THE DROSOPHILA GUSTATORY RECEPTOR GENES: THE MOLECULAR BASIS OF TASTE PERCEPTION AND CODING

by

Natasha Thorne

Department of Molecular Genetics and Microbiology
Duke University

Date:_______________________
Approved:

____
Hubert Amrein, Ph.D., Supervisor

____
Robin Wharton, Ph.D.

____
Steven Crews, Ph.D.

____
Hiro Matsunami, Ph.D.

____
Dan Tracey, Ph.D.

____
Fan Wang, Ph.D.

Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor
of Philosophy in the Department of
Molecular Genetics and Microbiology in the Graduate School
of Duke University

2007

ABSTRACT

THE DROSOPHILA GUSTATORY RECEPTOR GENES: THE MOLECULAR BASIS OF TASTE PERCEPTION AND CODING

by

Natasha Thorne

Department of Molecular Genetics and Microbiology
Duke University

Date:________________________
Approved:

____
Hubert Amrein, Ph.D., Supervisor

____
Robin Wharton, Ph.D.

____
Steven Crews, Ph.D.

____
Hiro Matsunami, Ph.D.

____
Dan Tracey, Ph.D.

____
Fan Wang, Ph.D.

An abstract of a dissertation submitted in partial
fulfillment of the requirements for the degree
of Doctor of Philosophy in the Department of
Molecular Genetics and Microbiology in the Graduate School
of Duke University

2007

Copyright by
Natasha Thorne
2007

Abstract

Discrimination between edible and contaminated foods is crucial for the survival of animals. In *Drosophila*, a family of gustatory receptors (GRs) expressed in taste neurons is thought to mediate the recognition of sugars and bitter compounds, thereby controlling feeding behavior. Using the Gal4/UAS system, we have characterized the expression of *Gr* genes in detail, and find that gene expression falls into two distinct groups. The first group, which make up the majority of the genes analyzed, are co-expressed with *Gr66a*, and functional ablation of taste neurons expressing these genes demonstrates that these neurons mediate sensitivity to bitter substrates. A second, distinct group of taste neurons, express *Gr5a* and mediate sensitivity to trehalose. We find that these two sub-populations of taste neurons – attractive-type and avoidance-type - project afferents to discrete areas of the primary taste center in the CNS. These results demonstrate how bitter and sweet taste are coded for in the periphery and indicates that information about different taste modalities is initially segregated in the CNS. We have also used the Gal4/UAS system to thoroughly characterize the expression profile of a cluster of six *Gr*s – *Gr28a* and *Gr28b.a-b.e*. We find that these receptors are expressed not only in taste neurons, but other sensory neurons, as well as neurons in the CNS. RNA *in situ* hybridization confirms this unusual expression pattern. In order to explore the function of these *Gr*s, these genes have been deleted using ends-out homologous recombination, to produce Δ28 mutant flies. Initial behavioral

experiments with Δ28 mutant flies suggest that these receptors may play a role in detecting aversive substrates and/or modulate avoidance responses to these substrates.

To my family – Mom, Dad, and Shawn – for never doubting.
To my dearest friends – Dianne, Medina, Caroline, Patrick, Renee, and Greg – for good laughs, strong shoulders, and huge hearts.
To Jake – for being Jake.

Contents

Abstract iv

List of Tables xiv

List of Figures xv

1. Introduction to Taste Detection and Perception 1

1.1 Chemosensation 1

1.2 Taste and olfaction 2

1.3 The mammalian taste system 3

1.3.1 Molecular basis of taste detection in mammals 4

1.3.2 Understanding taste perception in mammals 6

1.4 The Drosophila taste system 7

1.4.1 Assaying taste behavior in *Drosophila* 11

1.4.2 Electrophysiological studies: *Drosophila* taste neurons detect salts, sugars, bitter compounds, and water 13

1.4.3 *Drosophila* taste mutants provide genetic basis of taste detection in early studies 17

1.5 The Drosophila gustatory receptor (Gr) gene family 19

1.5.1 Discovery of the *Gr* gene family 19

1.5.2 The difficulty in studying *Gr* genes and the *Gal4/UAS* system 23

1.5.3 Using the Gal4/UAS system to determine *Gr* expression profiles 24

1.5.4 *Gr5a* is functionally defined as a trehalose receptor 26

1.6 Thesis research projects: taste detection and perception in Drosophila and characterizing the expression of Grs 27

1.6.1 Project One: Taste coding and perception 28

1.6.2 Project Two: Characterizing the expression of a highly conserved *Gr* gene cluster - the *Gr28* genes 29

1.6.3 Project Three: Determining the function of the *Gr28* genes 30

2. *Gr*s are expressed in avoidance- or attractive-type taste neurons 32

2.1 Introduction 32

2.2 Results 35

2.2.1 Expression map of *Gr* genes in the labial palps 37

2.2.2 Many *Gr* genes are coexpressed in some GRNs 41

2.2.3 The trehalose receptor GR5a defines a largely distinct set of GRNs 43

2.2.4 *Gr66a*- and *Gr5a*-expressing neurons mediate different taste perceptions 51

2.2.5 Axon projections of GRNs expressing specific *Gr* genes 54

2.2.6 Unilateral versus bilateral projections of labellar GRNs 60

2.2.7 Ascending afferents from sensory neurons of the legs directly innvervate the SOG 62

2.3 Discussion 65

2.3.1 Gustatory neurons mediating avoidance behavior are defined by subtle differences in the *Gr* gene code 66

2.3.2 *Gr5a*-expressing GRNs mediate sweet taste perception 70

2.3.3 A taste map in the brain 73

2.3.4 Similar logic of taste perception in insects and mammals 75

2.4 Acknowledgments 77

2.5 Experimental Procedures 78

2.5.1 Genetics/fly strains 78

2.5.2 Immunofluorescence 79

2.5.2.1 Labellum 79

2.5.2.2 Brains and thoracic-abdominal ganglia 80

2.5.2.3 Confocal microscopy 80

2.5.3 Labellar cell counting 81

2.5.4 Feeding-preference assays (FPAs) 81

2.5.5 Ablation of labial palps 82

2.6. Confirmation that Grs are expressed in attractive- or avoidance-type taste neurons 83

2.7 New developments in Drosophila taste 86

2.7.1 Drosophila taste neurons are hard-wired to mediate avoidance- or attractive-type feeding behavior and the *Gr*s a given neuron expresses determine its activation profile 86

2.7.2 Gr66a is required for a response to the bitter compound caffeine 88

2.8 Novel roles for Grs and new insight into how Grs may function 89

2.8.1 Gr21a and Gr63a function as a CO_2 receptor: precedent for alternative *Gr* function and heterodimeric interactions between *Gr*s 89

2.8.2 Drosophila olfactory receptors not only form heterodimers, but they may have unexpected topology and may not function as GPCRs 90

3 The highly conserved *Gr28* genes are expressed not only in taste neurons, but in non-chemosensory neurons and neurons in the CNS, suggesting a non-traditional role for these receptors 92

3.1 Introduction 92

3.2 Results 97

3.2.1 The *Gr28* genes 98

3.2.2 Expression of the *Gr28* genes using the Gal4/UAS system 101

3.2.2.1 Expression in adult taste tissues 102

3.2.2.2 Atypical *Gr* expression in sensory neurons 107

3.2.3 Projection of sensory neurons to the CNS and expression in the brain 112

3.2.3.1 Gr28.a and Gr28b.e 114

3.2.3.2 Gr28a 114

3.2.3.3 Gr28b.d 116

3.2.3.4 Gr28b.c 118

3.2.3.5 Gr28b.b 121

3.2.4 Larval expression 124

3.2.5 Non-neuronal expression of *Gr28* genes 127

3.2.6 Endogenous RNA expression of *Gr28* genes in non-chemosensory cells 128

3.3 Discussion 135

3.3.1 Expression of Gr28b.b and Gr28b.c in higher-order chemosensory neurons . 138

3.3.2 A role for GR proteins in the metabolism of nutrients? 140

3.3.3 Expression of *Gr28* genes in non-chemosensory neurons 141

3.4 Materials and Methods 142

3.4.1 Gene conservation/amino acid identity 142

3.4.2 Fly lines used 143

3.4.3 Production of transgenic Gal4 lines 143

3.4.4 RT-PCR 144

3.4.5 RNA fluorescent *in situ* hybridization of embryos and adult CNS 145

3.4.6 Production of *Gr28* genomic rescue line 146

3.4.7 Confocal microscopy 148

3.4.8 X-Gal staining 148

3.5 Acknowledgements 148

3.6 Future directions 149

4 Determining the function of the *Gr28* genes: putative function for cells expressing *Gr28*s and production of *Gr28* deletion animals and *Gr28* gene knock-out animals 150

4.1 Introduction 150

4.1.1 Cell function as a clue to gene function 150

4.1.2. P-elements and *piggy-Bac*-based deletions 153

4.1.3 Ends-out gene targeting as a method to produce gene deletions 155

4.2 Materials and methods 158

4.2.1 VR1 behavioral assays 158

4.2.2 Producing *piggy-Bac* based deletions 160

4.2.3 Production of 26kb genomic construct to rescue genes upstream of the *Gr28* locus 164

4.2.4 Ends-out homologous recombination 166

4.2.4.1 Donor construct production 166

4.2.4.2 Crossing scheme to produce deletion lines via homologous recombination 167

4.2.4.3 PCR reactions to determine presence or absence of Δ28 deletion 172

4.2.4.4 Southern blots of genomic DNA from putative Δ28 deletion lines 175

4.2.5 Production of a a genomic *Gr28* rescue line 181

4.2.6 Δ28 30i, Δ28 54B3, and control lines are backcrossed into the same genetic background for behavioral experiments 184

4.3 Results ... 186

4.3.1 Driving expression of VR1 in *Gr28b.c*-expressing cells causes wandering 3rd instar larvae to avoid capsaicin ... 186

4.3.2 Seven homozygous lethal lines with 50kb deleted at the *Gr28* locus are produced using *piggy-Bac* transposable elements ... 190

4.3.3 Two Δ28 deletion lines are produced using homologous recombination ... 192

4.4 Discussion ... 192

4.4.1 *Gr28* genes may play a role in detecting aversive tastants ... 192

4.4.2 Homologous recombination proves to be a better method than *piggyBac*-mediated FRT-recombination at producing a Δ28 deletion ... 193

4.5 Acknowledgments ... 195

5 Determining the function of the *Gr28* genes: initial behavioral experiments ... 196

5.1 Introduction ... 196

5.1.1 Introduction to larval assays ... 197

5.1.2 Introduction to adult assays ... 199

5.2 Materials and Methods ... 200

5.2.1 Larval lifespan ... 201

5.2.2 Larval position assay ... 202

5.2.3 Larval foraging assay ... 203

5.2.4 Nociception assays ... 204

5.2.5 Adult residence assays ... 205

5.3 Results ... 207

5.3.1 Mutant larvae have normal development time ... 207

5.3.2 Δ28 mutant larvae may have enhanced sensitivity to caffeine ... 208

5.3.3 Δ28 mutant larvae display typical foraging behavior 214

5.3.4 Δ28 mutant larvae are not hyper- or hypo-sensitive to nociceptive thermal heat 216

5.3.5 Δ28 mutant adults may have enhanced sensitivity to aversive substrates 216

5.4 Conclusion and future directions for determining the function of the Gr28 genes 222

6 Summary and conclusions 227

References 233

Biography 249

List of Tables

Table 1: Peripheral expression of *Gr*s..38

Table 2: Co-expression of various *Gr* genes...42

Table 3: Percent amino acid identity to putative ortholog..101

Table 4: Expression of *Gr28* genes in adult taste tissue..103

Table 5: Expression of *Gr28* genes in adult non-chemosensory neurons in the periphery and CNS...107

Table 6: Expression of *Gr* genes in larvae..125

Table 7: Lines that have donor construct targeted to 2nd chromosome and may have *Gr28* deletion – first round of homologous recombination...170

Table 8: Lines that have donor construct targeted to 2nd chromosome and may have *Gr28* deletion – second round of homologous recombination...172

Table 9: List of transgenic *Gr28* rescue insertion lines and their chromosomal location..183

Table 10: Preference for capsaicin in larvae expressing VR1 under the control of *Gr* drivers..188

Table 11: *piggyBac*-based lines with putative 50kb deletion of *Gr28* locus........................191

Table 12: Summary of larval preference assays using lines that have not been backcrossed to w^{1118}..212

Table 13: Adult residence assay experiments on lines that have not been backcrossed to w^{1118}..222

List of Figures

Figure 1: Organization of the *Drosophila* taste system..9

Figure 2: Structure of a taste bristle..11

Figure 3: The two choice feeding assay and proboscis extension assay.............................12

Figure 4: Functional organization of taste sensilla in the labial palps as determined by electrophysiology...15

Figure 5: Organization of the *gustatory receptor* gene family...22

Figure 6: Expression of *Gr22e* in GRNs of various taste organs using the Gal4/UAS system...25

Figure 7: Organization of the taste sensory organs and processing centers in the head...34

Figure 8: Expression of *Gr*s in the labellum..40

Figure 9: *Gr5a* expression in the labellum...46

Figure 10: *Gr5a* is expressed in a largely non-overlapping set of neurons in the labellum...48

Figure 11: Sectioning through labellum of a *Gr5a*/*Gr22e* double-driver line shows expression in both large and small neurons...50

Figure 12: Trehalose and caffeine sensitivity is mediated by neurons expressing different *Gr* genes...52

Figure 13: Distinct axonal projection patterns of the brain of GRNs expressing different *Gr* genes...57

Figure 14: Expression of *Gr59b* in the labellum, and axonal projections in the SOG of *Gr59b*- and *Gr28be*-expressing neurons...59

Figure 15: Distinct ipsilateral and contralateral contribution of gustatory receptor neurons expressing *Gr5a, Gr66a,* or *Gr22b*...61

Figure 16: Taste neurons of the legs that express *Gr66a* project to the SOG, whereas neurons of the legs that express *Gr32a* project beyond the SOG to a location in the protocerebrum....64

Figure 17: Model of taste coding in the labellum....67

Figure 18: Representation of avoidance and feeding neurons in S, I, and L-type taste bristles of the labellum....72

Figure 19: Functional organization of taste sensilla in the labial palps according to molecular analysis....76

Figure 20: Schematic of the *Gr28* locus on the second chromosome of *Drosophila melanogaster*....99

Figure 21: *Gr28* exprssion in the labellum revealed with the Gal4/UAS system....104

Figure 22: Expression of *Gr28* genes in non-chemosensory neurons....108

Figure 23: Axonal projection patterns in the CNS of gustatory receptor neurons (GRNs) expressing *Gr28a, Gr28b.a,* and *Gr28b.e.*....115

Figure 24: Axonal projection pattern of *Gr28b.d*-expressing GRNs to the CNS....117

Figure 25: Axonal projection pattern to the SOG and antennal lobes of peripheral sensory neurons and neurons of the CNS expressing *Gr28b.c*....120

Figure 26: *Gr28b.b* is expressed in numerous neurons of the CNS that target the SOG and antennal lobes....123

Figure 27: Both *Gr28b.b* and *Gr28b.c* are expressed in abdominal md neurons of larvae....126

Figure 28: *Gr28b.b* is expressed in oenocytes in adults....127

Figure 29: RT-PCR analysis indicates expression of *Gr28* genes in non-taste tissue....129

Figure 30: RNA *in situ* hybridization using antisense RNA complementary to *Gr28b* genes reveals expression in the CNS and embryo....133

Figure 31: No signal is seen when the full-length *Gr28b.c* antisense probe is hybridized to Δ28 knock-out embryos....135

Figure 32: Schematic of ends-out gene targeting....158

Figure 33: *Gr28* locus showing location of *piggyBac* elements used to create deletion....160

Figure 34: Crossing scheme for creating a deletion using *piggyBac* elements....161

Figure 35: *Gr28* locus showing upstream and downstream rescue constructs to replace genes lost in producing *piggyBac* deletions....165

Figure 36: Five kilobases of homologous upstream and downstream sequence was cloned into the CMC105 vector to make the donor construct....166

Figure 37: Crossing scheme to produce Δ28 30i deletion line via homologous recombination....168

Figure 38: Crossing scheme to produce Δ28 54B3 deletion line via homologous recombination....171

Figure 39: Schematic of donor construct targeting and recombining with homologous sequence of endogenous locus resulting in replacement with mini-*white* gene....173

Figure 40: PCR reactions of the "30i" line that contains the Δ28 deletion....174

Figure 41: PCR reactions of the "54B3" line that contains the Δ28 deletion....175

Figure 42: Schematic of the target donor construct and the *Gr28* locus....177

Figure 43: Southern blots showing "30i" line appears to have *Gr28* correctly targeted by donor construct....178

Figure 44: All three lines from the first round of homologous recombination lack *Gr28a* and the common exons of *Gr28b* as determined by genomic Southern blots....179

Figure 45: Southern blots showing "54B3" line appears to have *Gr28* correctly targeted by donor construct....180

Figure 46: Southern blots using the probes against *Gr28a* and *Gr28b* common exons show that the "54B3" line also has a deletion for these genes....181

Figure 47: Schematic of coverage of the *Gr28* rescue construct 183

Figure 48: Preference for 0.1mM capsaicin in larvae expressing VR1....189

Figure 49: Schematic of set-up for larval position assay to test for larval preference....203

Figure 50: Schematic of larval foraging assay set-up....204

Figure 51: A single snapshot of all six residence assay plates was taken every 10 seconds by a video recorder....206

Figure 52: Eclosion time for Δ28 30i animals is similar to that for both wild-type animals and animals heterozygous for the deletion....207

Figure 53: Eclosion time for Δ28 54B3 30i animals is similar to that for both wild-type animals and animals heterozygous for the deletion....208

Figure 54: Larvae avoid 10mM caffeine over time....209

Figure 55: Average larval preference over time for each genotype....210

Figure 56: Larval foraging assay....215

Figure 57: Adult residence assay of 50mM fructose plus 10mM caffeine versus water using fed flies....217

Figure 58: Adult residence assay of 50mM fructose plus 10mM caffeine versus water using starved flies....218

Figure 59: Adult residence assay of 1M trehalose versus water using fed flies....220

Figure 60: Adult residence assay of 2mM AITC versus water using fed flies....221

Figure 61: Taste signal transduction for sweet, bitter, and salt compounds....230

1. Introduction to Taste Detection and Perception

1.1 Chemosensation

Our interaction with the environment is mediated by our sensory systems. Our senses of vision, olfaction, audition, gustation, and touch not only add texture to our experiences with the environment, but they provide valuable information that allow us to act appropriately within the context of our environment. Chemosensation includes the sensory processes of gustation, olfaction and pheromone detection. These are considered the most primitive of senses, being found in some fashion in all living organisms, as they are required to detect and sequester food sources, as well as avoid toxins and poisons. In higher organisms, especially mammals, chemosensation in the form of phermonal cues aids in interaction with other individuals, such as is required when finding a mate, identifying and taking care of offspring, and maintaining dominance and territory in group-living situations. Chemosensory signals, in the form of chemicals in the environment, are detected by specialized tissues in higher organisms – such as the tongue and nose of mammals – that harbor cells or neurons that express receptors that bind to a specific chemical, causing activation of the receptor, and subsequent activation of the cell. These cells transmit this sensory activation to the brain

to produce a specific behavioral output dependent upon the identity of the small molecule.

The organization of chemosensory systems in mammals and *Drosophila* are strikingly similar. Both have tissues specifically dedicated to detecting either odorants or tastants; tissues with cells that express seven-transmembrane domain receptors that interact with the odorant or tastant ligand to elicit cellular activation and subsequent transmission of the sensory signal. Due to the parallels between *Drosophila* and mammalian taste and olfactory systems, in addition to the genetic, molecular, and electrophysiological tools that exist for *Drosophila*, this member of the insect family is being used extensively to explore the molecular basis of chemoperception and map the neuronal circuitry that bridges sensory perception and behavioral output.

1.2 Taste and olfaction

Olfaction begins with the detection of volatile molecules – odorants – such as isoamyl esters, which are small molecules that make a banana smell like a banana (Jordan et al., 2001). Humans can detect and differentiate approximately 10,000 different odors, and all of this is mediated by olfactory neurons that line the olfactory epithelium of the nose (Prasad and Reed, 1999). Gustation is the perception of soluble molecules, like fructose, which make the banana taste sweet. These soluble substrates are detected in humans and other mammals by taste cells located on the tongue and pharynx. Through this sense, we are able to perceive the qualities of sweet, bitter, salty, sour, and

savory (umami). *Drosophila* have taste preferences only slightly different from our own, and are surprisingly attracted to and repulsed by the same taste qualities as humans. Sweet and savory taste are associated with, and therefore have come to signify, substrates that offer nutrition and are considered a food source. At the opposite end of the spectrum is repulsion, characterized by a bitter taste, that is often associated with toxic chemicals and spoiled food. Though humans have learned to enjoy the bitter taste from caffeine in coffee, generally the behavioral output after sensing something bitter is to reject and avoid it. Since *Drosophila* and mammals share many of the same taste preferences, and have similar behavioral outputs to these tastes, it is not surprising that recent work has shown that their taste systems operate in a similar manner, especially at the molecular level.

1.3 The mammalian taste system

Mammalian taste receptor cells of the tongue are clustered into groups to make up a taste bud or papillae. Until quite recently, it was commonly thought that specific areas of the tongue were dedicated to detecting different types of tastants (a "tongue map"), in which, for example, the tip of the tongue is more sensitive to sweet taste whereas the very back of the tongue detects bitters (Chandrashekar et al., 2006). It is now known that most taste buds found in a variety of locations on the tongue can respond to different classes of tastants (Chandrashekar et al., 2006), and that this tongue taste map does not exist. There is also still debate amongst some electrophysiologists

and molecular biologists as to whether individual taste cells can detect different classes of tastasts. Some groups using electrophysiological and calcium imaging techniques claim that a specific taste cell is responsive to both sweet and bitter tastants (Caicedo et al., 2002). A taste quality is thus decoded as being sweet or bitter by the synchronous activation of multiple cells, a theory called "across-fiber" coding (Chandrashekar et al., 2006). It is the alternate theory that individual taste neurons respond only to one tastant type, ie sweet or bitter. This "labeled-line" theory of taste coding is more convincing due to recent advances in understanding the molecular basis of taste detection in mammals (Chandrashekar et al., 2006) .

1.3.1 Molecular basis of taste detection in mammals

In mammals, taste is mediated by GPCRs. These GPCRs are expressed on the cellular surface of taste cells, where they are in contact with the external environment via taste pores of the taste bud. The GPCRs that interact with ligands that are considered attractants – sugars and amino acids – are the T1Rs, which form functional hetero- and homodimers (Zhao et al., 2003). The traditional "sweet" receptor is a heterodimer composed of T1R2+T1R3 (Nelson et al., 2001), and the umami, or L-amino acid receptor is a heterodimer composed of T1R1+T1R3 (Nelson et al., 2002). The interaction of a specific ligand with a particular protein domain of the TIR is thought to allow the detection and activation of the receptor by a wide variety of sugars and sweetners (Xu et al., 2004). The T1R1+T1R3 amino acid receptor detects a broad range of L-amino acids in

most mammals, except in humans, where it appears to be specifically responsible for the detection of monosodium glutamate and aspartate (Chandrashekar et al., 2006). Although both the sweet and amino acid receptor have the same general behavioral output – attraction – these heterodimeric receptors are expressed in distinct taste receptor cells (Chandrashekar et al., 2006). Bitter tastants are detected by the GPCR family of T2Rs, of which there are about 30 (Adler et al., 2000; Matsunami et al., 2000), significantly more than the number of sweet or amino acid receptors. Each individual receptor detects a specific bitter tastant; for example, T2R5 mediates sensitivity to cycloheximide (Chandrashekar et al., 2006). All T2Rs are expressed in the same bitter taste cells, indicating that although mammals can detect a variety of different bitter compounds, they lack the ability to discriminate between them (Adler et al., 2000).

