 3: A schematic of the UAS/GAL4 system in *Drosophila*. Promoter fusion to the GAL4 coding sequence yields tissue-specific expression of GAL4. Coexpression of a UAS transgene can be used to drive expression of a transgenic 'tool' in GAL4-positive cells [6].

The library of *UAS* transgenic tools includes transgenes that can label cells (*LacZ, GFP*), silence neuronal activity (*TnT*), or even ablate tissue (*Reaper*) [6]. This resource, or the analogous bacterial *LexA* transcription factor/*Lexop* enhancer sequence system, can be used to analyze expression of a gene and even glean phenotypic data from cell

inhibition/ablation or overexpression studies. Another useful technique available to researchers is homologous recombination, first describe by Rong and Golic (figure 4). In *D. melanogaster*, this proceeds through the creation of a recombinogenic linear double-stranded DNA fragment. This fragment is generated through the use of an inducible transgene containing a FLP recombinase under the control of a heat shock promoter, which catalyzes recombination between FLP recombination target (FRT) sites, and a site-specific endonuclease, *I-sceI*. Incorporation of the FRT sites and the cut site into the donor construct (insert), coexpression of the heat-inducible transgene, and subsequent heat shock can be used to produce recombinants [7].

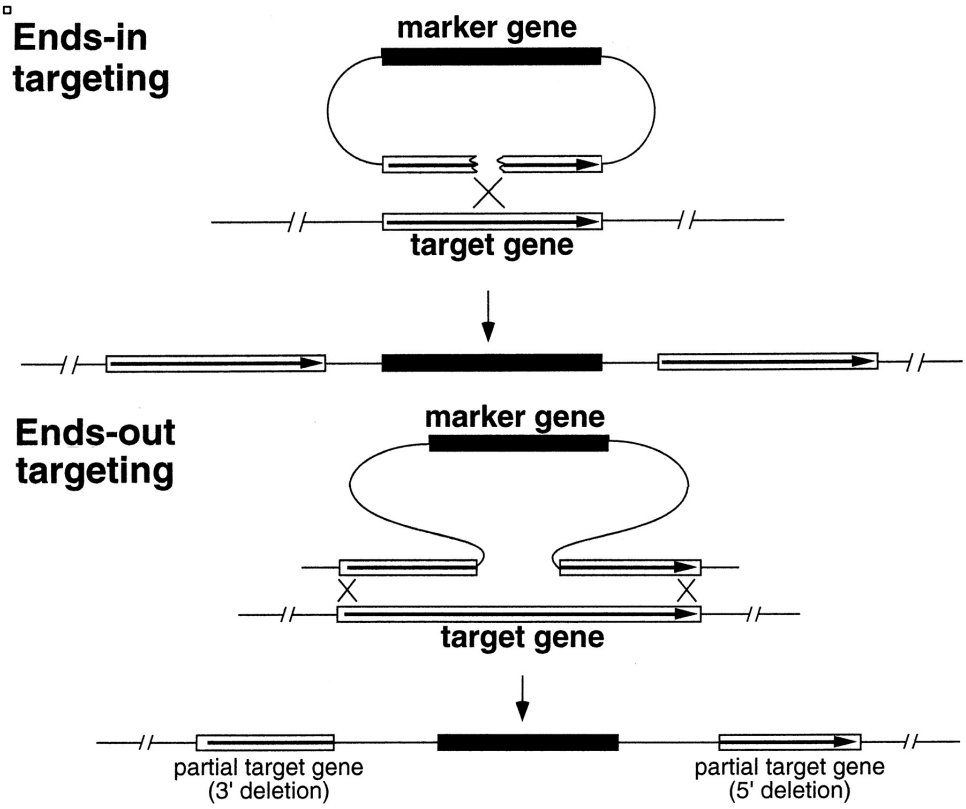

Figure 4: Homologous recombination in *Drosophila*. As described by Rong and Golic, showing both the double-stranded DNA fragment produced and the resulting recombinant after integration into the chromosome [7].

It is worth noting that the ends-out targeting strategy creates a partial deletion of the target locus. This has been utilized to make "knockin" *Drosophila* strains, with deletions of target genes. These knockin strains can be used for phenotypic studies to deduce the purpose of the interrupted gene [8].