Although sweet, umami, and bitter taste perception are mediated by different GPCRs that are expressed in discrete and non-overlapping subsets of taste receptor cells, they all signal through identical intracellular signaling cascades (Zhang et al., 2003). All taste signaling through these GPCRs involves activation of the heterotrimeric G-proteins gustducin or $G\alpha_{12}$, phospholipase Cβ2 (PLCβ2), and eventually the transient receptor potential (TRP) protein TRPM5 (Chandrashekar et al., 2006; Perez et al., 2002; Zhang et al., 2003). The identity of downstream signaling components in taste transduction had also been a matter of controversy, with groups arguing for the existence of multiple signaling pathways for different taste qualities in the same taste receptor cell. Since it

has been shown that only one taste quality (either sweet, umami, or bitter) is coded for by an individual cell, in addition to an experiment in PLCβ_2-KO mice that rescued only bitter taste by expressing PLCβ_2 only in bitter taste cells (Zhang et al., 2003), much credit has been lost from these arguments. This does not argue against the possibility of other signaling pathways impinging upon and modulating taste transduction (Chandrashekar et al., 2006).

Salt and sour taste are detected by cells distinct from those that detect bitters, sweets, and umami. It is thought that salt detection is mediated by sodium ion currents through amiloride-sensitive sodium channels, but a salt receptor has not be identified (Chandrashekar et al., 2006). Recent studies have found that a TRP-channel family member – PKD2L1 – is involved in detecting sour ligands, and that cells that express this TRP channel are distinct from those that detect other tastants (Huang et al., 2006; Ishimaru et al., 2006; LopezJimenez et al., 2006).

1.3.2 Understanding taste perception in mammals

Expression of T1Rs, T2Rs, and PKD2L1 in non-overlapping populations of taste receptor cells, and the selective rescue of bitter taste by driving PLCβ_2 in T2R-expressing cells in PLCβ_2 knock-out animals, give weight to the labeled-line model of taste coding in the periphery (Chandrashekar et al., 2006), in which a taste cell codes for a single taste modality, and that detection of a tastant, for example, bitter, is coded for by activation of bitter-sensing cells at the periphery. Further studies add support for this label-line

model, demonstrating that an individual taste cell is "hard-wired" for attraction or repulsion and that it is the receptors that it expresses that determine to what it responds. As an example, expression of a bitter taste receptor in T1R-expressing (sweet) taste cells causes an animal to be selectively attracted to the cognate bitter taste ligand (Mueller et al., 2005).

Although molecular biology has offered many answers to how tastants are detected at the periphery, and given clues as to how taste is initially coded, an interesting and vital component to understanding taste perception in mammals remains: the identification of higher order neurons that make up the taste circuit. Ultimately, how taste detection at the periphery results in even the relatively simple behavioral output of feeding or avoidance is not known (Chandrashekar et al., 2006). This is where studies in *Drosophila* may prove informative, not only because they are easier to manipulate genetically, but also because the taste circuit is somewhat simpler. Unlike in mammals, where taste receptor cells (which are specialized epithelial cells) couple to second order neurons that project axons to the CNS, *Drosophila* taste receptor cells are neurons themselves, and project their axons directly to the primary taste center in the CNS.

1.4 The Drosophila taste system

The majority of the information about the taste system in *Drosophila* is a reprint, as allowed by the journal in which the manuscript was published:

Amrein, H., and Thorne, N. (2005). "Gustatory perception and behavior in *Drosophila melanogaster*." Current Biology 15: R673-R684.

The gustatory (or taste) system of insects is complex and, unlike to mammals, not restricted to a single taste organ (Dethier, 1976; Stocker, 1994). The main taste tissue in *Drosophila* is the labellum, which is located at the distal end of the proboscis and is generally considered the equivalent of the human tongue (Figure 1). Each palp is covered with 31 stereotypically arranged taste bristles (sensilla). Based on morphological criteria, these bristles fall into three classes - small (s), intermediate (i) and long (l) - and contain 2 (i) or 4 (s and l) taste sensory neurons (Stocker, 1994). The two palps close off the entrance to the pharynx. During active feeding, the labial palps open and expose additional, poorly characterized sensilla - the taste pegs- which make contact with the food as it enters the pharynx. Three separate taste cell clusters, the labral sense organ and the ventral and the dorsal cibarial sense organs, line the interior wall of the pharynx and "monitor" the food as it is ingested. The exact role of these internal taste organs is not known, but they might serve either as sensors for harmful substances that, if activated, elicit a "regurgitate" response, or alternatively, verify desirable substances and promote sucking reflexes.

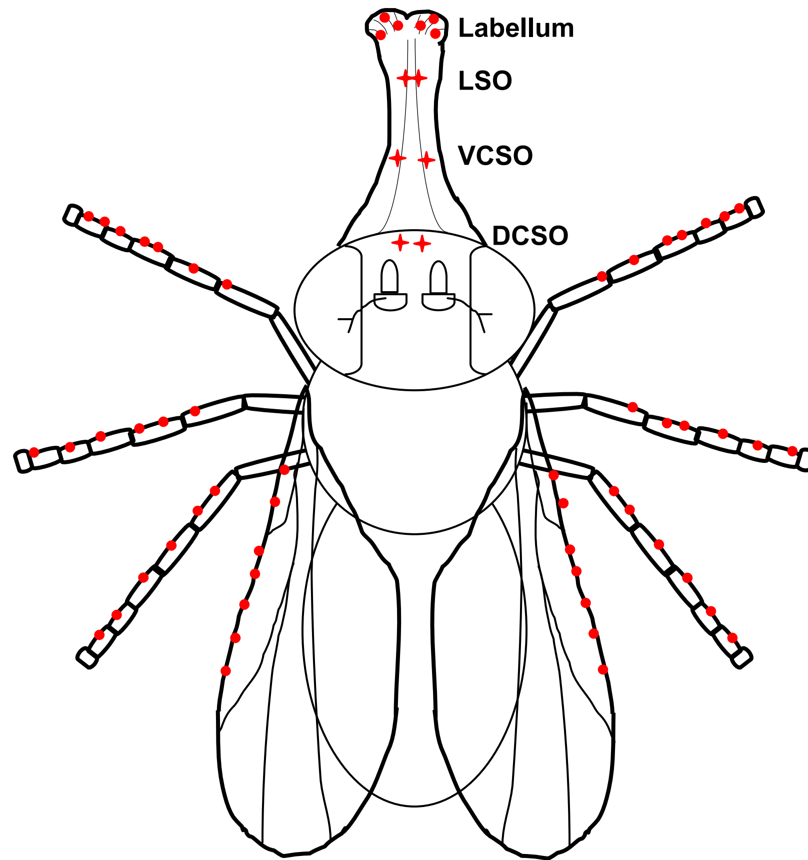


Figure 1: Organization of the *Drosophila* taste system. Red circles indicate the location of taste bristles and red stars indicate taste sensilla of the pharynx.

Additional taste bristles, interspersed between the more abundant mechanosensory bristles, are located on the legs and the anterior wing margins (Lienhard and Stocker, 1987; Nayak and Singh, 1983; Stocker, 1994) (Figure 1). Each leg contains at least 30 taste bristles and the anterior wing margin of each wing has an additional 40 taste bristles (Hartenstein, 1993; Stocker, 1994), bringing the total number of taste sensilla on the body to about 260. The wide distribution of taste cells throughout

the fly's body underscores the critical role that chemosensory stimuli represent in the fly's world.

Interestingly, the number of taste bristles of the forelegs appears to be sexually dimorphic - males have a signficantly greater number of taste bristles on their forelegs compared to females (50 compared to 37, respectively) (Meunier et al., 2000; Nayak and Singh, 1983). Courtship behavior of adult male flies may suggest a role for these bristles (Bray and Amrein, 2003). During the courtship sequence the male taps the female's abdomen, presumably to sample pheromone chemicals secreted and deposited on the abdomen (Hall, 1979; 1994).

Taste bristles house the dendrites of gustatory receptor neurons, and have a terminal pore at the tip to allow direct access tastants found in the external environment to the taste neuron (Figure 2) (Nayak and Singh, 1983). The space between the dendrite and the inner surface of the bristle is filled with lymph, a secretion from the support cells that are associated with each taste sensillum (Morita, 1992). Taste sensilla lymph likely plays a similar role to that of olfactory sensilla lymph – enhancing the solubility and modulating the accessibility of ligands to their cognate receptors, at least partially through "odorant binding proteins" that are found in the lymph (Pelosi, 1998; Steinbrecht, 1998). Indeed, several "odorant binding proteins" are also found to be associated with taste sensilla (Galindo and Smith, 2001; Pikielny et al., 1994; Shanbhag et al., 2001).

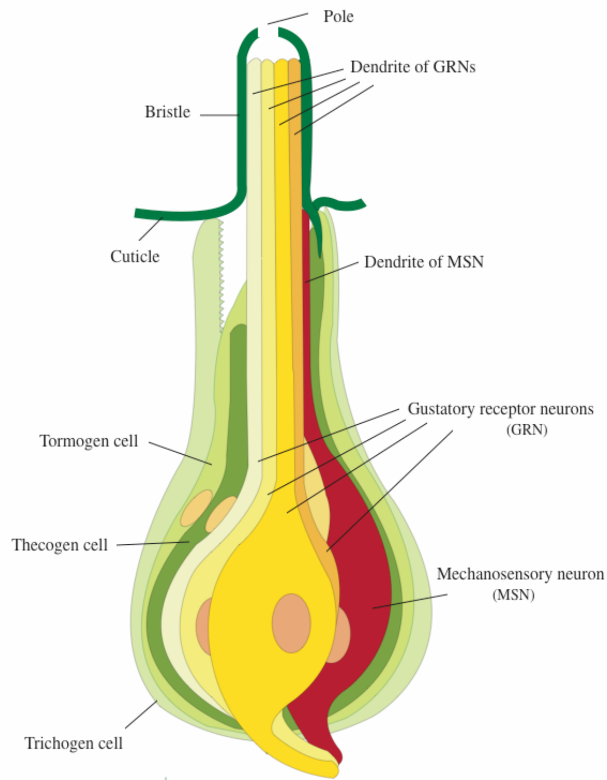


Figure 2: Structure of a taste bristle.

1.4.1 Assaying taste behavior in *Drosophila*

Drosophila taste behavior, at its most basic level, is similar to our own – they are repulsed by and avoid eating toxic, often bitter-tasting, substances, and are attracted to and feed upon sweet-tasting foods. In order to measure taste behavior in the fruit fly, several simple behavioral assays, crucial for a quantitative assessment of taste at the organismal level, have been developed (Figure 3). Attraction or aversion to soluble chemicals can be evaluated based on the adult fly's feeding behavior (the two-choice preference test) or by a robust reflex behavior that involves a poorly characterized

neural circuit (the proboscis extension reflex assay). The two-choice preference test, first developed by Tanimura and co-workers (1982), is a simple but powerful assay for measuring feeding behavior (Figure 3A). To promote feeding, flies are first starved for 24 to 32 hours, after which they are given the choice of two food substrates for 60 to 90 minutes while in the dark. The two food substrates are colored with "tasteless" red or blue chemical compounds, which, once ingested, are easily seen in the gut through the semi-transparent abdomen of the fly (Figure 3A). In this way, it is easily determined from which food substrate(s) the fly ate. A feeding preference index (PI) is calculated, indicating the preference for either one of the two substrates.

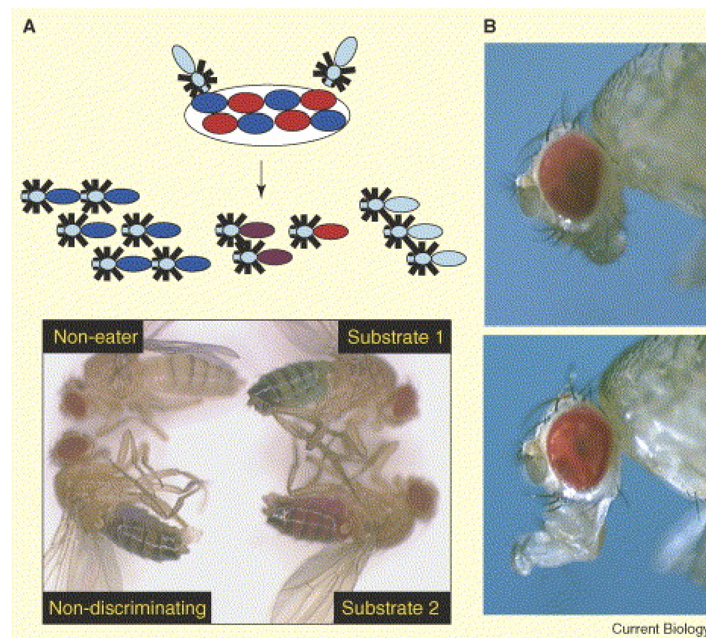


Figure 3: (A) The two-choice feeding assay allows adult flies to choose to feed on two different substrates, that contain dye of two colors. After feeding, it is easy to tell which substrate the flies have eaten based on the color of the food found in their abdomen. (B) The proboscis extension reflex assay. When the tarsal segments of the

fly's leg, or the proboscis itself, is touched with a sweet substance, the proboscis extends (bottom panel).

Unlike the two-choice feeding test, which measures taste perception at the level of the organism, the proboscis extension reflex is a more direct measure of the taste response of specific GRNs (Dethier, 1976). In this assay, flies are immobilized and observed under a binocular lens as specific taste hairs, usually located on the legs, are brought into contact with a test solution. Attractive substances, such as sucrose or trehalose, elicit a large increase in the frequency of proboscis extension (Figure 3B), a behavior that occurs spontaneously, but increases with feeding activity. Proboscis extension can also measure whether repulsive (bitter) compounds are detectable. To do this, an attractive substance (i.e. sucrose) is mixed with a bitter compound. If a substrate is detected as repulsive, there is a significant reduction in frequency of proboscis extension (Dethier, 1976).

1.4.2 Electrophysiological studies: *Drosophila* taste neurons detect salts, sugars, bitter compounds, and water

Many of the initial studies of taste used electrophysiology to measure responsiveness of taste neurons to various tastants. Taste neurons are relatively easy to access if electrophysiological recordings are taken from an entire taste bristle. Initial electrophysiological investigations focused on single sensilla recordings of the labial palps and leg (Dethier, 1976; Falk et al., 1976; Fujishiro et al., 1984; Wieczorek and Wolff,

1989). In this technique, an electrode is inserted into the base of a taste bristle, which is bathed in a solution that can be rapidly exchanged. Electrical activity of the neurons within the sensillum is recorded in the form of voltage spikes (action potentials) of cells before and during application of various chemical compounds (Dethier, 1976; Falk et al., 1976; Fujishiro et al., 1984; Wieczorek and Wolff, 1989). It is important to note that this technique records the activity of all (two to four) neurons within the sensillum simultaneously. Based on distinct characteristics of spiking patterns – which reflect the cells' distinct physiological and molecular nature – it is inferred which cell (if any) within a sensillum is stimulated upon exposure to specific ligands.

The main observations from these early electrophysiological investigations can be summarized as follows (Figure 4): A typical sensillum (the most studied being of the L-type) is composed of distinct sensory neurons, one of which responds (best) to water (W cell), one to sugars (S cell), one to low salt (L1 cell) and one to high salt (L2 cell) concentrations (Dethier, 1976; Fujishiro et al., 1984; Hiroi et al., 2002; Rodrigues and Siddiqi, 1981; Wieczorek and Wolff, 1989). Even though each sensilla appears to contain only one S cell, electrophysiological data suggests that this cell possesses distinct receptor sites (i.e. receptor proteins; see below), recognizing different sugars (a site specific for fructose and a site for most other sugars) (Tanimura and Shimada, 1981). I-type sensilla, which house only two chemosensory neurons, have both a sugar and high salt sensitive cell. S-type sensilla are more difficult to access and recordings from only a

couple have been performed, revealing a similar neuronal composition as L-type sensilla (Figure 4)(Hiroi et al., 2002).

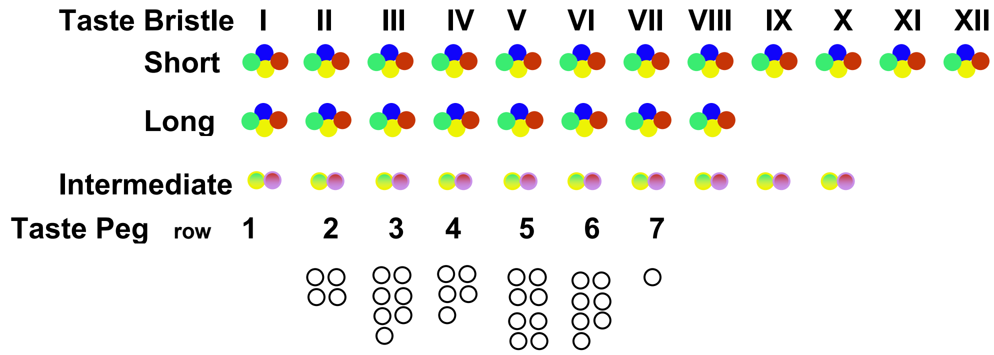


Figure 4: Functional organization of taste sensilla in the labial palps as determined by electrophysiology. Colors of neurons correspond to substrates to which they respond. Sugar, green; water, blue; low salt, yellow; high salt, red; bitter, purple; not characterized, white.

More recent electrophysiological investigations by the Tanimura laboratory have revisited the specificity of GRNs in various types of taste bristles and – not surprisingly - a more complex picture that is better aligned with the complex expression profile of the putative *gustatory receptor* genes (see below), has begun to emerge (Hiroi et al., 2002; Hiroi et al., 2004; Meunier et al., 2003). First, S-cells (sugar cells) from the three sensilla types show significant differences in spike frequency when stimulated with various sugars: for example, the S-cell in L-type sensilla is two- to three-fold more sensitive to sugars when compared to the S-cell of S-type sensilla. Second, the S-cell of S-type sensilla appears to detect only a subset of sugars, responding only to sucrose and fructose, but not glucose and trehalose (Hiroi et al., 2002).

Another recent study from the Tanimura lab (Meunier et al., 2003) was the first in depth investigation of GRNs' responsiveness to various bitter compounds. It is well established that herbivorous insects, such as the caterpillar *Manduca sexta*, possess gustatory neurons that are activated by harmful compounds, eliciting feeding inhibition (Glendinning et al., 2001; Glendinning et al., 2002). However, surprisingly little was known about "bitter taste" perception in *Drosophila* until recently, when Meunier and co-workers (2003) recorded from 18-28 taste sensilla located on the prothoracic leg and identified six sensilla that housed a neuron activated by bitter compounds. Specifically, the six sensilla fall into two groups: four sensilla were activated when stimulated with quinine but not berberine, and two sensilla were activated by berberine but not quinine. All six sensilla showed similar responses to denatonium and strychnine, two other bitter tasting compounds. Most interestingly, the bitter-sensing cell within these six sensilla was found to correspond to the L2 cell, which is known to be activated by high concentrations of NaCl - another repulsive stimulus. Thus, the L2 cell is a widely tuned neuron that responds to various, chemically diverse, repulsive compounds (see below). Many other prothoracic taste bristles, however, did not appear to house an L2 cell that was activated by the bitter tasting compounds used in this study. Interestingly, however, the firing pattern of the W- and S-cells (in sensilla of the tarsal segments to water or sugar) was significantly inhibited in the presence of quinine. These findings indicate that the detection of chemical compounds avoided by the fly are mediated

through (at least) two different mechanisms, one that leads to the activation of avoidance neurons (L2 cells) and one that leads to the inhibition of neurons involved in the detection of attractive substrates (S cells), such as sugars.

Finally, Hiroi and co-workers (2004) also investigated the spiking/firing pattern of labellar neurons stimulated by bitter-tasting compounds by focusing on I-type sensilla, which possess only two neurons, facilitating experimental interpretation of the firing patterns. They found that one of the two neurons responded to low salt concentrations as well as to sugars and that the other cell responded to high salt concentrations as well as bitter compounds (Figure 4). Thus I-type sensilla are associated with a taste neuron that has composite properties of L1 and S cells and another taste neuron that has properties of an L2 cell. This is the first time that a neuron was found responsive to both sugars and salt concentration, and is evidence that two basic types of taste neurons exist – those that detect repulsive tastants (bitters and high salt) and those that detect attractive tastants (sugars and low salt).

1.4.3 *Drosophila* taste mutants provide genetic basis of taste detection in early studies

There have been numerous genetic approaches for identifying genes involved in taste perception (Arora et al., 1987; Isono and Kikuchi, 1974; Lindemann, 1996; Morea, 1985; Rodrigues and Siddiqi, 1981; Singh, 1997; Tanimura et al., 1982). Many mutations show fairly broad and pleiotropic taste phenotypes and likely affect other sensory

modalities as well. For example, many of these genes could affect a variety of processes including signaling of neuronal activity within the cell, or the development and differentiation of specific types of GRNs. Curiously, the one mutation/variation that has been directly linked to a gene was *tre* (Tanimura et al., 1982), which was later found to encode the trehalose receptor GR5a (see below). The absence of linkage between other identified phenotypes and specific genes might reflect a lack in effort by the researchers or inherent difficulty and complexity of the mapping process per se, as the phenotypes are usually fairly subtle and often influenced by the genetic background.

Many gustatory mutations, such as *gustC* and *gustM*, affect the behavioral perception of different types of chemicals including sugars, salts and quinine (a bitter tasting compound) (Morea, 1985; Rodrigues et al., 1991). However, some have a relatively specific phenotype and affect only the perception of pyranose (*gustA*) (Singh, 1997), salt (*gustB* and *gustE*), or bitter compounds (*gustD*) (Arora et al., 1987; Singh, 1997). Identification of the *gustatory receptor* (*Gr*) gene family, members of which have definitively been found to be tastant receptors, came years later (Clyne et al., 2000) (see below). Interestingly, some mutations with relatively restricted phenotypes map in regions that harbor one or several *Gr* genes; for example, *gustC* and *gustE* (10E), *gustM* (93C/D) and *gustR* (64B/C) are in relative close proximity of *Gr* gene clusters (*Gr10a/b*, *Gr64a-f* and *Gr93a-d*) at these cytological positions (Singh, 1997). Thus, it remains to be seen whether any of these mutations map to a *Gr* gene.

1.5 The Drosophila gustatory receptor (Gr) gene family

Significant breakthroughs in our understanding of olfactory perception and coding in both mammals and invertebrates were achieved with, and were a direct consequence of, the identification of the genes encoding the olfactory receptors (Buck and Axel, 1991; Clyne et al., 2000; Gao and Chess, 1999; Vosshall et al., 1999). Similarly, the cloning of two gene families encoding mammalian G-protein coupled taste receptors, the *T1R*s and *T2R*s, has provided a molecular basis of bitter and sweet taste perception in mammals, respectively (Adler et al., 2000; Chandrashekar et al., 2006; Chandrashekar et al., 2000; Hoon et al., 1999; Matsunami et al., 2000; Nelson et al., 2001). Hence, major efforts were directed toward the identification of the *Drosophila* taste receptor genes.

1.5.1 Discovery of the *Gr* gene family

The putative taste receptor gene family was first described by Clyne and co-workers (2000). These authors developed an algorithm for the identification of DNA sequences encoding transmembrane proteins, which was 'trained' to distinguish G-protein coupled seven-transmembrane domain receptors from other multi-transmembrane domain proteins. Subsequent analysis revealed the presence of 43 related, putative candidate *gustatory receptor* (*Gr*) genes, 19 of which were shown by RT-PCR to be expressed in at least one of several tissues containing taste neurons. Later, Scott and co-workers (2001) extended the family to 56 genes using the candidate genes

identified by Carlson's group through reiterative database searches. Expression analysis of several of these genes confirmed their specific expression in GRNs of the labial palps and legs, as well as the internal taste organs and the terminal taste organ of larvae. A different strategy was employed by Dunipace and collaborators (2001), who observed that BLAST searches with the *Drosophila odorant receptor* sequences identified numerous, previously unknown seven- transmembrane domain receptor proteins. Using these new sequences for reiterative BLAST searches, they identified a novel gene family that turned out to correspond to the genes described by Clyne and co-workers (2000). Additionally, Gal4 analysis of some of these candidate taste receptor genes revealed expression in taste neurons throughout the fly's body, a result also observed by Scott and co-workers (2001). Once the entire *Drosophila melanogaster* genome sequence was released, the chemosensory gene repertoire of the fruit fly was systematically classified (Robertson et al., 2003). It contains almost 130 genes which fall into two large subfamilies, the *Or* genes (61) and the *Gr* (68) genes (Figure 5). Overall, the sequence similarity between different gustatory receptors is fairly low (8-12 % amino acid identity), even lower than that of the diverse olfactory receptors. The non-classical *Or83b* gene, which appears to encode a co-receptor for other (ligand-binding) olfactory receptors (Larsson et al., 2004), represents the evolutionary link between the two sub-families. About two-thirds of *Gr* genes appear as clusters harboring up to six genes; often, individual genes are separated by only a few hundred nucleotides. Clustered genes share much higher sequence similarity

to each other than to the remaining *Gr* genes (up to 70%). In three of these loci, the genes are alternatively spliced: promoters control transcription of unique 5' exons that are spliced to common 3'exons, generating transcripts that encode receptors with identical carboxy-, but different amino, termini. Unlike in mammals, *Drosophila* does not appear to possess a distinct pheromone receptor gene family, but instead appears to have recruited specific members of the *Or* and *Gr* gene family for these social behaviors (Bray and Amrein, 2003; Kurtovic et al., 2007).