Previous larval *Gr* studies

Although much has been done in regards to studying the larval olfactory system [4,9], the exploration of larval gustation is in its infancy. Analyses using *Gr-GAL4* transgenes have characterized the expression patterns of a number of Grs, but interestingly, the *psGrs Gr64a-f*, *Gr61a*, and *Gr5a* have not been reported to show expression in the larval stage of the life cycle. It has been suggested that larvae may detect sugars using other genes [10]. Additionally, while some behavioral studies have been performed, and it is known that larvae respond positively to sugars [11], there have been no phenotypic analyses of *psGr* knockin or deletion mutant larvae that we know of.

Given that no obvious gene targets have been identified, we sought to characterize the genes responsible for larval sugar gustation. Knockin *GAL4* strains, which are both simultaneously a null for the gene studied and a tool for *GAL4* expression analysis, are generally believed to more faithfully reflect expression of genes, since they are placed in the native locus rather than inserted at a random location. Expression analysis using knockin *GAL4* strains may yield new data about the expression of *psGrs* in *Drosophila* larvae. Using *GAL4* knockins previously constructed by the Amrein laboratory, we set

out to perform such an analysis to search for expression of these genes. Additionally, knockin/deletion mutants were challenged in the larval two-choice assay [11] with the goal of comparing their sugar preference to the wild-type animal to reveal the possibility of psGr function in larval sugar detection.

CHAPTER II

METHODS

Analysis of *psGr* expression

Expression of larval *Grs* was investigated by crossing *UAS-mCD8:GFP* or *UAS-nGFP* flies with each of the *Gr-Gal4* strains found in table 1. In the case of *Gr5a*, expression analysis was performed by crossing the $Gr5a^{LexA}$ knockin strain with a *Lexop-Cd2GFP* strain.

Table 1: *psGRGAL4* lines used for expression analysis.

Genotype	Description
Gr61a-GAL4	*Gr61a* promoter fused to the *GAL4* coding sequence
Gr64f-GAL4	*Gr64f* promoter fused to the *GAL4* coding sequence
Gr43a ^{GAL4} knockin	Coding sequence of *Gr43a* replaced with *GAL4*
Gr64a ^{GAL4} knockin	Coding sequence of *Gr64a* replaced with *GAL4*
Gr5a ^{LexA} knockin	Coding sequence of *Gr5a* replaced with *LexA*

Four to five days after performing the cross, larval progeny were collected from the vials by removing a scoop of food and straining it through a metal screen under tap water. First-instar larvae were mounted on microscope slides in Phosphate Buffered Saline/Triton X-100 solution and screened for fluorescence under a Nikon Eclipse Ti-U microscope. Images were processed with NIS-Elements AR software.

Behavioral assays

To measure the gustatory preference of various mutant larvae, we used a modified version of an established larval two-choice assay [11]. To minimize age variability in the animals, approximately 100 adult flies were placed in a bottle on standard cornmeal food and allowed to mate for 6-12 hours at 25^{o}C, after which they were removed. Larvae were collected and tested between 84 and 100 hours after adult removal. This constrained the possible ages of the larvae from approximately 90 to 110 hours. This narrow age range ensures that all larvae are in the feeding stage of the third instar and minimizes variation in behavior. Larvae were collected from bottles by scooping food into a large petri dish filled with tap water and separated from the substrate using paintbrushes. 12-15 larvae were placed into the prepared dishes, and the number of larvae on either side recorded at specific time points. Larvae found within approximately one centimeter of the centerline, or clinging to the sides or lid of the dish were included in the total, but did not count towards either side. For each data point, larval preference for a given sugar was calculated with the following formula: Preference = (nSugar-nPure)/nTotal, where n represents the number of larvae counted. This equation returns a value between -1 and +1, the former indicating a complete avoidance of the tastant, the latter indicating a complete preference for the tastant, and a value of 0 indicating that the larvae do not discriminate between the tastant and pure agar. Gustatory preferences were averaged over multiple trials and graphed as boxplots using Statistica software. We used the Mann-Whitney U-test to show a statistically significant deviation in the median values of different datasets. To prepare the assay plates, 60x15 mm petri dishes were filled with

10 ml of 1% agar and allowed to cool. Once solidified, the agar was cut into halves using a razor blade, and one half removed. The second half of the dish was filled with agar containing a sugar at a desired concentration. Plate preparation and larvae collection were performed simultaneously, and the assay began immediately after the agar plates had cooled.