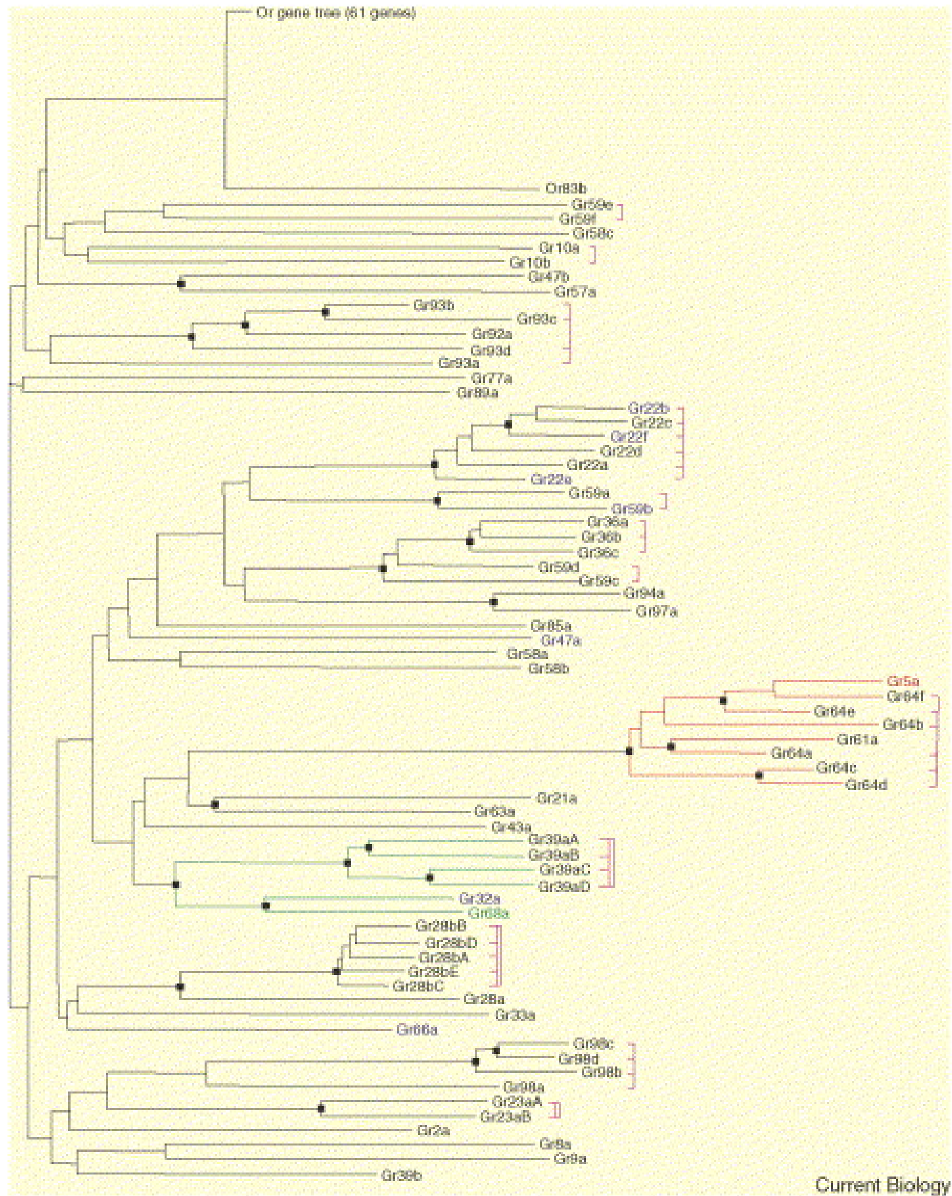


Figure 5: Organization of the *gustatory receptor* gene family. The *Gr* genes are structurally related to the *Or* genes, and the direct link between the two families is *Or83b*. Black boxes indicate branches with 75-100% bootstrap support. Other *Gr* genes with known expression – possibly involved in bitter taste perception – are shown in blue.

1.5.2 The difficulty in studying *Gr* genes and the *Gal4/UAS* system

The rapid progress in elucidating *Drosophila* olfactory coding was possible not only because of the many powerful molecular genetic tools that are routine in this system, but also because of the relative simplicity of the olfactory system, the manageable number of *Or* genes to be analyzed, and the structural features of olfactory sensory neurons (i.e. their direct physical connection with the primary processing centers in the CNS through their axons). Yet, despite the structural similarity of olfactory and gustatory neurons and the close relationship and similar sizes of *Gr* and *Or* gene families, establishment of gene expression profiles for *Gr* genes and determination of axonal projections of GRNs to primary processing centers in the brain turned out to be rather challenging. Unlike *Or* mRNAs, *Gr* mRNAs are found at exceedingly low levels in taste neurons, making RNA *in situ* hybridization an unreliable method and requiring more laborious, indirect detection strategies (Clyne et al., 2000; Dunipace et al., 2001; Scott et al., 2001). Moreover, taste neurons are widely distributed on many appendages throughout the body of the fly, many of which are not amenable to *in situ* hybridization methods at all (i.e. wings and legs). In addition, GRNs from these different appendages project to distinct CNS structures, the SOG in the brain as well as the abdominal thoracic ganglion. These projection centers are far less structured than the antennal lobes (Stocker, 1994), complicating the interpretation of axon tracing experiments from GRNs.

Progress was made possible by employing the bimodal Gal4/UAS expression system: a putative *Gr* gene promoter is used to drive expression of the yeast transcriptional activator Gal4 (Gal4 driver), which tightly regulates the expression of a Gal4 dependent *UAS-reporter* (Brand and Perrimon, 1993). This system has the distinct advantage over RNA *in situ* hybridization experiments in that it allows visualization of different structures of the *Gr*-expressing neuron via GFP targeting to the nucleus (*UAS-nucgfp*), the dendrites (*UAS-mCD8::GFP*), or the axons and the axon terminals (*UAS-nsynaptobrevinGFP*), depending on the molecular nature of the specific UAS reporter used. Even though the Gal4 system usually reproduces endogenous gene expression accurately, it should be kept in mind that it is an indirect method, and hence, it is possible that in some rare cases, a *Gr* expression profile obtained with *p[Gr]-Gal4* drivers might not precisely represent that gene's endogenous expression.

1.5.3 Using the Gal4/UAS system to determine *Gr* expression profiles

Initial expression studies using the Gal4/UAS system revealed a rather complex expression profile of the *Gr* gene family as a whole (Dunipace et al., 2001; Scott et al., 2001). For example, at least one gene, *Gr22e*, was found to be expressed in some, but not all, GRNs of each organ containing taste sensilla, whereas expression of most other *Gr* genes were found to be restricted to a few neurons in one or two taste organs (Figure 6) (Dunipace et al., 2001; Scott et al., 2001). All genes tested were found to be expressed in a fraction of GRNs (between 1 and 5%), but no detailed comparison between different *Gr*

genes were performed and it remained unclear whether and to what extent co-expression of different Gr genes occurred. These studies, as well as the identification of *Gr5a* as a trehalose receptor (Chyb et al., 2003), indicated that *Gr*s were the receptors for mediating taste and provided a promising start to understanding the role of *Gr*s in taste detection.

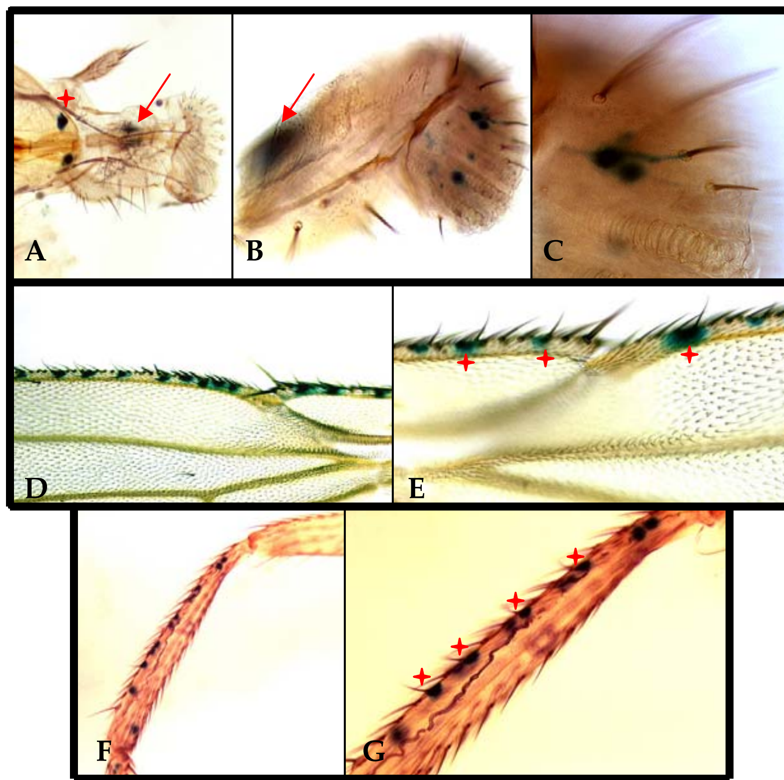


Figure 6: Expression *of Gr22e* in GRNs of various taste organs using the Gal4/UAS system. (A-C) show taste neurons of the labellum. (D+E) show taste neurons associated with the curved taste bristles of the anterior wing margin (red stars). (F+G) show taste neurons of the tarsal leg segments, also associated with curved chemosensory bristles (red stars).

1.5.4 *Gr5a* is functionally defined as a trehalose receptor

One of the earlier taste mutants discovered - *tre* - had decreased sensitivity specifically to the normally attractive sugar trehalose (Tanimura et al., 1982). Behavioral responses to other sugars remained unaltered in these mutants. The cytological location of the gene that appeared to be responsible was mapped to the X-chromosome (Tanimura et al., 1982), but it took the identification of the *Gr* gene family to really confirm the exact identity of the trehalose gene as *Gr5a* (Dahanukar et al., 2001; Ishimoto et al., 2000; Ueno et al., 2001). Using transgenic flies that express a reporter such as lacZ or GFP under the control of the putative promoter of *Gr5a* via the Gal4/UAS system (discussed in more detail below), Chyb and colleagues (2003) demonstrated expression of *Gr5a* in taste neurons of the labellum and legs. In order to functionally confirm that *Gr5a* was indeed a trehalose receptor, they expressed *Gr5a* cDNA in heterologous cells (*Drosophila* S2 cells *in vitro*) and showed that intracellular Ca^{2+} levels increased upon cellular exposure to trehalose, indicating activation of intracellular signaling pathways, but not to other sugars. Notably, this experiment is the only time a group has been able to functionally classify a *Gr* using a heterologous expression system.

1.6 Thesis research projects: taste detection and perception in Drosophila and characterizing the expression of Grs

Prior to the start of my graduate work at the end of 2000, characterization of the taste system in *Drosophila* had been largely restricted to the cellular level. Taste neurons in *Drosophila* had been described electrophysiologically, showing their responsiveness to different taste substrates, and their afferents to the CNS had been traced using dyes and neuronal markers (Edgecomb and Murdock, 1992; Lienhard and Stocker, 1987; Murphey et al., 1989; Nayak and Singh, 1983; Rajashekhar and Singh, 1994; Shanbhag and Singh, 1992; Stocker and Schorderet, 1981) to roughly identify and characterize the primary taste center in the *Drosophila* brain – the tritocerebrum/SOG. Molecular work in the taste field had just begun, with the discovery of the *Gr* gene family and initial descriptions of *Gr* expression in taste neurons (Clyne et al., 2000; Dunipace et al., 2001; Scott et al., 2001). Importantly, *Gr5a* was identified as a trehalose receptor (Dahanukar et al., 2001; Ueno et al., 2001). It was clear that the role of the *Gr* gene family in taste was an important key in determining the molecular basis behind taste detection. It was still unclear, however, how taste was coded in the periphery and how tastant detection led to appropriate behavioral output, such as feeding or avoidance. The role *Gr*s played in taste detection and perception was equally unclear.

Research that I conducted during my graduate career can be divided into three main projects described below.

1.6.1 Project One: Taste coding and perception

Initial experiments that I conducted in the lab indicated that the majority of *Gr*s the lab had analyzed –including *Gr66a* and *Gr22e* (Dunipace et al., 2001) - produced a very similar axonal projection pattern in the SOG. The only receptor that gave a different axonal projection pattern was *Gr5a*. Knowing that *Gr5a* was a receptor for trehalose (Dahanukar et al., 2001; Ueno et al., 2001), a sugar and attractive substrate, we hypothesized that the other receptors (*Gr66a* and *Gr22e*) may be involved in detecting a different type of tastant, bitter compounds, for example. To address this, we first characterized the peripheral expression of eight *Gr*s (including *Gr66a* and *Gr22e*), showing that most were co-expressed in subsets of taste neurons in the labellum. Interestingly, *Gr5a* was the only receptor of this group not co-expressed with any of the other *Gr*s. We then mapped the axonal projections of these primary taste neurons to the CNS, showing that *Gr5a*-expressing neurons produced a unique axonal projection pattern in the tritocerebrum/SOG compared to the other *Gr*-expressing neurons. Using two-choice feeding assays, we found that functionally ablating *Gr5a*-expressing neurons diminishes the preference for trehalose, and functionally ablating neurons expressing the other receptors (*Gr66a*/*Gr22e*) significantly reduces the sensitivity to caffeine, such that flies feed off of caffeine-contaminated food sources that they usually avoid.

This project thus demonstrated that primary taste neurons can be characterized as either avoidance- or attractive-type taste neurons, with the majority of the *Gr*s expressed in avoidance-type taste neurons. Functional ablation of avoidance-type neurons causes desensitization to bitter-compounds.

1.6.2 Project Two: Characterizing the expression of a highly conserved *Gr* gene cluster - the *Gr28* genes

Initially this project set out to identify *Gr*s that were expressed in taste neurons near the female ovipositor. Though taste neurons have not been characterized in this area, purportedly, there are chemosensory bristles near the ovipositor, proposed to be involved in sensing a suitable environment for a female to lay her eggs (Stocker, 1994). To date, no *Gr*s have been shown to be expressed in such taste neurons. In an effort to find *Gr*s expressed here, however, we came across a group of *Gr*s that were expressed in non-chemosensory neurons of the abdomen. Two of these genes belonged to a gene cluster, highly conserved in most *Drosophila* species, the *Gr28* genes. Expression analysis of all six *Gr28* genes revealed that they had an unusual expression pattern in non-chemosensory neurons and neurons in the CNS. RNA *in situ* hybridization confirmed these results in a number of tissues, including neurons of the pars intercerebralis of the CNS, which are higher-order taste neurons.

This project thus offers detailed expression analysis of a subfamily of *Gr* genes, and confirms expression of *Gr*s in non-taste neurons, providing further evidence that

members of the *Gr* gene family are recruited for roles beyond tastant detection. Additionally, this project helps characterize higher-order neurons in the taste circuit, and demonstrates, via *in situ* hybridization, that members of the *Gr28* gene family are expressed in these neurons.

1.6.3 Project Three: Determining the function of the *Gr28* genes

By the time most of the expression analysis had been done on the *Gr28* genes, it had been found that at least two members of the *Gr* gene family were involved in the detection of something other than tastants. *Gr21a* and *Gr63a*, which are both expressed in neurons of the antennae, were found to mediate CO_2 detection in *Drosophila* (Jones et al., 2007; Suh et al., 2004). In addition, Bray and Amrein (2003) found that *Gr68a* is expressed in neurons that mediate pheromone detection, with the implication that *Gr68a* is a pheromone receptor. Because of the precedence for *Gr*s to be recruited for roles beyond traditional taste receptors, we proposed that the *Gr28* genes may also have functional roles not limited to tastant detection, and that this could explain the unusual expression pattern of the *Gr28* genes. Especially interesting is the expression of the *Gr28* genes in higher-order taste neurons of the CNS that may also express *Drosophila*-like insulin peptides (DILPs) and in neurons associated with the aristae that serve as hygro/thermoreceptors. The possibility that these receptors may serves as internal nutrient receptors influencing DILP secretion, or be the long sought-after water receptor,

along with the evidence that these genes are highly conserved, led us to develop animals lacking this gene cluster.

Towards this end, I produced animals that lack the *Gr28* genes usng two methods – ends-out homologous recombination and FLP-FRT-based deletions using Exelixis *piggyBac* insertion lines. I successfully created two separate knock-out lines using homologous recombination, and created at least seven FLP-FRT-based deletion lines. Only very preliminary behavioral experiments have been done to analyze these *Gr28* knock-out animals for phenotypes. These preliminary experiments suggest that the *Gr28* genes may play a role in aversive substrate detection and/or modulation of avoidance behavior to these aversive substrates.

2. *Gr*s are expressed in avoidance- or attractive-type taste neurons

The majority of this Chapter is a reprint, as allowed by the journal in which the manscript was published.

Thorne, N., Chromey, C., Bray, S., and Amrein, H. (2004). "Taste perception and coding in *Drosophila*." Current Biology 12: 1065-1079.

2.1 Introduction

Taste is a vital sense for animals. Sensory cells located in the taste organs, such as the tongue of mammals or the labial palps of many insects, are dedicated to differentiating between a multitude of structurally diverse chemical compounds, some associated with nutrients and others with potentially harmful toxins. For example, humans are able to recognize hundreds of different soluble substrates, all of which are classified into the five basic taste qualities of bitter, sweet, umami, sour and salty. Two distinct receptor families have been shown to mediate three of these taste qualities: the T2R protein family of about 30 to 40 G-protein-coupled receptors (GPCRs) recognizes a diverse array of substrates that are perceived as "bitter-tasting" (Adler et al., 2000; Chandrashekar et al., 2000; Matsunami et al., 2000), and three distinct GPCRs, the T1Rs, recognize amino acids and sugars and provide the molecular basis for umami and sweet

taste (Damak et al., 2003; Max et al., 2001; Montmayeur et al., 2001; Nelson et al., 2002; Nelson et al., 2001; Zhang et al., 2003; Zhao et al., 2003). Many insects, including Drosophila, have taste preferences for these same ligands; for example, the fruit fly prefers foods that include sugars and amino acids, but avoids foods that contain compounds perceived as bitter (Dahanukar et al., 2001; Glendinning et al., 2001; Glendinning et al., 2002; Meunier et al., 2003; Tanimura et al., 1982; Tompkins et al., 1979; Ueno et al., 2001).

In *Drosophila* only a single gene family – the gustatory receptor (*Gr*) genes - has been proposed to mediate many, if not all, taste qualities (Bray and Amrein, 2003; Clyne et al., 2000; Dunipace et al., 2001; Matsunami and Amrein, 2003; Scott et al., 2001). The Gr genes are almost exclusively expressed in taste neurons (called gustatory receptor neurons or GRNs), which are associated with taste bristles and pegs located at the tip of the labellum (the labial palps), in three clusters inside the pharynx, and numerous taste bristles on the legs and the anterior wing margin (Figure 7) (Bray and Amrein, 2003; Dunipace et al., 2001; Scott et al., 2001). Expression analysis of a few Gr genes using the Gal4/UAS system revealed that each gene is expressed in a small subset of neurons, typically comprising less than 4% of GRNs, suggesting the possibility that each Gr gene is expressed in distinct, non-overlapping populations of cells (Dunipace et al., 2001; Scott et al., 2001).

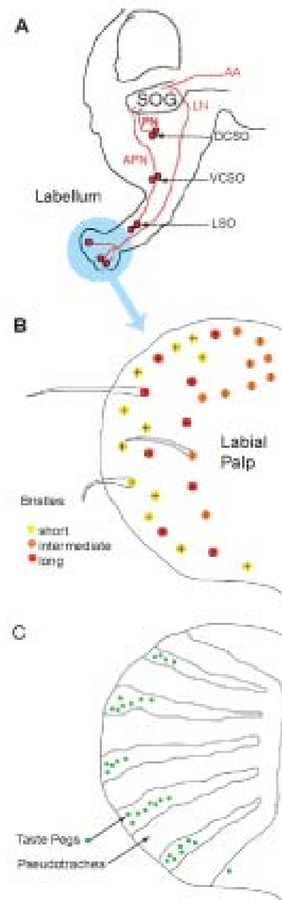


Figure 7: Organization of the taste sensory organs and processing centers in the head.

Two of the sixty Gr genes, *Gr5a* and *Gr68a*, have been characterized and were shown to function as a sugar and a putative pheromone receptor, respectively (Bray and Amrein, 2003; Dahanukar et al., 2001; Ueno et al., 2001). *Gr5a* encodes a receptor for the sugar trehalose, a metabolic component of yeast and therefore a major food component of many Drosophila species (Dahanukar et al., 2001; Ueno et al., 2001). *Gr68a* encodes a male-specific receptor that is required for the tapping step during courtship and is likely

to encode a pheromone receptor for a long-chain hydrocarbon (Bray and Amrein, 2003). Therefore, we proposed that most fly GR proteins are chemosensory receptors for a wide range of soluble ligands that may mediate both pleasant (sweet), repulsive (bitter) responses, as well as pheromone signals (Bray and Amrein, 2003; Matsunami and Amrein, 2003).

To expand our understanding of taste coding in the peripheral taste organs and the brain, we carried out a more detailed expression analysis of the *Gr* gene family, determined the taste property of classes of GRNs expressing specific (sets of) *Gr* genes and investigated the projection pattern of such neurons to the tritocerebrum and the subesophageal ganglion (SOG) - the first relay center of taste processing in the brain. Our results show that labellar Gr genes fall into two distinct groups. We found that seven of the *Gr* genes analyzed are partially co-expressed in overlapping sets of neurons. The projection patterns to the tritocerebrum/SOG of neurons expressing these receptors are therefore very similar, and behavioral studies suggest that these GRNs may mediate aversive taste response. An eighth *Gr* gene (*Gr5a*) is largely, and possibly exclusively, expressed in a different set of neurons than the other seven *Gr* genes, has a very different projection pattern in the brain, and mediates trehalose sensitivity.

2.2 Results

The labellum, considered the main taste organ in *Drosophila*, has approximately 62 chemosensory bristles (sensilla) that are arranged in a stereotyped pattern. These

sensilla are morphologically identified as short (S), intermediate (I), and long (L) (Shanbhag et al., 2001) (Figure 7). S- and L- bristles house dendrites of four chemosensory neurons, whereas I-bristles are associated with two chemosensory neurons (Shanbhag et al., 2001). To determine expression of *Gr* genes in these chemosensory neurons, we and others have employed the Gal4/UAS system (Brand and Perrimon, 1993). This indirect method of expression analysis has been proven far superior to RNA *in situ* hybridization due to low levels of *Gr* transcripts per cell and the wide distribution of taste neurons in tissues not amenable to sectioning procedures (Dunipace et al., 2001; Hiroi et al., 2002; Scott et al., 2001). The Gal4/UAS analyses revealed that a given *Gr* gene is expressed in a small number of chemosensory neurons per labial palp (Table 1), and, in each case, in only one neuron per chemosensory bristle. Tanimura and coworkers (Hiroi et al., 2002) also demonstrated an association of specific *Gr* genes with certain bristles of the labellum. They found that the majority of receptors examined were expressed in a single of the four neurons of S-type sensilla. For example, several *Gr* genes were strongly expressed in a single neuron associated with three S-type sensilla (S1, S3, and S6).

Several issues with broad implications for taste coding, however, remain to be elucidated. For example, it is still not known whether some *Gr* genes are co-expressed in the same neurons, and if so, to which extent. Similarly, it is not known what kind of taste properties are mediated by GRNs expressing these receptors. Finally, experiments to

visualize axonal targets in the CNS of neurons expressing individual *Gr* genes have not been performed in any detail. To further advance our understanding of *Drosophila* taste perception, we addressed these questions: we determined the number of neurons expressing novel and previously characterized *Gr* genes, investigated their extent of co-expression, visualized the projection patterns of GRNs expressing these genes, and determined how taste perception was affected in flies lacking specific sets of GRNs.