CHAPTER III

RESULTS

Screening for larval *psGr* expression

To investigate the possibility that larvae detect sugars with the *psGr* genes, we used two transgenic *psGr-GAL4* constructs, along with two *psGr* $^{GAL4/LexA}$ constructs coexpressed with *UAS-GFP* or *Lexop-GFP* to screen for expression of these genes, reasoning expression of these genes indicated a functional role in larval gustation. Additionally, we tested a *Gr43a* ^{GAL4} knockin construct, given data indicating that Gr43a acts as a fructose sensor in mature flies, despite not belonging to the *psGr* subfamily of genes [12]. Screening for the prescence of GFP in two transgenic *Gal4* constructs and three *Gal4/Lexa* knockin constructs revealed expression for one *psGr* and *Gr43a* in *D. melanogaster* larvae (figure 5). *Gr5a* showed expression in the larval segmental nerves, and screening for *Gr43a* revealed expression in six gustatory neurons, two of which are located in the DPS, and have been previously reported [10]. Although the anatomical location of the four remaining *Gr43a* neurons has not been confirmed, we hypothesize that they are located in the DPS and larval brain from their general location. Additionally, data from *Gr43a* expression in adult flies supports the hypothesis that this receptor is expressed in the brain [12].

Larval two-choice assays

To investigate the functionality of these gustatory receptors in larval sugar detection, we examined the sugar preference of larvae from different genetic backgrounds in the larval

two-choice assay. Our initial experiments established the preference of wild-type larvae for a variety of common sugars/tastants (figure 6). Larvae show a robust response to both fructose and glycerol at comparable concentrations, but the response to glucose and sucrose was notably weaker. Given the strong phenotypes for glycerol and fructose, we targeted these sugars in future experiments.

Based on previous work with *ΔGr64a-f* adults with proboscis extension assays [13], we tested *ΔGr61a,ΔGr64a-f* larvae for a glycerol defect, but found no significant deviation from wild-type glycerol preference (data not shown.)

Additionally, we tested *ΔGr43a* and *ΔGr61a,ΔGr64a-f* larvae for a fructose defect (figure 7). Although *ΔGr61a,ΔGR64a-f* larvae were indistinguishable from the wild-type, *ΔGr43a* larvae showed a defect in fructose preference. Given these results, we proceeded to test *ΔGr43a,ΔGr61a,ΔGr64a-f* combination mutants, hereafter referred to as the 'triple mutant'. Triple mutant larvae were shown to exhibit a near-zero preference for fructose (figure 8), a more severe phenotype than the *ΔGr43a* larvae.

A

Driver	*GR61aGal4*	*GR64fGal4*	*ΔGR64aGal4*	*ΔGR43aGal4*	*ΔGR5aLexa*
Fluorescence	-	-	-	+	+
Neurons	N/A	N/A	N/A	Six 2- DPS 2- PPS 2- brain?	16-18 (segmental nerves)

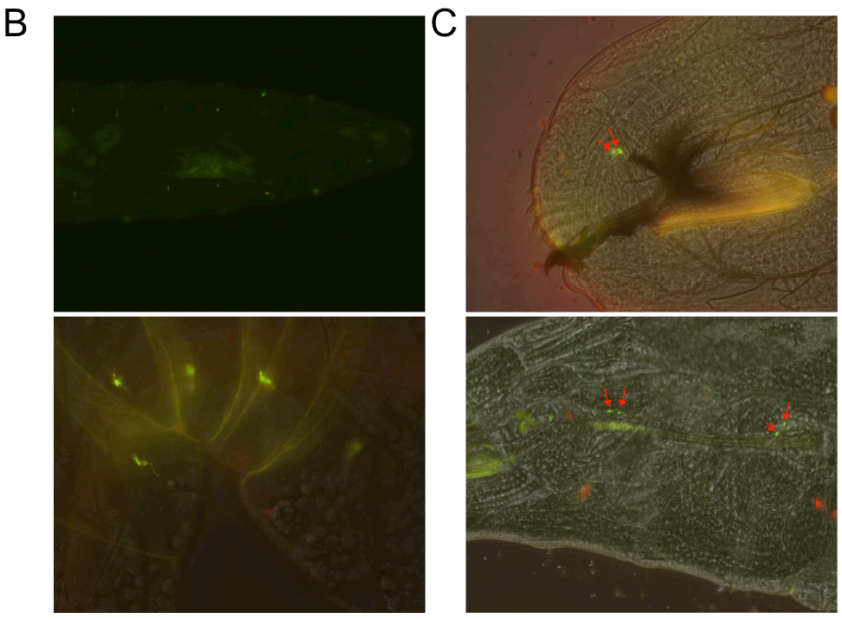

Figure 5: Expression of *psGrs* in *D. melanogaster* Larvae. (A) A table of transgenic constructs used to screen for *psGr* expression by crossing to a *Lexop-Cd2GFP, UAS-mCD8:GFP,* or UAS-nGFP strain and examining larval progeny. (B) Representative photographs of *ΔGr5aLexA/ Lexop-Cd2GFP* larvae at 10x (top) and 40x (bottom) magnification. (C) Representative photographs of *ΔGr43a/UAS-nGFP* larval head (top) and lower oesophagus (bottom).