2.2.1 Expression map of *Gr* genes in the labial palps

Gal4 drivers (*p[Gr]-Gal4*) for eight *Gr* genes, *Gr5a*, *Gr22b*, *Gr22e*, *Gr22f*, *Gr28b*, *Gr32a*, *Gr59b* and *Gr66a*, were combined with a *UAS-nucGFP* reporter gene encoding a green fluorescent protein tagged with a nuclear localization signal and images of optical sections through the entire labellum were collected using a confocal microscope after anti-GFP antibody staining (for details, see Experimental Procedures, this chapter). By using the map generated by Tanimura and coworkers as a guide (Hiroi et al., 2002), our detailed analysis of confocal stacks allowed us to more accurately determine the organization, number and location of neurons expressing each of these genes (Table 1 and Figure 8). The expression patterns fell into two broad classes: *Gr5a*, described later in greater detail, which was expressed in a large number of neurons throughout the entire labial palp, and the majority of other *Gr* genes whose expression was restricted to relatively few neurons.

Table 1: Peripheral expression of *Gr*s. We determined the number of GFP-positive neurons in at least five to ten stained labella in two independent lines for each driver. In many cases, our average is comparable to the number of strongly staining neurons as determined by Hiroi et al. (2002). Also included are labellar neuron counts made by the groups that originally characterized the expression pattern of most of these receptors (Dunipace et al., 2001; Scott et al., 2001). Expression of *Gr*s in pharyngeal neurons was confirmed by X-Gal staining of *p[Gr]-Gal4;UAS-lacZ* flies. The expression of *Gr*s in the legs was determined by using *p[Gr]-Gal4; UAS-nucgfp* flies.

	Neurons stained per labial palp						Averages		
Receptor	This study	Hiroi et al. (2002)	Scott et al. (2001)	Dunipace et al. (2001)	LSO	VCSO	1st leg	2nd leg	3rd leg
Gr22b	10				N	Y	2	0	0
Gr22e	14	14 (10s)		15	Y	Y	2	8	10
Gr22f	3	15 (2s)	3	4-8	N	N	0	0	0
Gr28b.e	13		9		N	Y	2	0	0
Gr32a	8	10 (6s)	6		N	Y	5	3	3
Gr59b	4	18 (4s)		2	N	N	0	0	0
Gr66a	22	22 (8s)	9	8	Y	Y	8	7	6
Gr5a	71				N	N	10	4	4
Gr5a C.	55				N/D	N/D	N/D	N/D	N/D

Of the group including the majority of *Gr* genes, *Gr66a* was expressed in the largest number of cells per palp (n = 22 ± 1). Significantly, only a single neuron per S- and I-type sensillum stained positive for this driver, an observation also made for *Gr22e* in previous studies (Dunipace et al., 2001). The neurons associated with S-type bristles, which are located more medially, appeared larger in size compared to the more laterally located neurons of I-type sensilla (Figure 8A+B). *Gr22b, Gr22e, Gr22f, Gr28be, Gr32a* and *Gr59b* were expressed in fewer neurons than *Gr66a* (Figure 8 and Table 1). Expression of these receptors appears more restricted to larger neurons associated mostly with S-type bristles. Taken together, our expression data are consistent with initial studies made by Axel's and our group (Dunipace et al., 2001; Scott et al., 2001) and confirm the more

detailed analysis conducted by Hiroi and co-workers (Hiroi et al., 2002) (Table 1). Thus, these expression studies provided the groundwork necessary to determine whether two or more *Gr* genes are actually co-expressed in the same neuron associated with an S-type bristle.

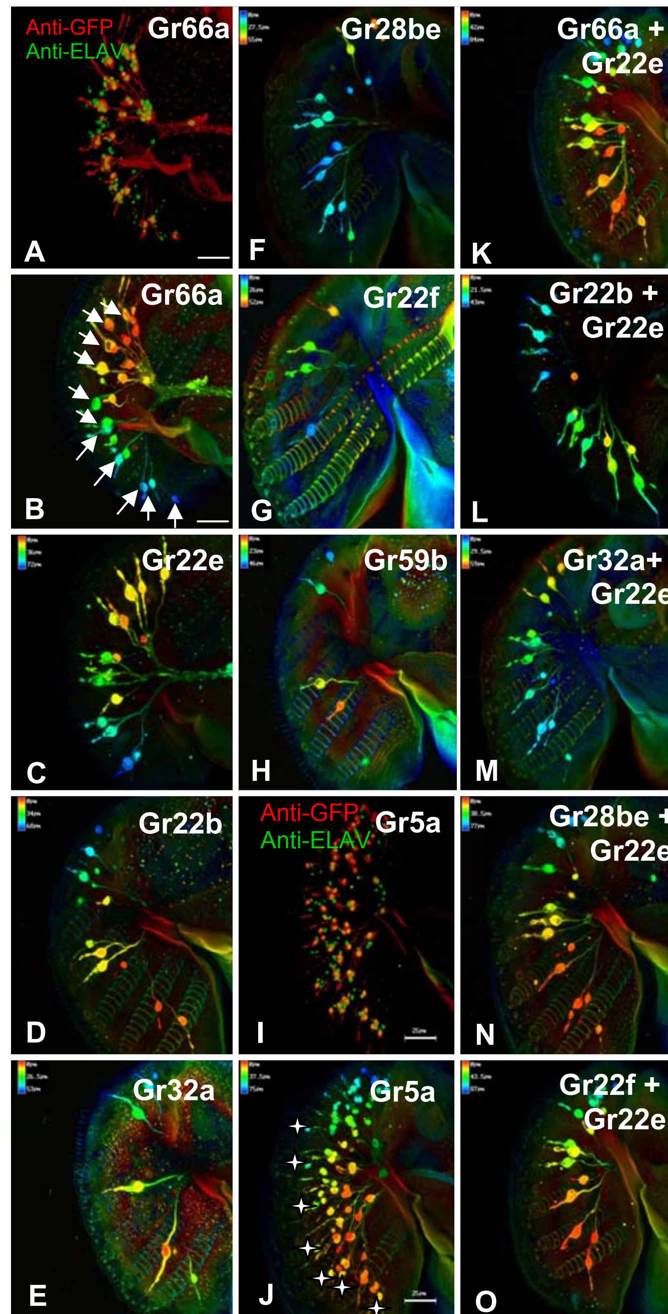


Figure 8: Expression of Grs in the labellum. Labella of p[Gr]-Gal4;UAS-nucgfp flies were dissected and stained with anti-GFP (red) and anti-ELAV (green) and visualized

with a confocal microscope. ELAV staining (green) colocalizes with anti-GFP (red) demonstrating that the Gr genes are expressed in neurons of the labellum (A+I). All images are optical stacks, with multicolored images created by using the depth-coding option included with the confocal software. Depth-coding was used to enhance the visualization of Gr-expressing (GFP-positive) neurons of the labellum. Of note, arrows in (B) show the large cell bodies of neurons that express most receptors and are associated with S-type bristles. Also, Gr5a is expressed in one neurons per taste peg sensilla (stars; J). Scale bars are all 25μm.

2.2.2 Many *Gr* genes are coexpressed in some GRNs

Ideally, co-expression of Gr genes may be addressed by labeling individual *Gr* gene-specific probes with different markers. However, expression levels of these genes are too low for reliable detection of transcripts by RNA *in situ* hybridization. We attempted to use the Gal4/UAS system along with a second reporter system, the tetracycline transactivator/tet-O reporter system (Bello et al., 1998). However, the sensitivity of this system proved to be too low to obtain reliable cell staining in the taste system (Bray and Amrein, unpublished data). Therefore, we sought to address the issue of co-expression by quantification of labeled cells using the Gal4/UAS system, an approach that seemed feasible, given the relative low number of cells in which a given receptor is expressed. We made transgenic fly lines expressing *UAS-nucGFP* under the control of two different Gal4 drivers and then counted and compared the number of labeled neurons to that of flies containing each driver alone (Figure 8 and Table 2). Surprisingly, in all cases where such double-driver experiments were carried out, the number of labeled cells expressing two drivers was close or equal to the number of labeled cells of flies containing the single driver with the higher cell count (Table 2). For

example, in flies that express either the *p[Gr66a]-Gal4* or *p[Gr22e]-Gal4* driver, an average of 22 and 14 neurons/labial palp are labeled, respectively (Figure 8B and 8C, Table 2). In flies that express both drivers, again approximately 22 neurons are detected per palp, which indicates that most if not all cells that express *Gr22e* also express *Gr66a* (Figure 8K). Similar results were obtained when *Gr66a* was compared to *Gr32a* (Table 2).

Table 2: Coexpression of various *Gr* genes. Average number of neurons expressing either of two *Gr* genes in the double-driver lines *p[Gr1]-Gal4/ UAS-nucgfp; p[Gr2]-Gal4/TM3*. If the number of neurons stained in the double driver line is equal to that of either of the single *Gr* drivers, then the two *Gr*s are co-expressed in the same labellar neurons. Counts were derived from at least five to ten stained labella/genotype.

	Neurons per palp			
Receptor	Single driver	+Gr22e driver	+Gr66a driver	+Gr5a driver
Gr22e	14±1		23±2	
Gr66a	22±1	23±2		
Gr22b	10±2	13±1		
Gr32a	8±1	14±1	23±3	
Gr28be	13±2	14±2		
Gf22f	3±1	13±1		
Gr59b	4±1	13±1		
Gr5a	71±11			
Gr5a C.	55±5			64±9

We next asked how expression of *Gr22e* relates to expression of *Gr22b, Gr22f, Gr28be* and *Gr59b* - receptors expressed in smaller numbers of neurons (Table 1 and Figure 8B to 8H). The number of labeled cells in the corresponding double driver lines was approximately equal to the 14 labeled neurons observed in palps of flies containing only *Gr22e*, suggesting that these *Gr* genes are also all co-expressed with *Gr22e* and – by

extension – with *Gr66a*. It is quite possible, however, that *Gr32a* and *Gr28be* are expressed in one or two neurons that do not express *Gr22e*.

The experiments above clearly indicate that the *Gr* genes examined thus far are expressed in overlapping sets of gustatory receptor neurons. The observed pattern of expression also shows that about eight to ten neurons express at least four of the receptors tested (*Gr66a, Gr22e, Gr28be, Gr32a*) and that a few neurons might express up to six receptors (*Gr66a, Gr22e, Gr32a, Gr28be, Gr22b*, and *Gr22f* or *Gr59b*); thus, a given neuron may be identified by a Gr gene code. A second novel observation derived from our analysis is the finding that the receptors analyzed thus far are expressed only in one of the four neurons of S- type sensilla and one of the two neurons of I-type sensilla. This finding is supported by the observation that only a single dendrite extends from the cell body to the bristle shaft (Figure 8A-H). Even in the few cases where two GFP-positive cells lay relatively close to each other, we have been able to track their dendrites into distinct bristles, ruling out the possibility that the corresponding neurons are associated with the same sensillum.

2.2.3 The trehalose receptor GR5a defines a largely distinct set of GRNs

The *Gr5a* gene encodes the only receptor with a known function in taste sensation (Dahanukar et al., 2001; Tanimura et al., 1982; Ueno et al., 2001), but its expression has not been analyzed in detail (Chyb et al., 2003). Hence, it is of primary

interest to determine the cellular expression profile of *Gr5a*. We created a *p[Gr5a]-Gal4* driver by cloning the putative promoter fragment of the *Gr5a* gene in front of the Gal4 coding sequence (see Experimental Procedures this Chapter) and analyzed its expression using the *UAS-nucgfp* reporter. Several aspects of the observed *Gr5a* expression pattern are strikingly different from that of the previously analyzed *Gr* drivers.

Visual inspection of confocal z-series unambiguously revealed that *Gr5a* is expressed in about three times as many neurons as the most abundant, previously analyzed driver (*p[Gr66a]-Gal4*; compare Figures 8A and 8B with 8I and 8J; Figure 9; Table 1). We found that accuracy of cell counts decreases with increasing number and density of GFP-positive cells (compare Figure 8A – 8H with 8I; Table 1), but were able to determine that a single labial palp harbors more than 55 *Gr5a* expressing neurons (72 ± 15). The most striking difference between *Gr5a*-expressing neurons and those expressing the seven other receptors is the difference in cell size: all GFP-positive neurons in flies with the *p[Gr5a]-Gal4* driver are similar in size (Figure 8I and 8J) and appear smaller than most or all GFP-positive neurons of drivers expressed in S-bristles, such as *p[Gr22e]-Gal4* (Figure 8C). Thus, in contrast to neurons expressing the other receptors, *Gr5a*-expressing neurons were distributed over the entire surface of the palp and appeared to all be of the same (small) size. Interestingly, *Gr5a*-expressing cells are often clustered in groups of two or three neurons, and rarely do we observe up to four neurons adjacent to one another (Figure 10E). When the dendrites of clustered neurons

are visible, they converge into the same bristle, indicating that these neurons belong to the same sensillum (Figure 10E and Figure 9C+D). Finally, about seven *Gr5a*-expressing neurons per labial palp were located between the pseudotrachea (Figures 8J and 10F). These cells are not associated with bristles but coincide with the location of taste pegs, providing direct support that these sensilla may have a function in chemosensory perception as suggested by their morphology (Shanbhag et al., 2001).

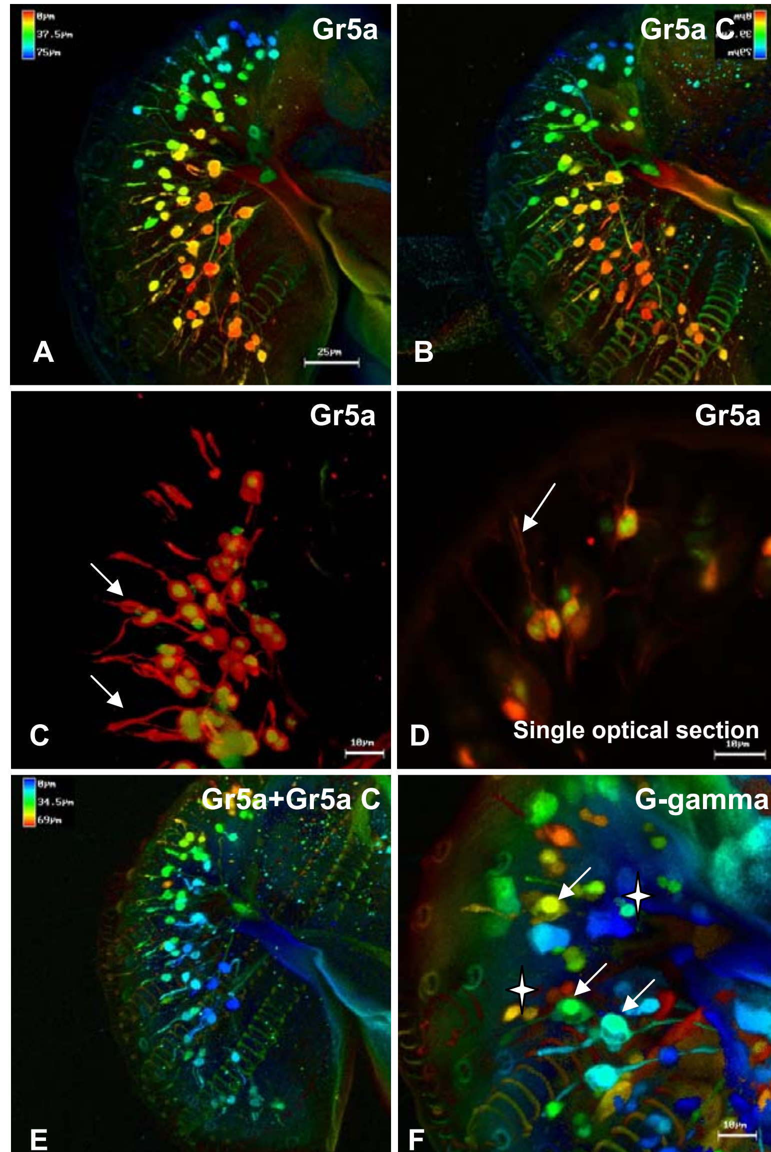


Figure 9: ***Gr5a*** **expression in the labellum. Two different driver lines (A+B) show slightly different numbers of labellar neurons that express** ***Gr5a*****. Our driver (A) shows expression in approximately 70 neurons, whereas the driver made by Dahanukar et al. (2001) (B) shows expression in about 55 neurons. Both** ***Gr5a*** **drivers are expressed in the same labellar neurons, however (E). Clusters of neurons expressing** ***Gr5a*** **are seen to project their dendrites into the same bristle shaft (C+D). G-gamma, a small G-protein expressed in labellar taste neurons (Ueno et al., 2006),**

and thought to be involved in cellular signaling, is expressed in both the larger neurons that express *Gr66a/Gr22e* and in smaller neurons that express *Gr5a*, further indication that two populations of taste neurons exist.

To investigate co-expression with the other *Gr* genes, we combined the *p[Gr22e]-Gal4* and *p[Gr5a]-Gal4* drivers within single flies. Due to the relatively large number and the high density of *Gr5a*-expressing cells (especially within clusters), cell counts were not sufficiently accurate, and we therefore used the difference in cell size as a criterion for co-expression. Analysis of each optical section from flies expressing *UAS-nucgfp* under the control of both these drivers revealed an average of nine large cells per palp (Figures 10A+D and Figure 11), whereas the same counting procedure applied to sections from flies with only the *p[Gr5a]-Gal4* revealed no large cells (Figure 8I+J; Figure 10B). Approximately nine large cells were also found in palps of flies with only the *p[Gr22e]-Gal4* driver (Figures 8C and 10C), strongly suggesting that at least these large cells do not express *Gr5a.* By extension, *Gr22b* and *Gr28b.e* expression is to a large extent also distinct from *Gr5a* expression, and *Gr32a, Gr22f*, and *Gr59b*, which appeared to be expressed exclusively in large neurons (Figure 8E,G, and H), may not be co-expressed with *Gr5a*. However, we cannot exclude the possibility that the few small cells that express some of these genes and about half of the (small) neurons expressing *Gr66a* also express *Gr5a*.

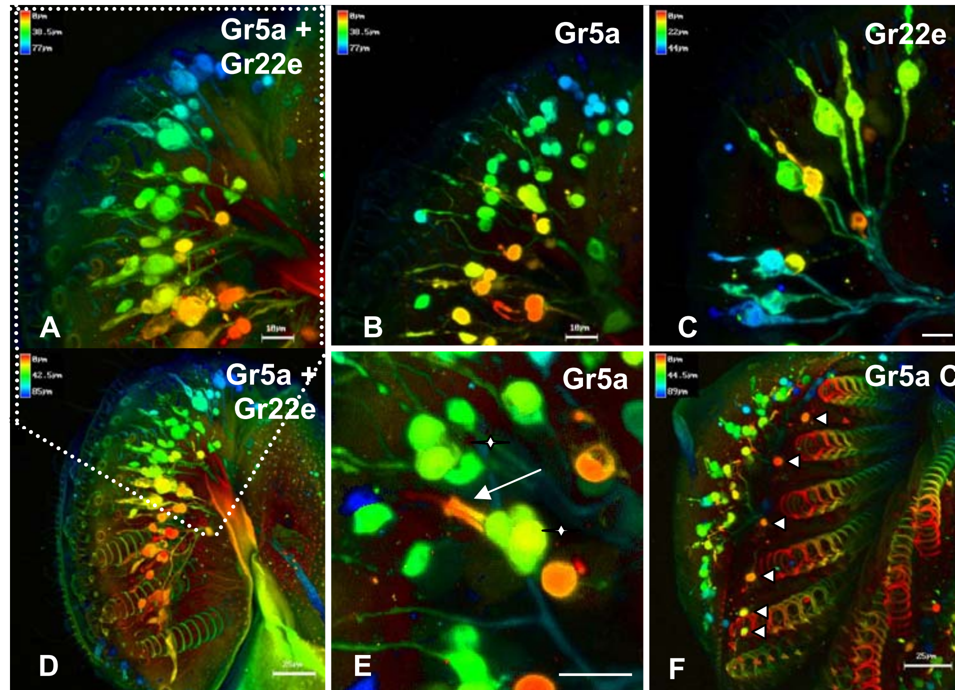
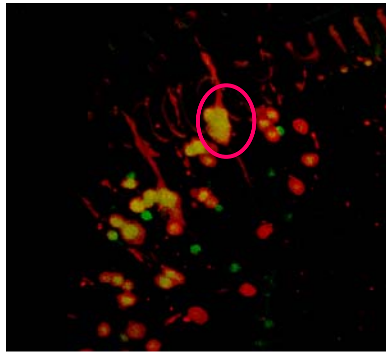
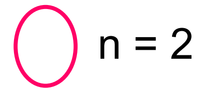


Figure 10: *Gr5a* is expressed in a largely nonoverlapping set of neurons in the labellum. (A-D) *Gr5a* is expressed in labellar neurons with cell bodies that appear signifcantly smaller than neurons that express *Gr22e* (C). This is especially obvious when comparing the single driver lines (B+C) to the double driver line (*p[Gr5a]-Gal4/UAS-nucgfp; p[Gr22e]-Gal/+)* (A+D). (E) Clusters of neurons expressing *Gr5a* (stars) extend their dendrites into the same bristle shaft. (F) *Gr5a* is also expressed in neurons associated with taste pegs, as indicated by arrowheads. Scale bars in (C+E) indicate 10µm.

We note that the expression of another Gal4 driver for the *Gr5a* gene, which contained significantly more upstream sequence, including a neighboring gene (*p[Gr5a_C]-Gal4*), was very similar to the expression seen for our *p[Gr5a]-Gal4* driver.

This driver was shown to be expressed in cells of the labellum, as might be expected for a genuine sugar receptor (Chyb et al., 2003). Direct comparison of the two drivers showed that they exhibited very similar expression profiles, the only difference being the somewhat lower cell count for *p[Gr5a_C]-Gal4* (Figure 9B+E and Table 2). In summary, these studies show that *Gr5a* is expressed in a different pattern than any of the previously analyzed *Gr* genes.




n = 2

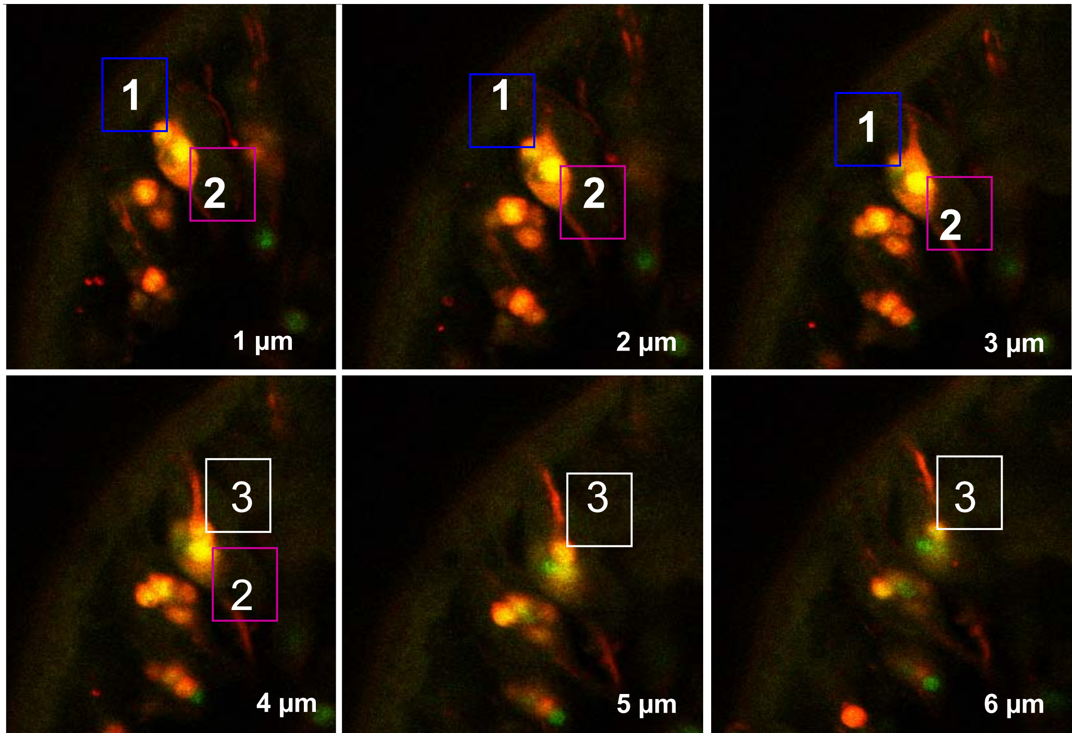

1
2
1 µm
1
2
2 µm
1
2
3 µm
3
2
4 µm
3
5 µm
3
6 µm

Figure 11: Sectioning through labellum of a *Gr5a*/*Gr22e* double driver line shows expression in both large (#2) and small neurons (#1+#3). In order to obtain accurate counts of the number of labellar neurons per palp, individual sections from a confocal stack were used for counting. What appears as only two neurons by the z-stack (top panel) actually is three neurons when counting section by section (in 1μm increments; other panels).

2.2.4 *Gr66a-* and *Gr5a*-expressing neurons mediate different taste perceptions

The expression analysis presented above raises the question as to whether the *Gr22e* group of genes mediate a distinct taste modality as compared to that expected for neuorns expressing *Gr5a*. Since Gr5a has been shown to encode a receptor for the sugar trehalose, we expect that inactivation of GRNs expressing this gene would result in reduction of sensitivity for trehalose. However, we would also predict that inactivation of GRNs expressing *Gr66a* or other *Gr* genes would not lead to any change in the perception for this sugar.

To investigate the function of these different subsets of neurons, we combined Gal4 drivers expressed in the two distinct sets of neurons with a UAS-tetanus toxin light chain (TNT) reporter. TNT specifically cleaves synaptobrevin, a protein essential for neurotransmitter release (DiAntonio et al., 1993), thereby rendering a TNT-expressing neuron functionally inactive. In combination with the Gal4 system, TNT has been used for inactivation of neurons both in the peripheral and central nervous system of *Drosophila* (Bray and Amrein, 2003; Heimbeck et al., 1999; Martin et al., 2002; Shanbhag and Singh, 1992; Sweeney et al., 1995). To control for genetic background and effects of

potential, *Gal4*-independent TNT expression, we used flies that contained the *UAS-tnt* reporter as controls. The effect on taste perception in these flies was measured using the two-choice feeding assay (Tanimura et al., 1982). The feeding preference is represented by preference index (PI), whereby a PI of 0 and 1 indicate complete feeding preference for one or the other substrate, respectively, and a PI of 0.5 indicates no (or equal) preference for either of the substrates (for details, see Experimental Procedure this Chapter and Figure 12).

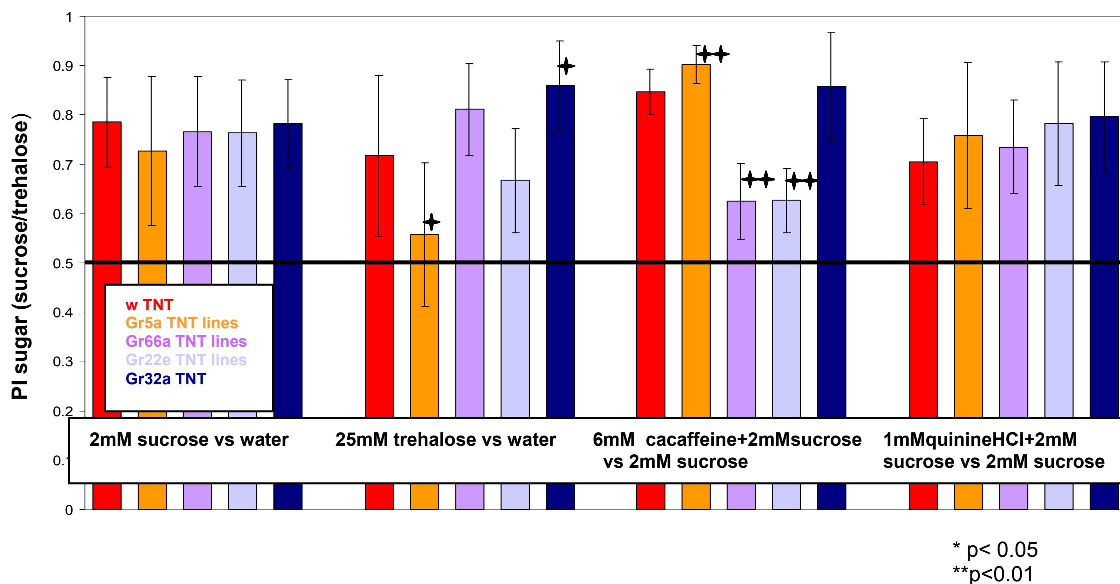


Figure 12: Trehalose and caffeine sensitivity is mediated by neurons expressing different *Gr* genes. A preference index (PI) of 0.5 indicates no preference, whereas a PI of 1 indicates absolute preferencefor the tested substrate. Our *p[Gr5a]-Gal4;UAS-tnt* lines are significantly less sensitive to 25mM trehalose compared to control flies. Both the *p[Gr66a]-* and *[Gr22e]-Gal4;UAS-tnt* lines have significantly reduced sensitivity to

6mM caffeine. No signficant difference in any assay was observed between different lines from the same construct; therefore, these experiments were pooled. A total of at least five different feeding assays were carried out for each line.

As expected, flies expressing *UAS-tnt* under the control of *p[Gr5a]-Gal4* showed a significant reduction in their preference for 25 mM trehalose containing agarose, when compared to control flies or flies with inactivated *Gr66a-*, *Gr22e-* or *Gr32a*-expressing neurons (Figure 12). We also tested these flies for the perception of sucrose. At the minimal concentration where this sugar is reliably detected (2mM), all flies, including those expressing TNT under the control of the *p[Gr5a]-Gal4* driver, continued to show a strong preference for the sucrose containing substrate. Thus, our behavioral assays indicate that *Gr5a*-expressing neurons mediate high sensitivity to trehalose but not sucrose. Second, neurons expressing *Gr66a*, *Gr22e* and *Gr32a* (and probably all other receptors we characterized; see above) are not necessary for detection of either sugar at the concentrations tested.

What then is the role of *Gr66a*-expressing neurons? Based on the difference in expression of *Gr66a* and *Gr5a* and the distinct projection patterns of these neurons in the brain (see below), these two sets of neurons may be dedicated to the perception of distinct taste qualities. In order to investigate this possibility, we tested flies to chemicals known to deter feeding in insects, such as caffeine, denatonium benzoate, quinine and berberine (Glendinning et al., 2001; Glendinning et al., 2002; Meunier et al., 2003; Tompkins et al., 1979). To promote feeding, we used an agarose-based medium that

contained 2mM sucrose and added the bitter compound to half of the feeding wells at the lowest possible concentration that provoked a reliable avoidance response (see Experimental Procedures this chapter). When control flies or flies lacking functional *Gr5a*- or *Gr32a*-expressing neurons were tested in this assay, profound avoidance of all tested compounds was observed. When flies lacking functional *Gr66a*- or *Gr22e*-expressing neurons were tested with the same substrates, caffeine failed to be avoided by these flies (Figure 12). These flies, however, continued to respond normally to other bitter compounds. Thus, these experiments suggest the 14 GRNs expressing *Gr22e*, or a subset of neurons within this group, are required for the detection of low concentrations of caffeine.

Our behavioral studies lead us to propose a model in which distinct subsets of *Gr66a*- and of *Gr5a*-expressing neurons mediate sensitivity to chemicals that result in two distinct behaviors: avoidance of caffeine that is perceived as bitter by humans, and attraction to a major food substrate, trehalose, a sweet tastant.

2.2.5 Axon projections of GRNs expressing specific *Gr* genes

The primary taste processing centers in the fly brain are the subesophageal ganglion and the tritocerebrum which receive direct input from all GRNs in the labellum, the pharynx and some GRNs in the legs (Edgecomb and Murdock, 1992; Lienhard and Stocker, 1987; Murphey et al., 1989; Nayak and Singh, 1983; Rajashekhar and Singh, 1994; Shanbhag and Singh, 1992; Stocker and Schorderet, 1981). Retrograde

labeling experiments of neurons associated with single labellar bristles have revealed a complex arborization pattern of axon termini in the *Drosophila* brain (Edgecomb and Murdock, 1992; Lienhard and Stocker, 1987; Murphey et al., 1989; Nayak and Singh, 1983; Rajashekhar and Singh, 1994; Shanbhag and Singh, 1992; Stocker and Schorderet, 1981). These investigations have also established that axons of chemosensory neurons located in the labellum project through the labial nerve (LN) into the SOG. In contrast, chemosensory neurons located in the pharynx send their axons to the tritocerebrum via the accessory pharyngeal nerve (APN) or the pharyngeal nerve (PN; Figure 7A).

How, then, is the activation of sensory neurons expressing *Gr5a* or *Gr66a* translated into distinct behavioral responses? It is obvious that a better understanding of this problem requires the correlation of receptor activation in the sensory epithelium (for example, the labellum) with specific processing centers (SOG/tritocerebrum) that are activated by the encounter of a specific taste stimulus. Similar studies have greatly advanced our knowledge of olfactory perception both in mammalian and insect model systems (Buck, 1996; Gao et al., 2000; Mombaerts et al., 1996a; Vosshall et al., 2000; Wang et al., 1998). The identification of neurons with a role in either promoting or preventing food intake allowed us to investigate this question in the fly taste system.

As a first step towards the creation of a functional taste map, we visualized axon targets of neurons expressing the *Gr* genes by combining the various Gal4 drivers with reporters encoding neuronal synaptobrevin-GFP fusion protein (nSYB-GFP), which

preferentially localizes to the synapse (Estes et al., 2000). The projection patterns of the eight *Gr* genes described here can be divided into three classes (I, II and III; Figure 13). The Class I projection pattern (*Gr22e, Gr66a, Gr22b, Gr32a,* and *Gr28be*) is characterized by afferents of the pharyngeal nerves terminating in the dorsolateral region of the tritocerebrum, with additional fibers derived from the labral nerves extending their axons towards the adjacent region in the SOG. A significant number of fibers derived from the labral nerve cross the midline medially in the SOG (Figure 13A and Figure 14). Nayak and Singh (1985) have characterized seven sensory neuron afferent types that enter the SOG. Using their descriptions as a guide, we believe that the Class I projection pattern is composed of Type-I, -IV, and –VI fibers, along with input from the pharyngeal nerves. Interestingly, we found that many of these Class I projection patterns in the SOG/tritocerebrum received significant input from ascending afferents from sensory neurons in the legs. Moreover, leg afferents expressing the Gr32a receptor appear to terminate in the protocerebrum posterior to the antennal lobe (Figure 16).

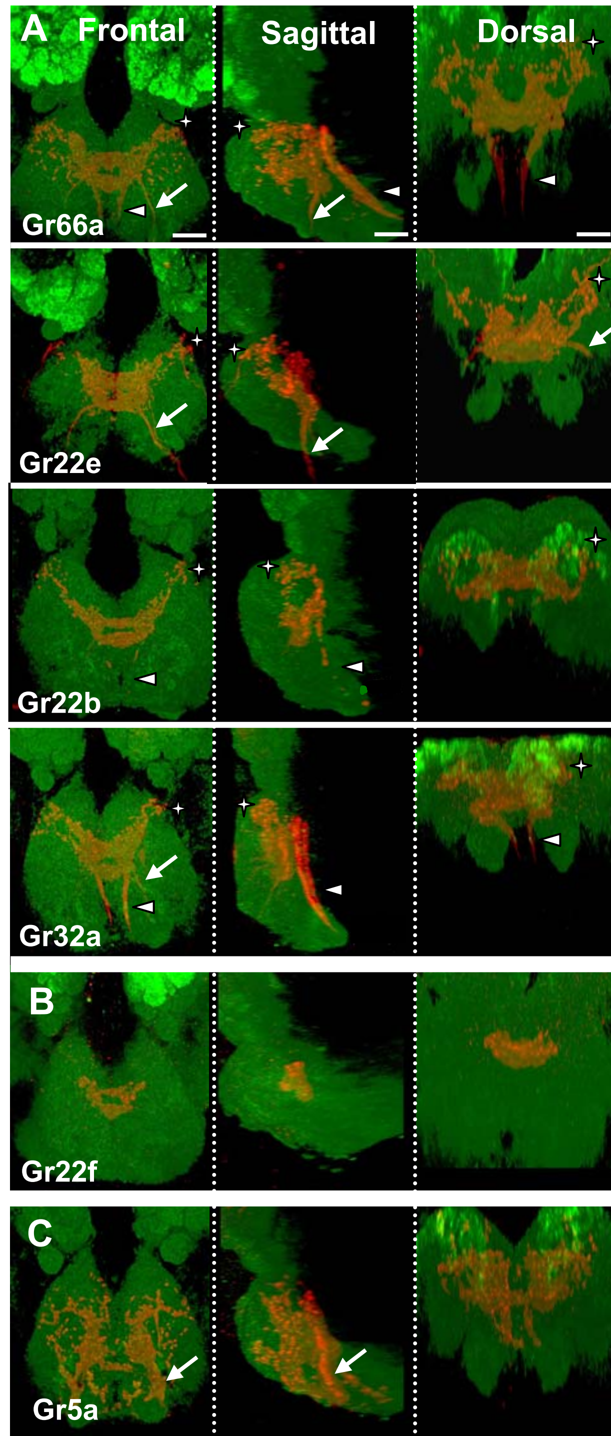

A
Frontal
Sagittal
Dorsal
Gr66a
Gr22e
Gr22b
Gr32a
B
Gr22f
C
Gr5a

Figure 13: Distinct axonal projection patterns in the brain of GRNs expressing different *Gr* genes. The axon termini of taste neurons from the labellum and pharynx expresssing a given *Gr* were visualized by using a *n-Synaptobrevin-GFP* reporter. All images are confocal stacks of whole-mount brains stained with anti-GFP (red) to visualize neuropil. All images are of identical magnification, with the scale bar equal to 25μm. The labellar input (arrow) and ascending afferent from the legs (arrowhead) is often strongly labeled. The pharyngeal input via the APN/PN is not visible, but terminations can be seen dorsolaterally (star).

Gr22f and *Gr59b* are expressed in a subset of *Gr22e*– expressing neurons of the labellum (Figure 8F, Figure 14, and Table 2) and are not expressed in pharyngeal sensilla, producing a "Class II" projection pattern (Figure 13B and Figure 14). The main difference between Class I and Class II projection patterns is a complete lack of anterolateral terminals derived from the pharyngeal input. In addition, fewer terminations are found in the SOG, which is expected based on the lower number of labellar neurons expressing these two genes. The Class II projection pattern is composed of the same labellum-derived afferent types as Class I – Types-I, -IV, and –VI, but lack the pharyngeal input.

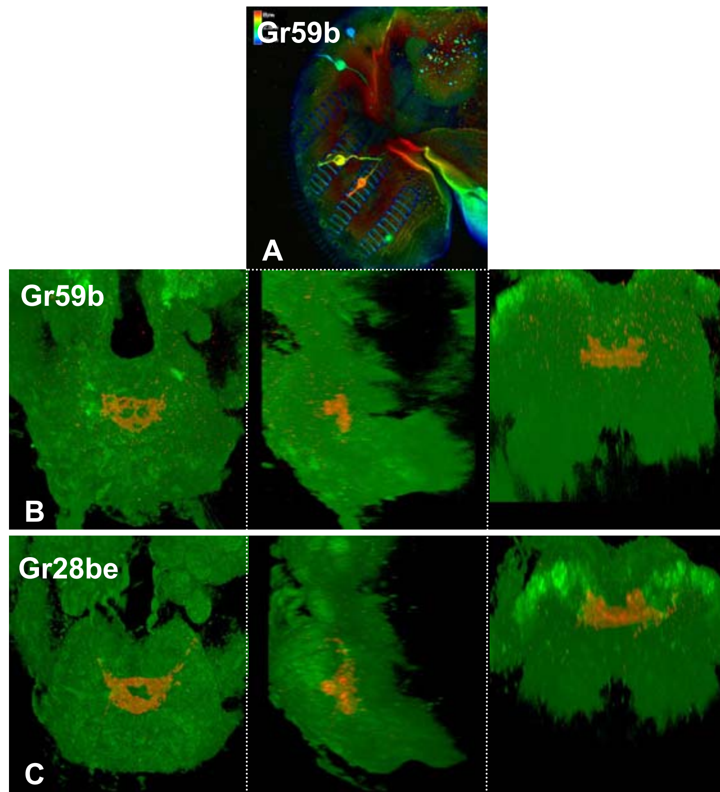


Figure 14: Expression of *Gr59b* in the labellum (A), and axonal projections in the SOG of *Gr59b*- and *Gr28be*-expressing neurons.

The last projection pattern type, Class III, is seen for only a single receptor, *Gr5a* (Figure 13C), and is entirely different from the Class I and II patterns. This observation further supports the conclusion from our co-expression analysis, which suggested that *Gr5a* is largely expressed in a distinct set of neurons. With peripheral head expression restricted to the labial palps, there is no pharyngeal component to the projection pattern. Labial nerve input appears to consist of mainly Type-II fibers.

Because the projection patterns are a composite of axon terminations from neurons of the labellum, pharynx, and forelegs that express a given receptor, it is not possible in these experiments to unequivocally determine which terminations arise from which neurons. Ablation experiments of labial palps and legs were performed to clarify this issue, yet some ambiguity remains.

2.2.6 Unilateral versus bilateral projections of labellar GRNs

Class I and II patterns originate from gustatory neurons of the labellum and have a significant number of medial fibers that project contralaterally (Figure 13A and 13B). In comparison, Class III fibers, which also originate exclusively from labellar neurons, lack this dense medial stain (Figure 13C). This observation suggests that projections from *Gr5a*-expressing neurons on the left or right labial palp terminate preferentially on the left or right side in the SOG, respectively, whereas labellar neurons expressing any of the other genes examined bifurcate and send axons both ipsi- and contralaterally, resulting in the intense medial stain.

To test this hypothesis, we surgically ablated the right labial palp, effectively killing sensory neurons of this palp (for details, see Experimental Procedure this chapter). Two weeks after surgery, anti-GFP antibody staining of flies expressing *UAS-nSyb-GFP* under the control of Gal4 drivers of *Gr66a, Gr22b* and *Gr5a* revealed the absence of staining of the right, but not left, labral nerve, indicating that the majority of sensory neurons of the right palp were killed (Figure 15). In *p[Gr66a]-Gal4* and *p[Gr22b]-*

Gal4 flies, no obvious difference in density of terminations was seen in the two halves of the SOG (Figure 15B and 15C). This finding supports the idea that labellar neurons generating Class I staining patterns in the SOG have similar numbers of termini in the ipsi- and contralateral side of the SOG (Nayak and Singh, 1985).

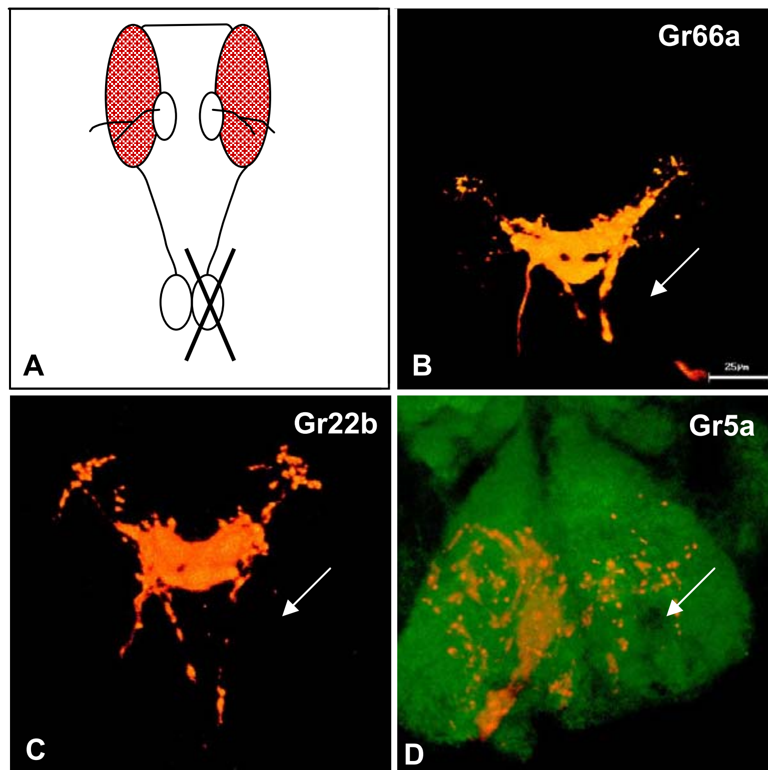


Figure 15: Distinct ipsilateral and contralateral contribution of gustatory receptor neurons expressing *Gr5a, Gr66a,* or *Gr22b*. The right labial palp was removed by mechanical ablation. Two weeks after ablation, the brains of flies of *p[Gr]-Gal4;UAS-nsybGFP* were stained in order to determine what effect loss of the LN had on the projection pattern for a *Gr*. Loss of the LN was evident in all cases (arrows in (B+D). (B+C) For *Gr66a* and *Gr22b*, no decrease in axon terminations was observed on the side of the SOG no longer directly innervated by the LN. (D) In the case of *Gr5a*, ablation of one LN resulted in a significant reduction in the number of termini seen in the corresponding half of the SOG.

Labial palp ablation in *p[Gr5a]-Gal4* flies revealed a different result. Lack of innervation by the right LN resulted in a significant reduction in terminations of *Gr5a* expressing neurons in the right half of the SOG (Figure 15D). The simplest interpretation of this result is that axons of *Gr5a*-expressing neurons from the left labial palp extend branches that terminate mostly in the left half of the SOG, a notion that is consistent with the comparatively small number of medial fibers (Figure 13C).

2.2.7 Ascending afferents from sensory neurons of the legs directly innvervate the SOG

In order to confirm the identity of afferents from taste neurons of the legs, we amputated one or two legs from adult flies, allowed time for deafferentiation, then dissected out both the CNS and thoracic-abdominal ganglion. We found that taste neurons of the forelegs that express *Gr66a* project afferents all the way to the SOG, and that removal of the right first leg causes significant dimunition in staining of the right ascending afferent into the SOG (Figure 16B-E). A slightly different result was found for taste neurons of the leg that express *Gr32a*. Unlike *Gr66a* these afferents do not contribute to the projection pattern in the SOG, but instead project dorsally past the SOG to terminate behind the antennal lobes (Figure 16G; asterisk). Upon amputation of the first and second right legs, a slight diminution in staining of the right ascending nerve was seen in the brain (arrow, Figure 16G), and the fibers from the first and second legs are not seen in the thoracic-abdominal ganglion (arrows, Figure 16H). Amputation of

the first or second right leg alone did not produce a significant decrease in staining of the right afferent, however the corresponding leg fibers in the thoracic-abdominal ganglion were always missing (Figure 16I, for example).