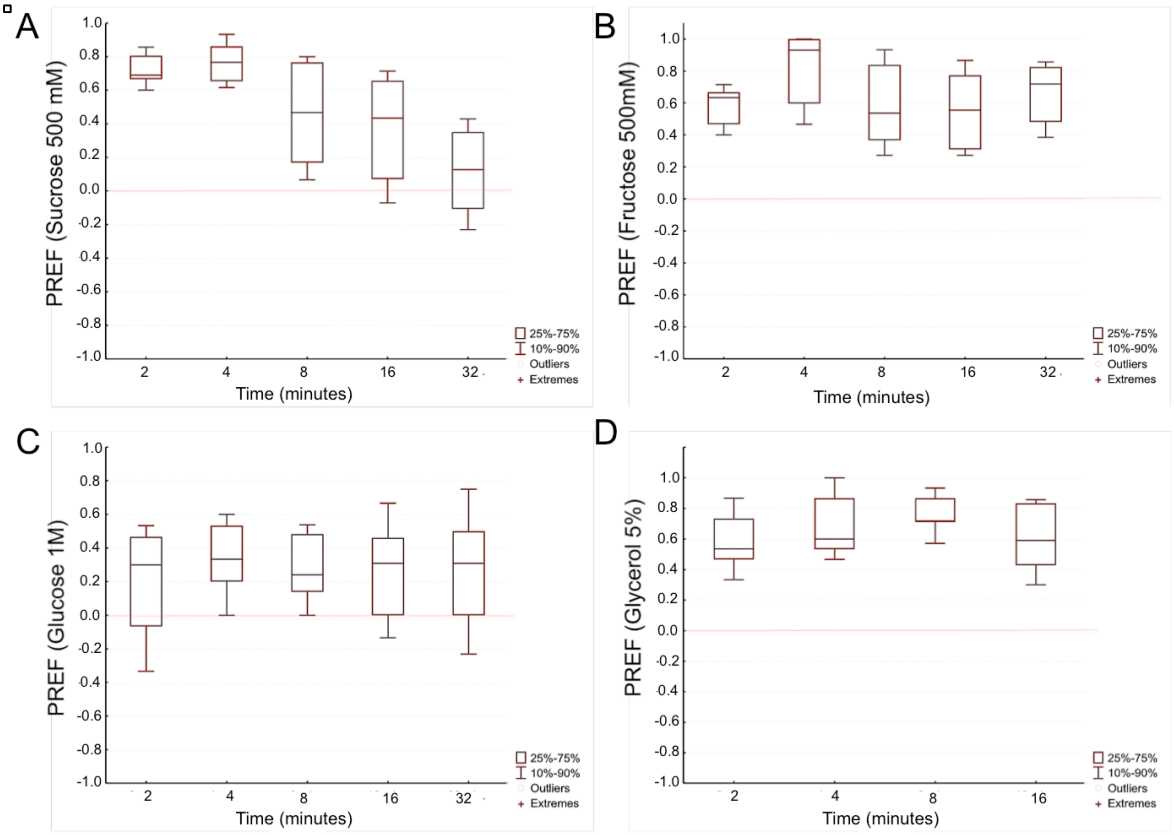

Figure 6: Sugar preferences of wild-type *D. melanogaster* larvae. W^{1118} control larvae tested with 5% glycerol (A), 500 mM fructose (B), 1 M glucose (C), and 500 mM sucrose (D).

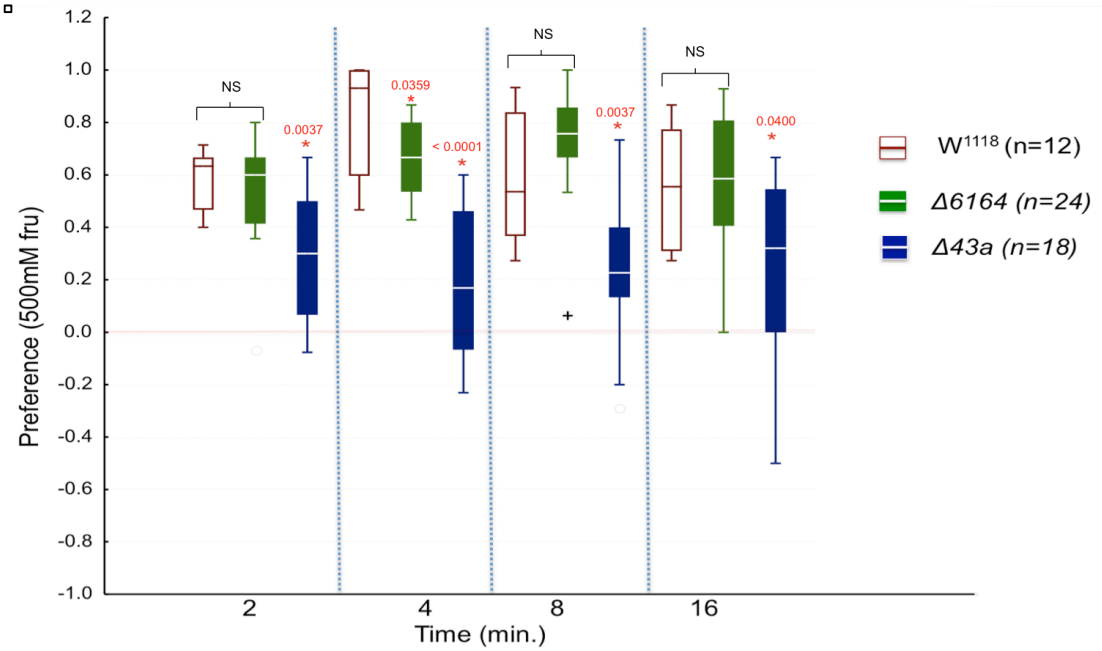

Figure 7: LTCA data for W^{118} control, *ΔGr61a,ΔGr64a-f,* and *ΔGr43a* larvae with 500mM fructose. Asterisk indicates p<.05 from the Whitney-Mann U-test for difference of medians.