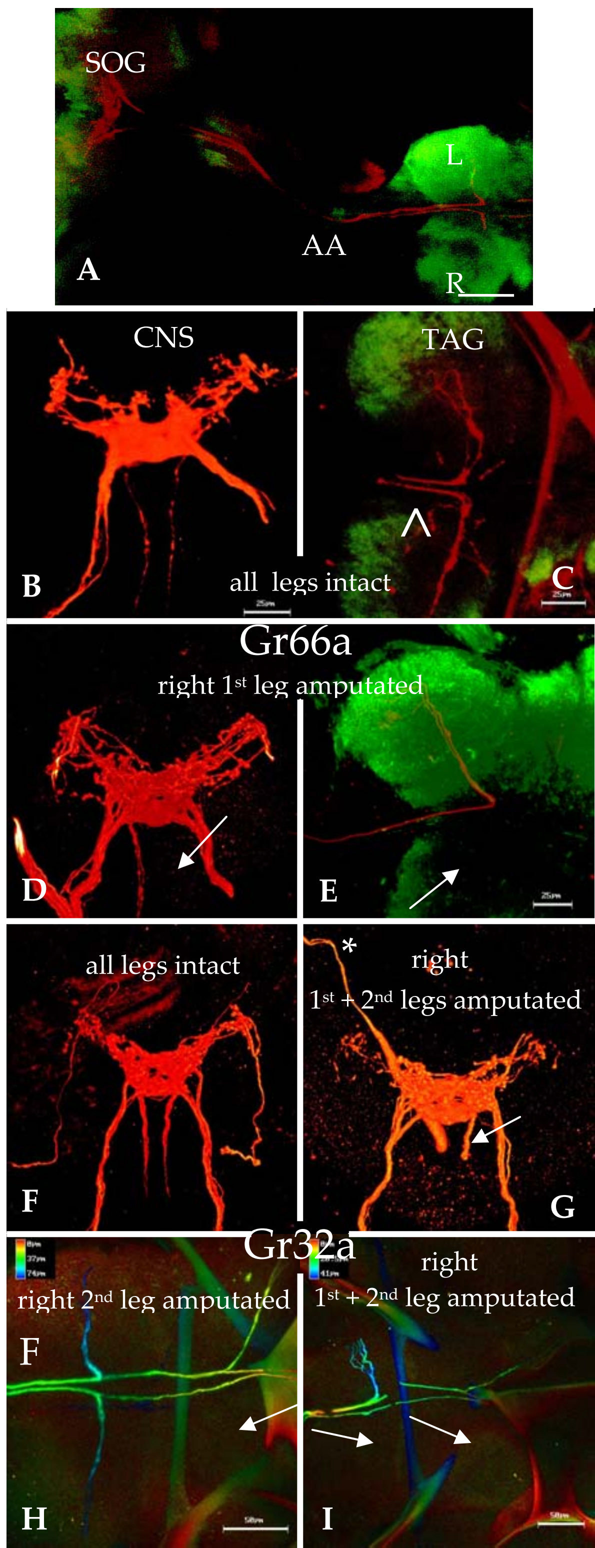

SOG
L
AA
A
R
CNS
TAG
B
all legs intact
C
Gr66a
right 1st leg amputated
D
E
all legs intact
*
right
1st + 2nd legs amputated
F
G
Gr32a
right 2nd leg amputated
right
1st + 2nd leg amputated
F
H
I

Figure 16: Taste neurons of the legs that express *Gr66a* project to the SOG (B-E), whereas neurons of the legs that express *Gr32a* project beyond the SOG to a location in the protocerebrum (F-I). When the right 1st and 2nd legs are surgically removed, only the afferent from the remaining legs remains (star, G).

These results indicated that *Gr66a* is expressed in taste neurons of the leg whose afferents terminate in the SOG, and likely provide gustatory information. Taste neurons of the leg that express *Gr32a* project their afferents elsewhere in the CNS, indicating that these neurons may have a role other than in tastant detection.

2.3 Discussion

A crucial determinant for discerning chemical cues present in the environment is embedded in the peripheral expression pattern of cell surface receptors in sensory epithelia. In the olfactory systems of Drosophila and mice, each olfactory receptor neuron expresses only one of 60 or one of approximately 1000 *Or* genes, respectively, enabling these animals to discriminate between hundreds or thousands of different odors (Buck and Axel, 1991; Clyne et al., 1999; Vosshall et al., 1999). In contrast, taste cells of the tongue allow mammals to distinguish only a few taste qualities: bitter, sweet, umami, salty and acidic taste (Lindemann, 1996). Lack of discrimination between the hundreds of diverse chemical compounds – all perceived as bitter – is thought to be caused by co-expression of the approximately 40 T2R receptors in a single set of taste cells (Chandrashekar et al., 2000; Zhang et al., 2003). Therefore, activation of the bitter taste cells by any one of the T2Rs is likely to generate a single activation pattern in taste

centers of the brain, leading to a similar, repulsive behavioral output. Associating primary taste centers in the mammalian brain with specific taste modalities has, as of yet, proved challenging.

Insect taste is still rather poorly understood, especially at the molecular level. *Drosophila melanogaster*, which exhibits remarkably similar taste preferences with humans, is the only insect for which candidate receptors have been characterized experimentally. The investigations presented here provide significant new insights into insect taste perception.

2.3.1 Gustatory neurons mediating avoidance behavior are defined by subtle differences in the *Gr* gene code

Initial expression studies (Dunipace et al., 2001; Scott et al., 2001) suggested that the fly gustatory receptors are not simply co-expressed in three sets of cells dedicated to bitter, sweet, and umami taste like the T2Rs, T1R2/T1R3 and T1R1/T1R3 receptors of mammals. Instead, these experiments suggested that they either are expressed according to the one receptor : one neuron hypothesis well-established for insect and mammalian olfactory systems, or they are expressed in partially overlapping sets of neurons. Our analysis supports the latter of these possibilities. We found that most labellar *Gr* genes (seven out of eight) are expressed in a single neuron of mostly S– and some I- type bristles. Most interestingly, our co-expression studies provide evidence that individual neurons express anywhere from one to six receptors (Figure 8K to 8O and Table 2). In

this way, S-bristle-associated neurons are defined by unique receptor gene codes, thereby outfitting the labellum with an array of sensory assemblies that may exhibit distinct - albeit overlapping - ligand specificities (Figure 17).

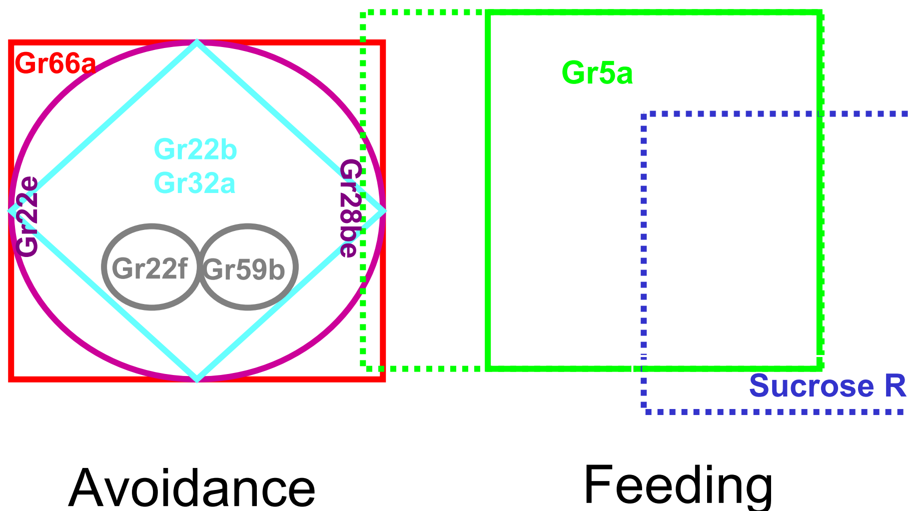


Figure 17: Model of taste coding in the labellum. Representation of coexpression and exclusive expression of *Gr* genes analyzed in this study. Most neurons that express *Gr22b, Gr32a, Gr28be, Gr22f,* and *Gr59b* also express *Gr66a* and *Gr22e.* The labellar neurons that express *Gr5a* define a discrete subset of taste neurons of the labellum. However, partially overlapping set(s) of neurons may express other sugar receptors (for example, a receptor for sucrose; blue-dotted square). Note that our analysis cannot exclude the possibility that a few (small) neurons expressing the other *Gr*s also express *Gr5a* (green-dotted square).

The functional implications of distinct neuronal receptor codes on taste perception are currently unclear and will require analysis of mutations of individual *Gr* genes. However, a general role for these neurons in feeding inhibition (“avoidance neurons”) can be inferred from experiments presented in this paper and supported through analogy with the mammalian taste system/receptors. First, avoidance neurons express the majority of analyzed Gr genes – and by extension – the majority of the genes

in the entire *Gr* gene family. In mammals, bitter taste receptors far outnumber the sweet taste receptors (40 : 3). Second, avoidance neurons associated with S-type bristles do not express the receptors for the sugar trehalose encoded by the *Gr5a* gene. In fact, avoidance neurons associated with S-type bristles have a distinct appearance compared to neurons expressing the *Gr5a* gene. In mammals, the sweet/umami taste receptors and the bitter taste receptors are expressed in distinct group of cells (Chandrashekar et al., 2000; Zhang et al., 2003). Third, inactivation of avoidance neurons has no effect on sucrose or trehalose sensitivity in flies, but significantly reduces their sensitivity to caffeine. Fourth, avoidance neurons and *Gr5a*-expressing neurons have distinct targets in the SOG, a feature consistent with the detection of different taste qualities by these neurons.

If the avoidance neurons have a general function in the detection of toxic or otherwise undesirable chemicals, what is the rationale for a complex and distinct *Gr* gene code among different groups of such neurons? We propose that the receptor code allows a fly to discriminate among different chemicals, which are in general avoided, but might have distinct consequences on their health if ingested. According to such a proposal, a fly encountering a food source rich in nutrients (sugars) but contaminated with toxic chemicals, may choose between feeding and avoidance, depending on the impact the particular toxic compound may have on its health. There is indirect evidence from feeding studies in *Maduca sexta* larvae that discrimination between the bitter

substrates caffeine and aristolochic acid does occur in insects, even though actual taste preference, adaptation, or both may contribute to this phenomenon (Glendinning et al., 2001). Thus, discrimination among toxic/bitter tasting compounds might be possible in insects including *Drosophila*.

It was somewhat surprising that the sensitivity to other compounds known to be avoided by insects – denatonium benzoate, quinine hydrochloride and berberine – was not affected in animals lacking Gr66a-expressing neurons. This may simply be explained by the presence of additional neurons expressing receptors that recognize these particular substrates. Alternatively, one or a few neurons co-expressing *Gr66a* along with a receptor for one (of these) ligand(s) might not have been completely inactivated by TNT. Finally, studies in rodents indicate that caffeine may directly affect neurons in the brain, circumventing activation of taste cells altogether (Antle et al., 2001; Frank et al., 2004; Fredholm, 1995; Fredholm et al., 1999). This is not likely to be the case in our experiments, because none of the *Gr* genes examined is expressed in the CNS.

Relatively few studies have investigated bitter taste sensitivity in insects, or *Drosophila*, in particular (Glendinning et al., 2001; Glendinning et al., 2002; Tompkins et al., 1979). Electrophsiological studies have identified bristles in the legs, but not the labellum of Drosophila, that respond to bitter-tasting chemicals (Meunier et al., 2003). However, S-type sensilla are notoriously difficult to record from, because their bristles are extremely difficult to access for this type of experiment (Hiroi et al., 2002).

2.3.2 *Gr5a*-expressing GRNs mediate sweet taste perception

Gr5a-expressing neurons represent more than half of chemosensory cells in the labellum, and appear to be associated with all sensilla types, including the taste pegs. In fact, association of *Gr5a* with taste pegs provides the best evidence yet that these sensilla have a specific chemosensory function in the detection of trehalose. Significantly, *Gr5a*-expressing neurons define a largely distinct set of neurons from the avoidance neurons (Compare Figures 8A to 8H with Figures 8I and 8J; see figures 10A and 10D). This observation is consistent with our results from behavioral investigations of flies lacking the function of specific sets of neurons. Specifically, inactivation of *Gr5a*-expressing neurons leads to a reduction in trehalose sensitivity, but the sensitivity to any bitter substrate tested was unaffected (Figure 12). These flies did not exhibit reduced sucrose sensitivity, another nutrient-relevant sugar for *Drosophila* (Figure 12). This result is somewhat unexpected, as electrophysiological investigations have led to the proposal that a single neuron in L-, I- and S-bristles is responsive to several sugars including trehalose and sucrose (the "sugar" neuron) (Dahanukar et al., 2001; Dethier and Goldrich-Rachman, 1976; Hiroi et al., 2002; Rodrigues and Siddiqi, 1981). According to these studies, sugar neurons may express a single, broadly tuned sugar receptor or – more likely - they may co-express several distinct sugar receptors, each of which recognizes a specific sugar (i.e. sucrose, trehalose, fructose, etc). This latter possibility is favored from genetic studies, which have shown that mutation in the *Gr5a* gene reduces

the sensitivity of flies to trehalose, but not to sucrose (Dahanukar et al., 2001; Tanimura et al., 1982; Ueno et al., 2001). However, the proposition of a single sugar neuron per bristle is also not consistent with our expression studies, which showed that two to three neurons within a bristle can express *Gr5a* (Figures 9 and 10E). We cannot exclude the possibility that the *p[Gr5a]-Gal4* drivers do not represent endogenous *Gr5a* expression, but we think this is unlikely to be the case for two reasons. First, several lines with our *p[Gr5a]-Gal4* show the same expression, and second, the *p[Gr5a_C]-Gal4* driver containing a much larger promoter fragment produces a similar expression profile, with many clusters of *Gr5a*-expressing neurons associated with the same bristle (Figure 9).

In order to realign the electrophysiological data with our expression analysis, we propose another explanation: the "sugar neuron" identified in electrophysiological studies expresses many (possibly all) distinct sugar receptors, including GR5a. However, one or two additional neurons per bristle express only a fraction, or possibly just one, of all sugar receptors present in the sugar neuron (Figure 18). Worth noting in this context is the fact that electrophysiological recordings are carried out at significantly higher substrate concentrations (up to 100 mM for sucrose and trehalose) than our behavioral experiments (2mM for sucrose and 25 mM for trehalose). Our model is also more consistent with recent experiments that noted different electrophysiological sugar responses among labellar sensilla (Hiroi et al., 2002).

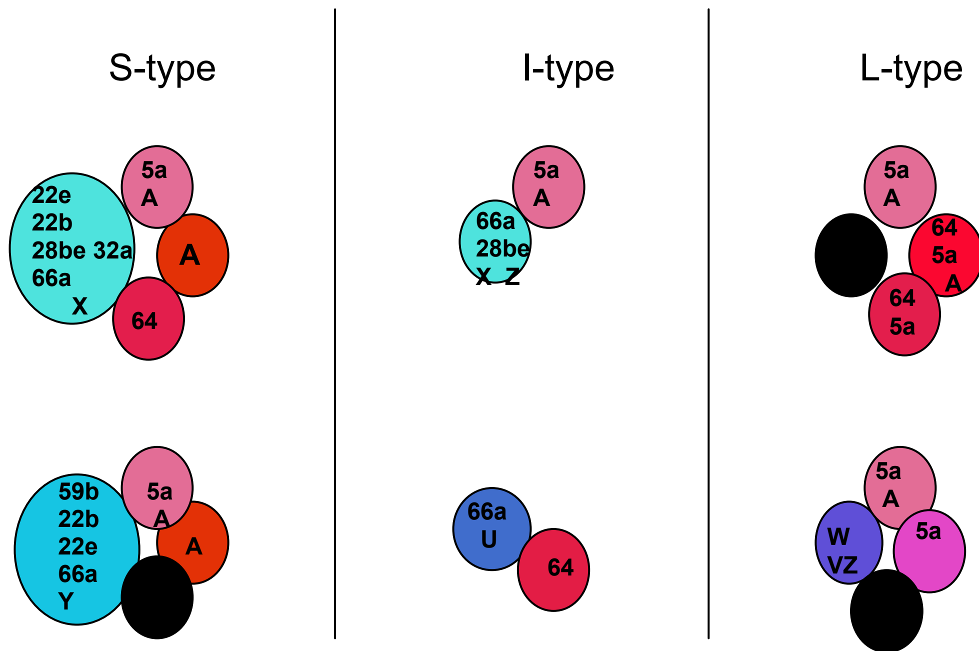


Figure 18: Representation of avoidance (blue hues) and feeding (red hues) neurons in S, I, and L-type taste bristles of the labellum. Different (sets of) avoidance and feeding neurons express different combinations of cognate receptors. Receptors for additional sugars and possible amino acids (Gr64, A) in feeding neurons and other bitter receptors (U-Z) expressed in avoidance neurons of all bristle types are proposed but have not been identified. Some neurons (black) might be dedicated to salt and/or sour perception.

Approximately 45 labellar neurons have not yet been associated with any *Gr* gene and some of these neurons might express putative candidate receptors for sucrose or additional sugars. These genes are likely to be encoded by members of the *Gr64* gene cluster, which share much higher sequence similarity with *Gr5a* than any other *Gr* genes (Dahanukar et al., 2001; Dunipace et al., 2001; Robertson et al., 2003; Scott et al., 2001; Ueno et al., 2001). *Gal4* drivers for two of these genes (*Gr64a* and *Gr64e*) were analyzed and found to be expressed in the pharyngeal taste organs, but not in the labellum (N.T. and H.A., unpublished observations). Whether these two receptors are indeed involved

in sugar detection remains to be seen, but we would predict that other *Gr* genes for sugars like sucrose and fructose would be expressed more broadly and in taste neurons of labellar bristles and pegs.

In summary, our expression and behavioral studies suggest two fundamentally different roles for neurons expressing non-overlapping groups of *Gr* genes (Figure 18) in the detection of substrates that lead to feeding or avoidance behavior. According to this new model, S- and I-bristles on the labial palps contain one avoidance neuron and one or more feeding neurons (depending on the number of neurons associated with the particular bristle). The avoidance neuron expresses multiple *Gr* genes, and avoidance neurons of different bristles express these *Gr* genes in different combinations (the *Gr* gene code). The feeding neurons, which appear morphologically smaller than the avoidance neurons, express an entirely different set of receptors that includes *Gr5a*, and possibly *Gr* genes encoding receptors for other sugars, amino acids and peptides.

2.3.3 A taste map in the brain

The different functions for GRNs expressing *Gr66a* and *Gr5a* are also supported by their different projection patterns in the brain. Neurons expressing *Gr66a*, or any of the partially co-expressed receptors, target similar regions in the SOG/tritocerebrum, though the number of termini differs significantly depending on the number of peripheral sensory neurons the *Gr* is expressed in (Figures 13A and 13B). For example, *Gr66a*-expressing neurons show a robust array of termini in the SOG/tritocerebrum,

whereas the termini of *Gr59b* and *Gr22f*-expressing neurons are significantly less numerous. In all cases, dense contralaterally projecting fibers provide extensive innervation of both halves of the SOG by labellar neurons, as demonstrated by labial palp ablation experiments (Figure 15).

An entirely different projection pattern is observed for feeding neurons that express *Gr5a*. Most strikingly, the axon termini of these neurons are distributed over a very large area of the SOG and extend into regions not innervated by avoidance neurons. A second striking difference is the poorly established contralateral connective between the two halves of the SOG, suggesting that neurons located in the right labial palp preferentially terminate in the right half of the SOG. This idea was tested and confirmed through ablation studies (Figure 15) and has interesting implications, namely that spatially restricted activation of neurons in one palp will preferentially stimulate the same side of the SOG; this could potentially allow for spatial discrimination of taste input in the brain. This feature might allow the fly to orient its labellum in the direction of a food source, identifying regions with high concentrations of trehalose or other sugars (Yetman and Pollack, 1987).

The distinct pattern of axon termini in the SOG of neurons required for feeding and avoidance suggests that these behaviors are mediated through different neuronal pathways. Anatomical studies in honeybees have identified second-order neurons that mediate synaptic activity of primary taste neurons to higher brain centers (Schroter and

Menzel, 2003). It will be interesting to see whether second-order neurons contacting synapses of avoidance and feeding neurons define different target regions in these higher brain centers.

2.3.4 Similar logic of taste perception in insects and mammals

Taste is an ancient sense, which exists in bacteria in the form of chemotaxis. Neuroanatomical and molecular comparison of taste systems between mammals and insects imply that this sense has evolved independently in these phyla (Matsunami and Amrein, 2003). In mammals, for example, taste ligands are perceived through sensory epithelial cells in the lingual epithelium of the tongue. These cells then activate secondary neurons that innervate taste centers in the brain. In insects, tastants are detected by primary sensory neurons that directly innervate the CNS. Moreover, insects have multiple taste organs (legs, wings, and in some cases, the female genitalia), for which no counterparts exist in mammals. Finally, sequence comparison of the *Gr* and *T1R*/*T2R* genes has failed to reveal any direct kinship between mammalian and insect taste receptors (Clyne et al., 2000; Dunipace et al., 2001; Scott et al., 2001).

However, a remarkable convergence of anatomical as well as molecular features of gustatory systems between mammals and insects (*Drosophila*) appears to emerge from our studies. The functional taste units, the taste buds in the tongue and the taste bristles of the labellum, are composed of 30 to 100 taste cells and two to four chemosensory neurons, respectively. Individual taste cells in each taste bud are dedicated to the

perception of sweet, umami, or bitter taste sensation, based on the T1R or T2R receptors they express. Similarly, our data indicate that taste bristles of the labellum contain neurons that either respond to repulsive or attractive stimuli, properties that are likely determined by the specific (set of) taste receptors they express (Figure 19).

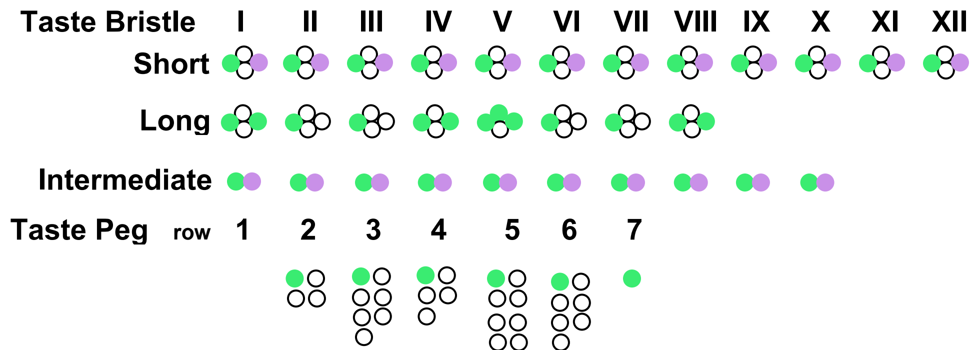


Figure 19: Functional orgaization of taste sensilla in the labial palps according to molecular analysis. Neurons that are sensitive to bitter substances and express at least *Gr22e* and *Gr66a* are shown in distinct shades of purple. The *Gr5a* gene, encoding a receptor for the sugar trehalose, is expressed in a distinct group of neurons, associated with each bristle type, as well as with taste pegs (shown in green).

Despite the sequence divergence of mammalian and insect taste receptors, we believe there are intriguing similarities at the molecular level as well. First, the number of taste receptors in mammals and *Drosophila* is very similar. The eight genes described here probably encode a significant number of the functional labellar taste receptors. Some of the 60 *Gr* genes are likely to encode taste receptors only expressed in the pharynx, legs and wings or might only be expressed in the larva (Dunipace et al., 2001; Scott et al., 2001). Other Gr genes are likely to function as pheromone receptors (Bray and Amrein, 2003), or might recognize internal ligands based on their restricted expression in the

CNS (N.T. and H.A., unpublished data). Considering these alternative functions for some *Gr* genes, we estimate that the fly has about 30 to 45 labellar taste receptors, a number close to the total number of T1Rs and T2Rs (~ 30 in humans and 45 in mice).

In addition to the similar size of the *Gr* and *T1R*/*T2R* gene families, taste receptors of mammals and *Drosophila* fall into similar functional groups. Only three mammalian T1R receptors are thought to be dedicated to the detection of attractive stimuli (sugars and amino acids/proteins), whereas the large majority - the T2Rs - are thought to be exclusively involved in the detection of repulsive (bitter) ligands. If our expression analysis is more or less representative of the entire *Gr* gene family, we might expect that 25 to 40 *Gr* genes will be expressed in the avoidance neurons, whereas just three to six are expected to be expressed in feeding neurons. Identification and analysis of *Gr* genes encoding receptors for known ligands, combined with biochemical analyses, should reveal whether additional molecular features are shared between the GRs and T1Rs and T2Rs, such as whether *Drosophila* also possess a specific receptor for amino acids and whether some receptors also function as multimers, as is proposed for mammalian T1Rs (Zhang et al., 2003; Zhao et al., 2003).

2.4 Acknowledgments

We would like to thank D. Kimbrough and M. Zhang for technical assistance, Drs. K. Scott, L. Vosshall, J. Carlson, the Bloomington and Kyoto stock centers for fly strains and R. Stocker and L. Vosshall for antibodies. We are indebted to Dr. R. Stocker for advice

and help with deciphering the projection patterns of GRNs to the brain. We also wish to thank Dr. H. Matsunami and members of the Amrein lab for suggestions during the course of this project. This work was supported by grants from N.I.H to H.A. (1RO1GM60234-01 and 1RO1GMDC05606-01).

2.5 Experimental Procedures

2.5.1 Genetics/fly strains

Drosophila stocks were raised on standard cornmeal-agar-molasses medium at 25°C. Transgenic Gal4-driver lines for *Gr66a, Gr22e, Gr22f,* and *Gr59b* were generated in a previous study by this lab (Dunipace et al., 2001). The transgenic driver lines for *Gr32a* and *Gr28b.e* were kindly provided by Kristin Scott (Scott et al., 2001). A *p[Gr5aC]-Gal4* line, with 8.5 kb upstream sequence to Gr5a was generously supplied by John Carlson and previously published in Chyb et al. (2003). A second *p[Gr5a]-Gal4* line, which used a 1 kb upstream fragment of *Gr5a* was generated by PCR from genomic DNA, using standard PCR protocol, with primers 5' TGGTACCAAATGCAATAACAATAAAAACGCGC 3' and 5' GGGATTCTAACGATTTGGATAGATTCACCTCG 3'. The PCR fragment was cloned directly into pGEMT then excised with ACC65I/NOT I and cloned into SM1 vector upstream of Gal4.

Lines homozygous for a driver and reporter (for example, *UAS-nucgfp; p[Gr22e]-Gal4*) were made and crossed to a driver line with Gal4 under the control of a different Gr promoter to produce double driver lines of genotype *p[Gr5a]-Gal4/UAS-nucgfp; p[Gr22e]-Gal4/+*. *UAS-tnt* lines were obtained from S. Sweeney.

2.5.2 Immunofluorescence

For all immunofluorescence experiments, expression of two lines for each *Gr*-driver was examined. In all cases, identical expression profiles were seen for both drivers of the same *Gr.*

2.5.2.1 Labellum

P[Gr-promoter]-Gal4 lines were crossed to *UAS-nucgfp* stocks. Progeny were aged for at least four days before dissection. In order to allow sufficient antibody penetration to the tissue, labella were dissected from the rest of the proboscis prior to incubation with primary antibody. Antibody staining was conducted as described by Laissue et al. (1999), except that 5% heat-inactivated goat serum was added to the blocking solution (protocol provided by Leslie Vosshall). Primary antibodies used were rabbit anti-GFP (Molecular Probes; A-6455) at a 1:1000 dilution and mouse anti-ELAV as a neuronal marker (provided by Leslie Vosshall) at a 1:10 dilution. Secondary antibodies used for visualization were goat anti-rabbit Cy3 (Jackson Immunoresearch Laboratories;

111-165-144) at a 1:500 dilution and goat anti-mouse ALEXA 488 (Molecular Probes; A-11017) at a 1:100 dilution.

2.5.2.2 Brains and thoracic-abdominal ganglia

P[Gr-promoter]-Gal4 lines were crossed to either *UAS-n-Synaptobrevin-GFP* flies (Estes et al., 2000) to visualize axon termini or *UAS-mCD8::GFP* flies (obtained from Bloomington Drosophila stock center; 5137) to visualize axonal projections of GRNs. The immunofluorescence protocol followed is similar to that for labellum staining. Primary antibodies used were rabbit anti-GFP (1:1000) and mouse anti-nc82 (kindly provided by R. Stocker), a neuropil marker, at a 1:10 dilution.

2.5.2.3 Confocal microscopy

A Zeiss LSM 410 confocal microscope with a KrAr laser was used for all immunofluorescence imaging. For all optical stacks produced, optical sections were taken at intervals of 1μM. Multi-colored depth-coded images from a single channel which revealed only GFP-expressing neurons were produced using Zeiss LSM software. Adobe Photoshop 6.0 was used to adjust the contrast in images when required. Color spectra were used for depth coding, with red indicating the first optical section (surface) and blue indicating the last (deepest) optical section or vice versa.

2.5.3 Labellar cell counting

Hard copies of all optical stack images were used to obtain reliable cell counts. In the case of *Gr5a*, in which a significant number of small neurons stained, it was necessary to divide each labellum into sections of higher magnification, and a count made for each section. In many cases, we found that the multi-colored depth-coded stacks aided in the visualization of stained neurons, and often these images were used in conjunction with the unaltered red/green images, which allowed us to identify stained cells as neurons due to anti-ELAV staining (green). In all cases at least five to ten well-stained labella were used to determine the average cell count. Additional labella (up to 30) were also viewed to confirm the staining pattern seen. Two driver lines for each Gr were always analyzed.

2.5.4 Feeding-preference assays (FPAs)

In all FPAs, 50-70 male flies aged between six to nine days were used. Again, for all assays, two *Gr* driver lines were tested. Flies were starved for 28 hours at 25°C in vials containing dampened Whatman paper. Flies were allowed to feed in the dark at 25°C for two hours. FPAs were always carried out at the same time of day. Cages with 6X6 well micro plates (Falcon; 353911) were used for the FPA. Wells contained alternating test solutions with red (0.2μg/mL per well sulforhodamine B; Sigma, S-9012) or blue (0.075μg/mL per well erioglaucine; Sigma, A-86,114-6) dye in agarose (Promega; V3121). After the assay, flies were immediately frozen, then sorted and counted based

on the color dye witnessed in their abdomen. The preference index (PI) for a red colored substrate, for example, is calculated as (n_{red} + 1/2n_{purple}) / n_{total}. The closer the PI is to 1, the higher the preference for the red-colored substrate, and the closer the PI to 0, the higher the preference for the blue-colored substrate. A PI of 0.5 indicates no (or equal) preference for the two substrates. Substrate color alone did not appear to affect feeding preference (data not shown). The following substrate combinations were tested: 2mM sucrose vs. dH2O, 25mM trehalose vs. dH2O, 6mM caffeine + 2mM sucrose vs. 2mM sucrose, 0.25mM denatonium benzoate + 2mM sucrose vs. 2mM sucrose, 1mM quinine hydrochloride + 2mM sucrose vs. 2mM sucrose, 1mM berberine + 2mM sucrose vs. 2mM sucrose, 100mM NaCl + 2mM sucrose vs. 2mM sucrose. All chemical compounds were obtained from Sigma.

Averages and standard deviations, and basic student t-tests to determine significance were used to analyze numerical data and to produce graphs (Microsoft Excel 2000).

2.5.5 Ablation of labial palps

The right labial palp of 1 to 3 day old flies was surgically ablated with a wolfram needle under a dissection microscope. Flies were aged for two weeks before their brain was dissected and stained

2.6. Confirmation that Grs are expressed in attractive- or avoidance-type taste neurons

Shortly after our findings indicated that the majority of *Gr*s were co-expressed in taste neurons of the labellum, another group confirmed our findings using a technically sophisticated approach (Wang et al., 2004). We determined co-expression of *Gr*s in labellar neurons by expressing a reporter (*UAS-nucgfp*) under the control of two *Gr*-promoter drivers (for example, *p[Gr22e]-Gal4* and *p[Gr66a]-Gal4*) and counting the number of GFP-positive cells. If the number of positive cells in the double-driver lines was equal to the number of GFP-positive cells in the single-driver line with the most expression, this indicated that the two *Gr*s were expressed in the same cells. We found that *Gr66a*-expressing labellar neurons expressed subsets of the other six receptors examined.

Wang and colleagues also used the Gal4/UAS system to study co-expression of *Gr* genes in taste neurons. They, however, also generated transgenic fly lines that directly expressed GFP under the endogenous *Gr* promoter. Because amplification of the GFP signal was deemed necessary due to low expression of these receptors, the *Gr* promoters drove expression of multiple copies of GFP (for example, *Gr66a-GFP-IRES-GFP-IRES-GFP*). Ultimately, they produced these types of transgenic lines for three *Gr*s: *Gr66a*, *Gr32a*, and *Gr47a*. This then allowed them to make animals that were transgenic both for this construct, as wells as for *p[Gr]-Gal4; UAS-CD2*. Antibody staining using

anti-GFP and anti-CD2 revealed that all the receptors analyzed were co-expressed in subsets of labellar neurons that express *Gr66a*. They also found that *Gr5a* was expressed in labellar neurons distinct from those expressing *Gr66a* and the other receptors. Not surprisingly, they also found that the axonal projection patterns of *Gr66a* and *Gr5a* in the tritocerebrum/SOG were different, similar to our findings.

Whereas we used the two-choice feeding assay to measure taste preferences in flies that lacked *Gr66a*/*Gr22e* and *Gr5a* neurons, Wang and coworkers (2004) used the proboscis extension assay, described in Chapter 1. Additionally, instead of functionally ablating taste neurons using UAS-TNT as we did, they drove expression of Diptheria toxin (DTI) under the control of *Gr* promoters, which actually kills the cell in which it is expressed. They found that ablation of *Gr5a*-expressing neurons decreases the proboscis extenstion frequency not just to trehalose, but to other sugars – sucrose and glucose – as well as low salt, indicating that *Gr5a*-expressing neurons may also express receptors for other sugars. They also found that ablation of *Gr66a*-expressing neurons did not decrease the frequency of proboscis extension, as it does in control animals, to bitter compounds including berberine, caffeine, denatonium, and quinine, indicating that they lacked sensitivity to bitter compounds. These flies had normal responses to sugars. Thus, their results confirmed our own: *Gr5a*-expressing neurons define attractive-type taste neurons, and that *Gr66a*-expressing neurons are avoidance-type neurons.

Although our results largely aligned with what was found by Wang and colleagues (2004), there were two important differences. Whereas we only found reduction in sensitivity to trehalose, but not sucrose, in flies without *Gr5a* neurons, they found that sensitivity to a number of different sugars was affected. Additionally, we found that only caffeine-sensitivity was affected in flies without *Gr66a* neurons, and they found that this decrease in sensitivity extended to other bitter compounds. These differences could be due to the type of behavioral assay used. The proboscis extension assay is a very direct method of testing whether a tastant is detected or not, and relies on a reflex response. The two-choice feeding assay is indirect, allowing the fly to feed and make feeding choices over the course of two hours. We believe the findings of Wang and coworkers are correct: *Gr66a*-expressing neurons mediate sensitivity to many bitter compounds, and *Gr5a*-expressing neurons detect many types of sugars. This makes sense based on the co-expression of many *Gr*s in a single avoidance neuron, which indicates that these neurons likely detect many noxious compounds.

2.7 New developments in Drosophila taste

2.7.1 Drosophila taste neurons are hard-wired to mediate avoidance- or attractive-type feeding behavior and the *Gr*s a given neuron expresses determine its activation profile

Further experiments from the laboratory of K. Scott (Marella et al., 2006) provided functional evidence that *Gr66a*-expressing neurons were activated by bitter compounds and *Gr5a*-expressing neurons by sweet compounds. To do this, they utilized a UAS reporter that monitors Ca^{2+} influx, a hallmark of neuronal activation, called G-CaMP. Upon cellular activation, in this case, upon application of bitter or sweet test solutions to the proboscis, a fluorescent signal is seen in the cells that express the G-CaMP reporter. They were able to monitor live activation by removing a small section of cuticle from the head capsule and directly viewing the CNS during stimulation of the poroboscis. By selectively expressing G-CaMP in *Gr66a*- expressing cells, they showed that these neurons are activated by a large number of bitter compounds (ten), but not to sugars or amino acids. Additionally, they found that *Gr5a*-expressing neurons were activated by numerous sugars, but not to bitter compounds or amino acids.

Because other *Gr*s are co-expressed in subsets of labellar taste neurons that express *Gr66a*, Marella and coworkers (2006) wanted to try to address whether these smaller subsets of neurons were sensitive to some bitter compounds and not others, which would indicate that flies potentially could discriminate between different bitter

compounds. To do this, they drove expression of G-CaMP in *Gr32a*- and *Gr47a*-expressing neurons and looked to see which bitter compounds caused cellular activation. They found that these cells were generally responsive to all the bitter compounds that *Gr66a*-expressing neurons responded to (with the exception of azadirachtin). This indicates that it is likely that individual *Gr66a*-expressing neurons are no different from one another in their responses to bitter compounds, and that discrimination between bitter compounds may not occur. Obviously, this experiment is limited by the number of bitter compounds tested, and so it is not entirely conclusive. It is not entirely clear why a bitter-sensitive neuron would co-express certain subsets of *Gr*s if not for discriminatory purposes, unless the number of labellar neurons a given bitter-sensing receptor expresses codes for strength of repulsion, such that the most toxic/bitter substances activate the most neurons, thereby eliciting the strongest avoidance response. It is also possible that the Gal4/UAS system is simply not showing the entire expression profile of the *Gr*s, and that *Gr*s expressed in avoidance-type taste neurons are actually expressed in all avoidance-type taste neurons of the labellum.

In order to demonstrate that neurons are hard-wired as avoidance-type or attractive-type taste neurons, they drove expression of a foreign receptor, the mammalian vanilloid receptor (VR1) which is activated by capsaicin, a substrate *Drosophila* are normally not sensitive to, in either *Gr66a*- or *Gr5a*-expressing neurons. Measuring neuronal activation via G-CaMP, they found that application of capsaicin on

the proboscis of flies expressing VR1 in *Gr66a*-expressing neurons caused activation of these neurons. Behavioral assays (residence assays) on these flies show that they actively avoid capsaicin. When VR1 is expressed in *Gr5a*-expressing neurons, however, the flies become attracted to capsaicin. These results indicate that the receptor(s) that a taste neuron expresses determine the ligands to which it is sensitive, and that the behavioral output depends on the type of neuron activated.

2.7.2 Gr66a is required for a response to the bitter compound caffeine

Shortly after demonstrating that *Gr66a* and other *Gr*s are expressed in taste neurons that mediate avoidance behavior in *Drosophila*, a group identified *Gr66a* as necessary for an *in vivo* response to caffeine. Moon and colleagues (2006) created a fly strain with *Gr66a* removed, via P-element excision. Electrophysiological recordings from sensilla of the labellum known to house taste neurons that usually express *Gr66a* showed that these Δ*Gr66a* flies did not have neuronal activation upon caffeine application. Additionally, in two-choice feeding assays, Δ*Gr66a* failed to avoid caffeine-contaminated food. Both electrophysiological and behavioral assays showed that *Gr66a* mutants specifically lack sensitivity to caffeine, and not to other bitter compounds or sweet tastants.

Moon and coworkers (2006) also tried to express *Gr66a* in a heterologous cell system to show specific cellular activation (via calcium imaging) in response to caffeine. The receptor failed to be expressed on the cell surface or to show cellular activation,

however, preventing the conclusion that *Gr66a* is a caffeine receptor. While *Gr66a* is necessary for a caffeine response, it is still possible it acts as a co-receptor.

2.8 Novel roles for Grs and new insight into how Grs may function

2.8.1 Gr21a and Gr63a function as a CO_2 receptor: precedent for alternative *Gr* function and heterodimeric interactions between *Gr*s

Studies by Suh coworkers (2004) found that when flies were stressed in a vial they left something behind that caused newly introduced flies to avoid the area in which the original flies were stressed. They later determined that one component of this "avoidance" substrate was CO_2, emitted by the stressed flies. Previously a group had identified a type of olfactory neuron found on the 3rd antennal segment that was responsive to CO_2 (de Bruyne et al., 2001), and it turned out that a gustatory receptor – *Gr21a* – was expressed in these olfactory neurons. These olfactory neurons projected their axons to a single glomerulus, V, in the antennal lobes (Suh et al., 2004).

Later, a different group (Jones et al., 2007) would show that another *Gr* receptor – *Gr63a* – was co-expressed in these CO_2-responsive olfactory neurons, and that both receptors were required for CO_2 detection. This indicates that *Gr*s may function as heterodimers, which is similar to findings in the *Drosophila* olfactory system, where *Or83b* is required for efficient *Or* function (Neuhaus et al., 2005). It remains unclear,

however, if either or both *Gr*s interact with CO_2 , or whether one serves as a co-receptor or chaperone.

2.8.2 Drosophila olfactory receptors not only form heterodimers, but they may have unexpected topology and may not function as GPCRs

Both *Gr*s and *Or*s belong to a larger chemosensory receptor superfamily in *Drosophila*. Based on their seven-transmembrane domain topology, and the fact that mammalian taste receptor and olfactory receptors function as G-protein-coupled receptors, it was presumed that the *Drosophila Ors* and *Grs* would function similarly. Evidence has been accumulating, though, that indicates that *Drosophila Or*s may not function as simple GPCRs. Although the *Drosophila Or*s and *Gr*s have little sequence similarity, *Or83b,* which is the most related to the *Gr*s, has been found to have some unusual properties.

Unlike mammalian olfactory receptors, which are expressed one per olfactory sensory neuron, *Drosophila* olfactory sensory neurons express two olfactory receptors – an OR that binds a specific odorant, and OR83b (Larsson et al., 2004). It has been found that OR83b is necessary for the proper function of other ORs *in vivo*, with olfactory neurons in *Or83b* mutant animals failing to respond electrophysiologically to odorants. This is most likely because ORs fail to be properly localized and stabilized at dendritic cell membranes, where they normally interact with odorant ligands (Larsson et al., 2004). Thus OR83b does not itself specify odorant ligand specificity, or determine to

which odorants a given olfactory sensory neuron is responsive to, instead it insures proper functioning of other odorant-specific ORs.

Further studies found that OR83b localizes and stabilizes other ORs to the cell membrane by direct interactions, forming an OR:OR83b heteromeric receptor complex (Benton et al., 2006). Interestingly, experiments to determine membrane topology of OR83b found that unlike the mammalian olfactory receptors (which are GPCRs) where the amino-terminus is extracellular, the amino-terminus of OR83b was intracellular. In addition, it appears that membrane loops once thought to be extracellular, were cytoplasmic, and it is through these loops that OR83b interacts with an OR (Benton et al., 2006). These experiments indicate that the topology of the *Drosophila* ORs may not be what was initially expected, and that they may not function as GPCRs. Because multiple GRs are also found in a single chemosensory neuron, and both GR21a and GR63a are required to mediate CO_2 sensitivity, it is possible that GRs also do not function as GPCRs, as once thought, and that they also form heteromeric complexes.

3 The highly conserved *Gr28* genes are expressed not only in taste neurons, but in non-chemosensory neurons and neurons in the CNS, suggesting a non-traditional role for these receptors

The majority of this Chapter has been submitted to The Journal of Comparative Neurology as the manuscript:

Thorne, N., and Amrein, H. (2007). Atypcial expression of *gustatory receptor* genes in sensory and central neurons. The Journal of Comparative Neurology (in submission).

3.1 Introduction

Taste is considered to be the perception of soluble substrates, detected in humans and other mammals by taste cells located on the tongue and pharynx. Through this sense, we are able to perceive the qualities of sweet, bitter, salty, sour, and savory (umami). *Drosophila*, a member of the insect family, have taste preferences only slightly different from our own, and are surprisingly attracted to and repulsed by the same taste qualities as humans. Sweet and savory taste are associated with, and therefore have come to signify, substrates that offer nutrition and are considered a food source. At the opposite end of the spectrum is repulsion, characterized by a bitter taste, and that is often associated with toxic chemicals and spoiled food. Though humans have learned to enjoy

the bitter taste from caffeine in coffee, generally the behavioral output after sensing something bitter is to be repelled by it and subsequently avoid it.

Insect taste is unique from that of mammals in one especially striking way – Drosophila taste is not restricted to the mouth, and it can "taste" with many appendages, thanks to taste neurons located on their wings, legs, and even genitalia. One taste tissue - the labellum - and the taste neurons it contains, has been the subject of extensive study and analysis in an effort to understand the cellular and molecular basis of taste perception. Considered the *Drosophila* equivalent of the mammalian tongue, the labellum is considered the key tissue in tastant perception. It is thought to be involved in triggering nutrient uptake or toxin repulsion based on the identity of the substrate perceived by taste neurons that are associated with taste bristles (sensillum) that project like whiskers from each labial palp. There are 31 taste bristles per labial palp, arranged in a stereotyped pattern and consisting of three morphologically different types – short (S), intermediate (I), and long (L) (Hiroi et al., 2002). Each bristle is associated with 2 (I) or 4 (S and L) taste neurons, such that each labial palp has over 100 taste neurons.

Internal taste neurons are found in the pharynx, and may be involved in re-enforcing the swallowing reflex upon food uptake or triggering regurgitation of contaminated food substances upon toxin detection. There are three paired clusters of sensilla - found on symmetrically opposing sides of the pharynx - associated with taste

neurons: the labral sense organ (LSO), the ventral cibarial sense organ (VCSO) and the dorsal cibarial sense organ (DCSO) (Stocker, 1994).

Tastant detection by sensilla of the legs is thought to be important in the fly's inital determination of whether a given substrate is toxic or nutritious. Stimulation of taste neurons of the legs elicits almost immediate extension of the proboscis if the subtrate is a sugar. A common behavioral technique that demonstrates this is the proboscis extension reflex assay. If a sugar solution is applied to the tarsal segments of the foreleg of a fly, there is a high likelihood that the proboscis will extend in response to nutrient detection. If a bitter contaminant is added to the sugar solution, the likelihood that the fly extends its proboscis decreases dramatically. These studies, as well as electrophysiological studies, indicate that flies can sense at least sweet and bitter compounds with taste neurons of the legs (Meunier et al., 2003). Taste bristles of the legs contain four gustatory neurons, and approximately 30 taste bristles are found on the tibiae and tarsi of all three sets of legs, with the first set of legs having a few additional taste bristles (Mitchell et al., 1999; Stocker, 1994). Additionally, male flies have 13-14 extra taste bristles on their forelegs, which are thought to play a role in sex-specific pheromone detection necessary for courtship and mating (Singh, 1997). Although the anterior wing margin of *Drosophila* contains an abundant number of chemosensory sensilla, little is specifically known about taste neurons of this tissue. In addition, female flies have chemosensory bristles located near their genitalia, which are thought to play a

role in substrate selection during oviposition, but little more is known about these putative taste neurons (Stocker, 1994).

Members of the gustatory receptor gene family are expressed in taste neurons of all the taste tissues described, with the exception of the female genitalia (Clyne et al., 2000; Dunipace et al., 2001; Scott et al., 2001; Thorne et al., 2004; Wang et al., 2004). Molecular studies that look at the expression of the Drosophila gustatory receptor gene family found that the majority of Grs analyzed to date (such as *Gr66a* and *Gr22e*) are co-expressed in taste neurons associated with S-type and I-type bristles (Hiroi et al., 2002). These receptors were expressed in a single neuron associated with an S- or I-type bristle (Thorne et al., 2004). Behavioral studies in which flies were starved and then given a choice to feed on sucrose or sucrose contaminated with a bitter substrate (two-choice feeding assay) indicated that when neurons expressing these receptors were functionally ablated (by driving expression of tetanus toxin under the control of a *Gr*-promoter), flies were no longer repelled by the bitter substrate, and fed from the contaminated food (Thorne et al., 2004). Similar studies using the proboscis extension reflex assay (and Diphtheria toxin neuronal ablation) indicated that these flies extended their proboscis significantly more frequently to a sucrose solution contaminated with a bitter substrate than controls, indicating that they had decreased sensitivity to bitter tastant detection (Wang et al., 2004). These studies also demonstrated that a trehalose receptor – *Gr5a* – is expressed in a different subset of taste neurons of the labellum, and that these neurons

mediate sweet taste sensitivity. Further studies have indicated that the taste neurons that express *Gr66a* – a caffeine receptor (Moon et al., 2006) - (and *Gr22e*) are hard-wired to mediate repulsive/avoidance taste behavior, and neurons that express *Gr5a* are hard-wired for attractive/feeding taste behavior (Marella et al., 2006).

Although expression of these *Gr*s is reported in unexpected sensory neurons, there is precedence for members of this gene family to be involved in functions beyond taste perception: *Gr21a* and *Gr63a* are expressed in olfactory neurons of the antennae and are thought to be a CO2 receptor (Jones et al., 2007; Suh et al., 2004), *Gr68a* is expressed in a sex-specific fashion in taste neurons of male forelegs and may play a role in pheromone detection (Bray and Amrein, 2003), and *Gr32a* may play a role in detecting inhibitory pheromones.

We have concentrated our efforts on describing the expression of a *Gr* gene cluster – *Gr28a* and *Gr28b.a-e*. This gene cluster is composed of a single gene – *Gr28a* – that produces one transcript and second gene – *Gr28b* - that produces five transcripts, due to five alternative first exons. Not only are these genes highly conserved among all *Drosophila* species, they have an extremely unusual expression profile. Members of this gene cluster are expressed in taste neurons, but some have additional expression in non-gustatory neurons. In this study, we have carefully examined the expression of each of these genes in great detail, which is the first time the expression of an entire gene cluster of *Gr*s has ever been described. This is also the first report of such an unusual expression

pattern for members of this gene family, with noted expression in the pars intercerebralis of the CNS, multidendritic neurons of the abdomen, and neurons associated with the arista. We have also confirmed this unusual expression via in situ hybridization, to show that the *Gr28* genes are expressed not only in cells outside the taste system in the embryo, but significantly, in putative neurosecretory cells of the CNS in the adult, which we believe are second-order neurons of the taste circuit.