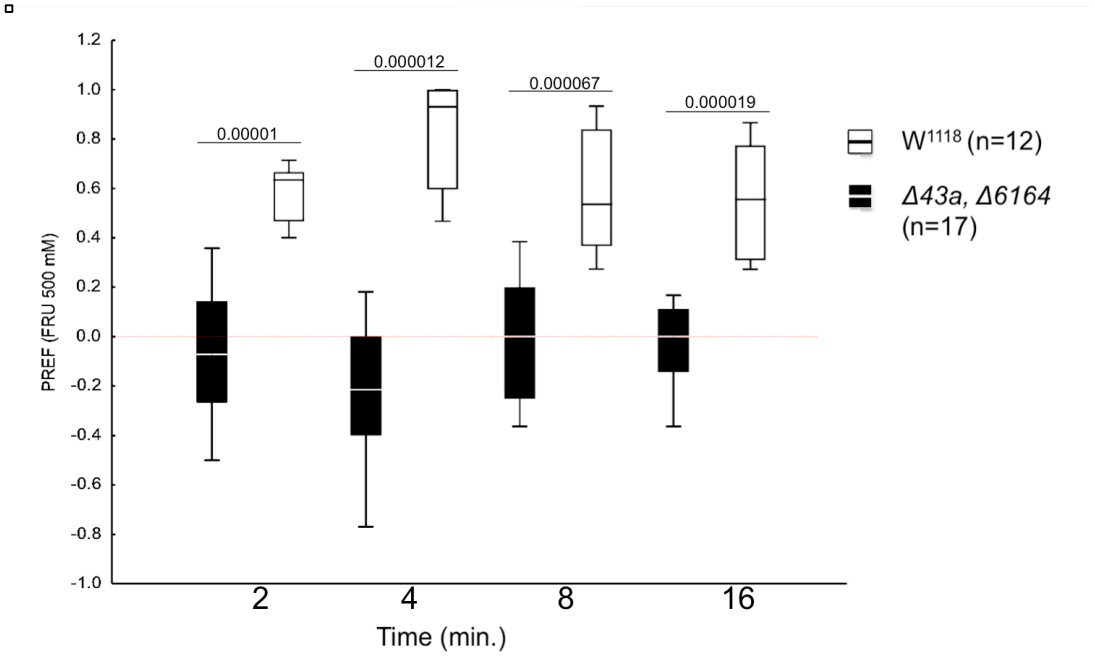

Figure 8: LTCA data for triple mutant larvae with 500mM fructose. P-values from the Whitney-Mann U-test for difference of medians are indicated above each dataset.

CHAPTER IV

CONCLUSIONS AND DISCUSSION

LTCA experiments with wild-type D. melanogaster indicate that larvae respond more robustly to fructose than other sugars tested here, and it follows suit that this tastant drives feeding in the major growth phase of the animal. Behavioral assays with *ΔGr43a* larvae indicate that Gr43a is the primary receptor for fructose sensation. Although costaining experiments or other preparations will be required required to confirm the anatomical location of the four newly discovered *Gr43a* neurons, we hypothesize from their relative position and from adult expression patterns that they reside in the brain and PPS, respectively. Expression of Gr43a in the brain may indicate that fructose serves as an internal nutrient sensor in the larvae, as has been found in the adult [12]. Although Gr43a appears to be the primary receptor, experiments with the triple mutant larvae indicate that one or more proteins encoded by the *Gr64a-f* gene cluster or *Gr61a* play a role in fructose sensation. One can speculate that these accessory genes function as low-affinity fructose receptors, with Gr43a acting as a high-affinity receptor. Alternatively, there is evidence that *Gr*s in *D. melanogaster* function as multimeric complexes, requiring coreceptors to bind ligands [14]. It is possible that fructose sensation requires *Gr43a* in conjunction with one or more proteins encoded by the *Gr64a-f* gene cluster or *Gr61a* to form functional receptors.

Analysis of *Gr5a* expression using the *LexA* knockout revealed a new subset of neural cells expressing this gene. These cells strongly resemble the putative glial cells

associated with the segmental nerves [15]. There are two possibilities for *Gr5a* expression in these cells, assuming that our assay reflects expression patterns accurately. Either these cells have a previously undiscovered gustatory role, given that Gr5a is an essential subunit of the primary receptor for the sugar trehalose [16], or Gr5a is serving an entirely different and previously uncharacterized function in these cells. Data from adult expression patterns, in conjunction with our larval data, indicates that the latter is more likely [17]. This calls into question the frequent use of *Gr5a-Gal4* as a driver for sugar-sensitive cells, as it appears that this receptor is not expressed exclusively in sugar-sensing neurons.

These experiments demonstrate the utility of the LTCA in deorphanizing chemoreceptors. This fast and effective method could be used to complement data from other phenotypic assays such as the proboscis extension assay (PER).

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