3.2 Results

All *Gr* genes analyzed to date show expression restricted to the peripheral chemosensory systems. Initially, RT-PCR analysis was used to associate expression of about twenty *Gr* genes with taste organs (Clyne et al., 2000). Subsequently, cellular expression studies using the GAL4/UAS system confirmed that of about 15 *Gr* genes, 9 are indeed expressed in taste neurons of the labial palps, the pharynx, the legs and the wings(Clyne et al., 2000; Dunipace et al., 2001; Hiroi et al., 2002; Marella et al., 2006; Scott et al., 2001; Thorne et al., 2004; Wang et al., 2004). Two *Gr* genes - *Gr21a* and *Gr63a* - were found to be expressed exclusively in small subsets of olfactory sensilla (Jones et al., 2007; Scott et al., 2001; Suh et al., 2004), and a third - *Gr22e* - has been found to be broadly expressed in the antennae (Dunipace et al., 2001), in addition to its broad expression in the gustatory system. Despite many efforts by different investigators, expression analysis by RNA *in situ* hybridization has been largely unsuccessful, and only a few *Gr* RNAs have been convincingly detected in taste tissue using this method (Jones et al.,

2007; Scott et al., 2001). The difficulty in reliably detecting *Gr* transcripts by *in situ* hybridization is probably due to the very low abundance of *Gr* mRNAs in taste neurons.

The expression analysis presented in this paper is based on an initial observation in RT-PCR experiments, which indicated that *Gr* transcripts were expressed in abdominal tissue. This analysis was then extended by analyzing their cellular expression with the GAL4/UAS system, which revealed that several members of the *Gr28* cluster appear to be more broadly expressed than any other *Gr* gene examined to date. Thus, we performed a detailed analysis of all six *Gr28* genes and report here both expected expression of these genes in gustatory sensory neurons, as well as wide-spread atypical expression in sensory and central neurons for four of them.

3.2.1 The *Gr28* genes

The *Gr28* gene cluster is comprised of six genes, relatively tightly clustered within a genomic region of approximately 13 kilobases (kb) (Figure 20). *Gr28a* is a independent transcription unit whereas the five remaining genes - *Gr28b.a, Gr28b.b, Gr28b.c, Gr28b.d* and *Gr28b.e* - are composed of their own 5' regulatory regions and unique first exons, which are spliced to common exons at the 3' end, an organization that is similar to two other Gr gene clusters (*Gr23* and *Gr39*) (Clyne et al., 2000; Robertson et al., 2003). The first exon of each of the five *Gr28b* genes is thought to encode transmembrane domains 1-4 of the receptor, with the two common exons coding for transmembrane domains 5-7 (Robertson et al., 2003).

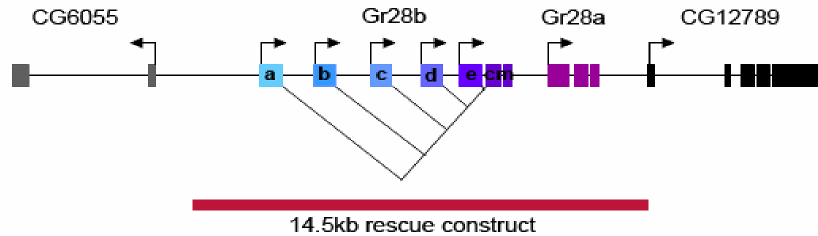


Figure 20: Schematic of the *Gr28* locus on the second chromosome of *Drosophila melanogaster.* Each of the the *Gr28b* genes contains a unique first exon, which is spliced to two exons shared by all five genes. *Gr28a* is an independent transcription unit, downstream of the *Gr28b* genes. Structure and orientation of the *Gr28* genes, as well as the upstream and downstream flanking genes, are conserved even in distantly related *Drosophila* species. The 14.5kb rescue construct, which includes all six genes, was used to increase the gene copy number (and amount of transcript) for RNA *in situ* hybridization experiments.

Gr genes encode a family of 68 seven transmembrane receptors that share little amino acid identity, ranging form 8-12% on average (Robertson et al., 2003); however, clustered *Gr* genes are usually much more similar to other members of the same cluster, which can reach 50% amino acid sequence identity (Dunipace et al., 2001). For example, GR28a shares between 28% and 35% sequence identity to the five GR28b proteins, and sequence identity of the unique amino terminus of the five GR28b proteins ranges between 14% and 35% (range of 56% to 64% amino acid identity when comparing the whole GR28b proteins). For comparison, GR66a shares 19% and 16% amino acid

sequence identity with GR28a and GR28b.b, respectively, and GR5a shares 15% identity with both these GR28 proteins. Likewise, GR5a and GR66a share only 13% sequence identity.

Gr28a and the alternatively spliced *Gr28b.b* genes are among the most conserved *Gr* genes within different *Drosophila* species or even among different Diptera. Amino acid sequence identity of Drosophila melanogaster GR28a and GR28b.b to the orthologs in *Drosophila similans* and *Drosophila pseudoboscura*, two close relatives, and *Drosophila virilis*, a more distant relative, is very high (between 85% and 99%) and exceeds that of most other GRs examined, such as GR66a (a caffeine receptor), GR5a (a trehalose receptor) and GR22e (see Table 3). Moreover, all these *Drosophila* species have retained the same gene structure for *Gr28b*, with distinct promoters and first exons that are spliced to two common exons. Likewise, the relative orientation of *Gr28a* and the syntenic flanking genes is conserved in all these *Drosophila* species. It has previously been noted that *Gr28b.a* is a pseudogene in *D. sechellia*, with multiple deletions in the coding sequence (McBride, 2007), which may indicate species-specific roles for some *Gr28* genes or redundancy in function. The *Gr28* genes appear to be represented by a single putative ortholog in *Anopheles gambiae* – *GPRgr33* (Hill et al., 2002). GR28b.b is the most similar member of the cluster to GPRgr33, sharing 34% amino acid identity. Thus, GR28b.b is more conserved to its ortholog in Anopheles than GR66a, GR5a or GR22e (Table 3). The most conserved GR is GR21a which is required for CO2 detection and

shares more than 60% amino acid identity with its *Anopheles* ortholog (GPRgr22) (Hill et al., 2002).

Table 3: Percent amino acid identity to putative ortholog. Orthologs in other *Drosophila* species were identified with BLASTP using *Drosophila melanogaster* amino acid sequence to query against the GLEANR protein prediction database. Percent amino acid identity to *D. melanogaster* sequence is indicated, as determined from ClustalW alignments. An ortholog to *Gr5a* was not found in the databases for *D. pseudo obscura* (*), and the percent identity listed is that of *D. melanogaster Gr5a* and the putative ortholog to *Gr64* in *D. pseudo obscura*. Orthologs in *A. gambiae* were found by performing peptide queries using the ENSEMBL database. The names of the *A. gambiae* genes are indicated in parentheses.

	D. similans	D. pseudo obscura	D. virilis	Anopheles gambiae
Gr28a	99	88	85	32 (GPRgr33)
Gr28b.b	99	96	93	34 (GPRgr33)
Gr21a	99	96	88	62 (GPRgr22)
Gr66a	95	84	79	29 (GPRgr2)
Gr22e	95	70	49	15 (GPRgr33)
Gr5a	69	44*	62	19 (GPRgr15)

3.2.2 Expression of the *Gr28* genes using the Gal4/UAS system

Four different *p[Gr28]-Gal4* insertion lines were used to determine the expression of each of the six *Gr28* genes. Since expression profiles of the same Gal4 construct can vary in different lines (due to integration in different regions of the genome) and may depend on effects of nearby enhancers or silencers, we considered expression 'genuine' only if it was observed in at least three of the four lines we analyzed. It is not uncommon, however, to find variable levels of the same expression pattern among

different insertion lines of a given driver. In such cases, we usually used the strongest driver for detailed analysis.

We use particular reporter genes to analyze different types of *Gr* expression. For example, UAS-nucleargfp (UAS-nucgfp) was generally used to determine expression in the labellum and legs, while UAS-n-synaptobrevinEGFP (UAS-nsybEGFP) was employed to map the axonal projection patterns in the CNS, and UAS-mCD8::GFP, which labels cellular membranes, was used to visualize the overall cell morphology – cell body, axons, and dendrites.

Only two of the six genes, *Gr28b.a* and *Gr28b.e*, had expression characteristic of "true" taste receptor genes, i.e. expression restricted to taste neurons. Expression of the other four *Gr28* genes was not restricted to the taste system, but included many different types of sensory neurons as well as neurons in the CNS and non-neuronal cells.

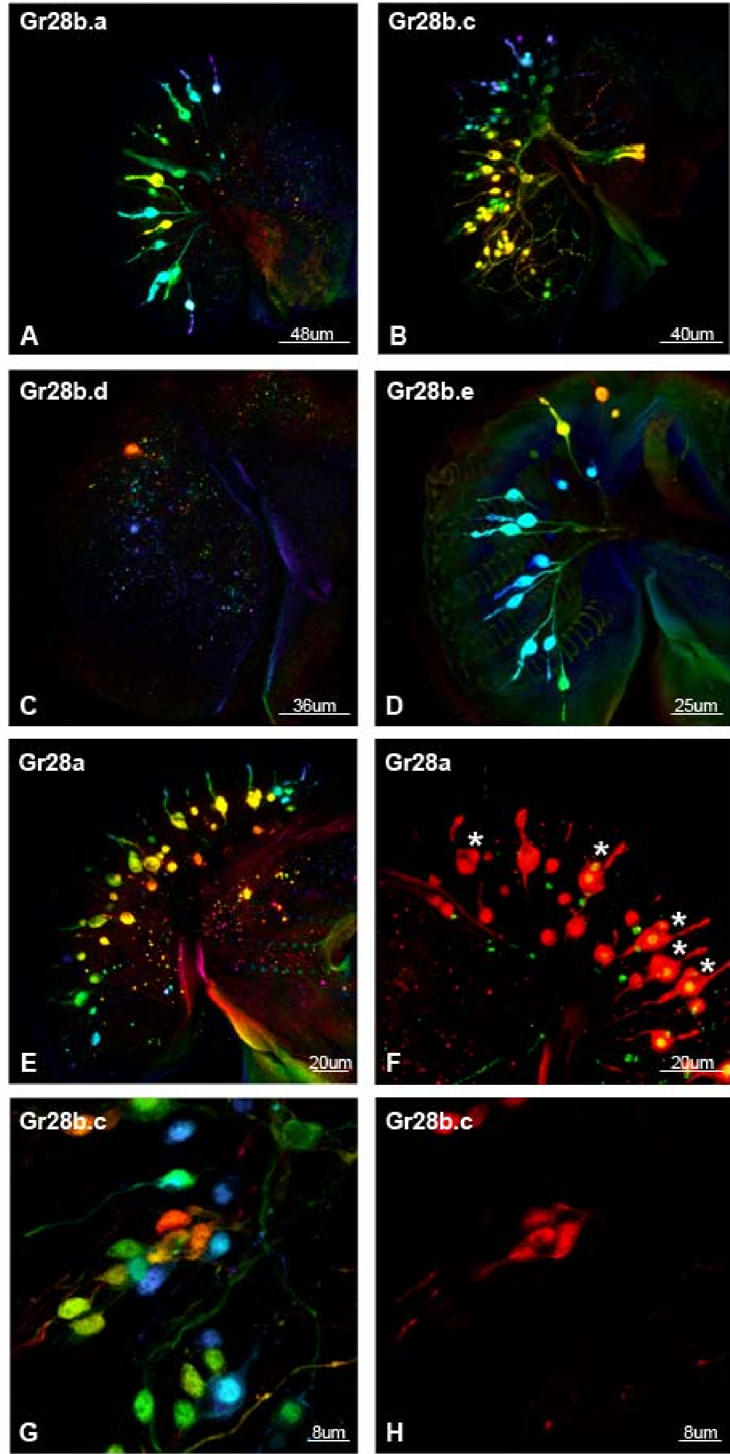
3.2.2.1 Expression in adult taste tissues

Most *Gr28* genes are expressed in taste neurons of the labellum, the pharynx, and the legs, but none are expressed in taste sensilla of the wings. Co-expression analysis of the *Gr28* genes with *Gr22e* in the labellum was carried out by comparing expression of flies with single *promoter[Gr]-Gal4* drivers to flies containing two *promoter[Gr]-Gal4* drivers (Thorne et al., 2004). The results of these expression analyses in adult taste tissue are summarized in Table 4.

Table 4: : Expression of *Gr28* genes in adult taste tissue. The numbers in each column indicate the number of neurons that were GFP-positive. "No" indicates no expression was observed. Cell count was based on Gal4/UAS expression from multiple driver lines and a total of at least 15 animals.

	labellum avg no. /palp	tested + found coexpressed in labellum with:	LSO	VCSO	DCSO	legs	wings
Gr28a	32	Gr22e	No	1	1	Yes; 1st set	No
Gr28b.a	13	Gr28b.e	No	2	1	Yes; 1st set	No
Gr28b.b	No	N/A	No	No	1	No	No
Gr28b.c	45	Gr22e	1	1	No	Yes; all sets	No
Gr28b.d	3	Gr22e	No	1	1	Yes; all sets	No
Gr28b.e	13	Gr22e+Gr28b.a	No	1	1	Yes; 1st set	No

Labellum: All *Gr28* genes, with the exception of *Gr28b.b* are expressed in taste neurons of the labellum (Figure 21). Two of these genes, *Gr28b.a* and *Gr28b.e,* show nearly identical expression, with each expressed in approximately 13 taste neurons per labial palp (Figure 21A+D; Table 4). We previously described expression of *Gr28b.e* in one neuron associated with each of the 12 S-type bristles found on each labial palp, and a single neuron associated with one or two of the I-type bristles (Thorne et al., 2004). This expression profile is identical to that of *Gr28b.a* and *Gr22e* (Thorne et al., 2004), as determined by co-expression analysis. It has been previously shown that *Gr22e*-expressing GRNs mediate bitter taste sensitivity (Marella et al., 2006; Thorne et al., 2004; Wang et al., 2004).



Gr28b.a
A
48um
Gr28b.c
B
40um
Gr28b.d
C
36um
Gr28b.e
D
25um
Gr28a
E
20um
Gr28a
F
20um
Gr28b.c
G
8um
Gr28b.c
H
8um

Figure 21: *Gr28* expression in the labellum revealed with the Gal4/UAS system. Immunohistochemistry of dissected labella from *Gr-Gal4/ UAS-nucgfp* flies, with an antibody against GFP, was used to visualize labellar neurons expressing a given *Gr* gene. Confocal stacks are depth-coded, resulting in multi-colored images. Drivers depicted in the panel are NT42aA48a (*Gr28b.a*), NT21B1 (*Gr28b.c*), Gr28a4B27a1 (*Gr28b.d*), Gr28a3AII (*Gr28b.e*), SF36S and SF36B1 (*Gr28a*). (A-E) Of the *Gr28* genes expressed in the labellum, all appear to be expressed in the relatively large cell-bodied neurons associated with S-type bristles. *Gr28b.d* (C) is expressed in the fewest (3) neurons per labial palp whereas *Gr28b.c* (B) is expressed in the greatest number (~ 45). For more details, see text. (F-H) *Gr28a* and *Gr28b.c* are both expressed in more than one taste neuron associated with a given bristle. For *Gr28a* (E+F), one larger neuron and a smaller neuron are often seen in close proximity (F; asterisks), projecting their dendrites into the same sensillum shaft. Clusters of several taste neurons express *Gr28b.c* (G), which is more obviously revealed in single section of a z-series (H).

Gr28a and *Gr28b.c* are also co-expressed with *Gr22e* (Table 4) in neurons associated with S- and I-type bristles (Figure 21). These two *Gr28* genes, however, are expressed in additional labellar neurons not expressing *Gr22e, Gr28b.a,* or *Gr28b.e*. On average, we find about 32 neurons per palp for *Gr28a* and 45 neurons per palp for *Gr28b.c* (Figure 21; Table 4). In S-type bristles, *Gr28a* is not only expressed in each of the characteristically large neurons also expressing *Gr22e* and *Gr28b.a/b.e* but often in several smaller neurons (Figure 21E+F), some of which may express *Gr5a,* a gene encoding a trehalose receptor (Dahanukar et al., 2001; Ueno et al., 2001). Of all the *Gr28* genes, *Gr28b.c* is expressed in the greatest number of labellar neurons (~ 45 per palp), including at least half of the morphologically large neurons associated with S-type bristles and expressing *Gr22e* (Figure 21B; Table 4). In addition, many *Gr28b.c* expressing neurons are associated with I- and L-type bristles. Some of these neurons appear in clusters,

indicating that a given sensilla contains more than one *Gr28b.c* expressing neuron (Figure 14G+H).

The *Gr* gene with the most restricted expression is *Gr28b.d* which is found in two to three taste neurons associated with S-type bristles. These neurons also express *Gr22e* and the several other *Gr28* genes (Figure 21C; Table 4). Finally, *Gr28b.b* does not show expression in labellar GRNs (or GRNs of other taste organs; data not shown).

Pharyngeal taste neurons -LSO, VCSO, DCSO: Only one of the *Gr28* genes is expressed in the LSO, VCSO, and DCSO – *Gr28b.c.* However, with the exception of *Gr28b.b*, all *Gr28* genes are expressed in the VCSO. Most are expressed in only one neuron of the VCSO, but *Gr28b.a* is expressed in two VCSO neurons (Table 4).

Legs: Expression levels of the *Gr28* genes in taste sensilla of the legs, even with the amplification afforded by the Gal4/UAS system, is weak. The only reliable method to determine location and number of neurons in the legs was to use the UAS-nucgfp reporter, when legs were viewed immediately after dissection using a fluorescent microscope. *Gr28b.a* and *Gr28b.e* are both expressed in two neurons, on the fifth tarsal segment in the forelegs, whereas *Gr28a, Gr28b.c* and *Gr28b.d* are expressed in at least two taste neurons of the fifth tarsal segment, with additional expression in non-chemosensory neurons of the leg (Table 4; see below). *Gr28b.b* does not appear to be expressed in the legs (data not shown).

Wings: None of the *Gr28* genes are expressed in neurons associated with chemosensory bristles of the wings (data now shown).

3.2.2.2 Atypical *Gr* expression in sensory neurons

The novel and most interesting aspect of this study is the observation that four of the six *Gr28* genes - *Gr28a, Gr28b.b, Gr28b.c* and *Gr28b.d* - show expression in peripheral sensory and neurons and neurons of the central nervous system (CNS) that are in many cases not associated with the gustatory system, suggesting novel roles in sensory perception and signaling for members of this gene family. A compilation of atypical *Gr28* expression is shown in Table 5.

Table 5: Expression of *Gr28* genes in adult non-chemosensory neurons in the periphery and CNS. The numbers in each column indicate the number of neurons that were GFP-positive. ** indicates that the cell counts reported for *Gr28a*, *Gr28b.c*, and *Gr28b.d* leg expression include taste neurons. "No" indicates that no expression was observed. "Yes" indicates expression in many neurons. Counts were based on Gal4/UAS expression from multiple lines and a total of at least 15 animals, except in the case of JON expression for *Gr28b.c* (two lines/five animals).

	maxillary palps	JONs	aristae	wings campaniform sensilla	legs stretch receptors	legs FCO	abdominal md neurons	abdominal oenocytes	CNS
Gr28a	No	No	No	1 (L3)	n=6-8**; 1st set	No	Yes	No	No
Gr28b.a	No	No	No	No	No	No	No	No	No
Gr28b.b	1	No	No	1 (L1); 1 (L3)	No	No	Yes	Yes	Yes
Gr28b.c	1	~5	No	1 (L1); 2 (L3)	n=5-10**; all sets	Yes	Yes	No	Yes
Gr28b.d	No	No	3	1 (L1); 1 (L3)	n=3-5**; all sets	Yes	Yes	No	No
Gr28b.e	No	No	No	No	No	No	No	No	No

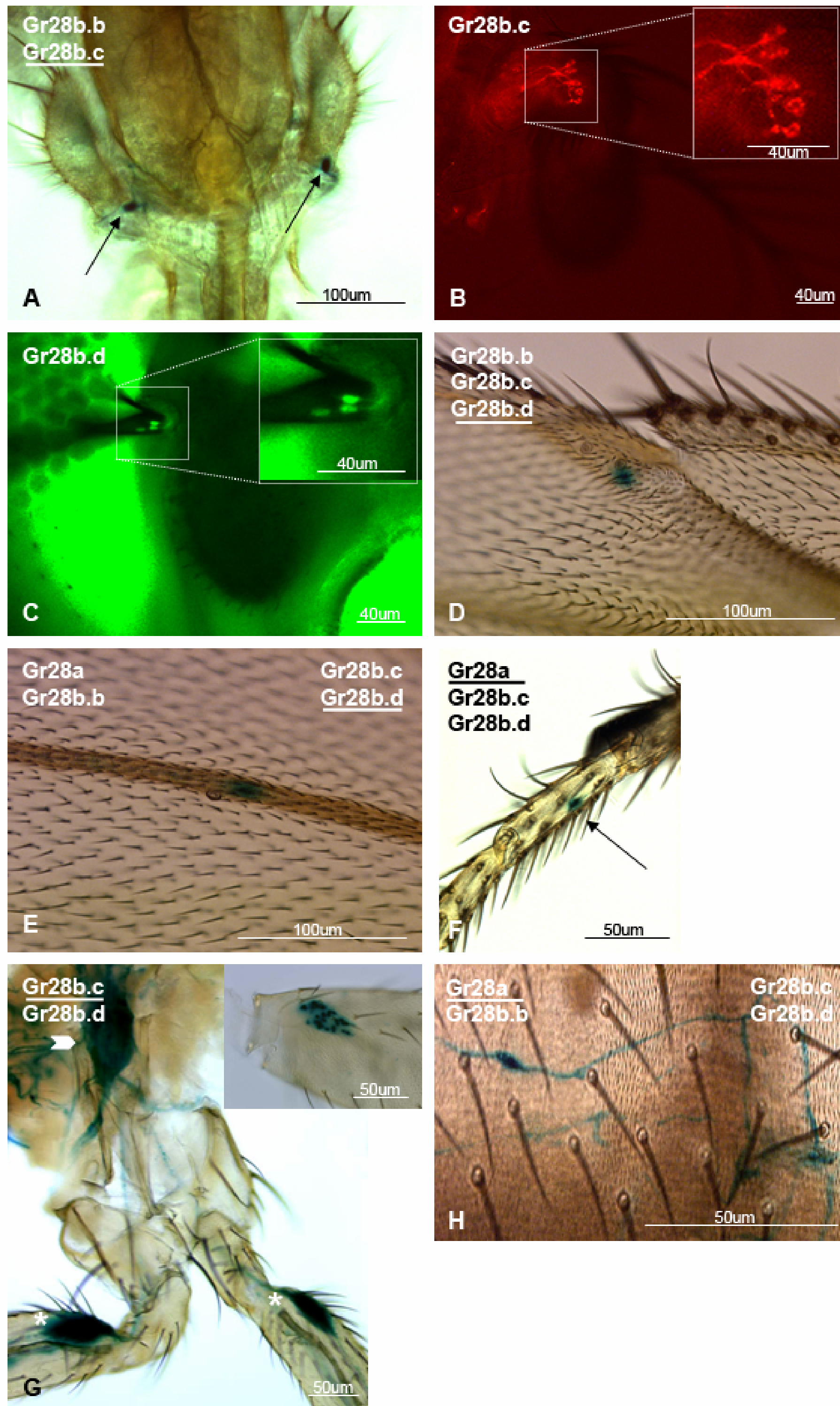


Figure 22: Expression of *Gr28* genes in non-chemosensory neurons. Expression is

revealed using indicated *Gr28-Gal4* drivers in combination with a UAS-lacZ (A, D-H) or UAS-GFP reporters (B and C) and visualized by X-Gal staining (A, D-H), anti-GFP antibody staining (B), or live GFP microscopy (C). All receptors expressed in a tissue are noted in each panel, with the receptor actually shown underlined (see also Table 5). Specific drivers seen in panels are indicated in genotype within parentheses. (A) A single neuron at the base of the maxillary palp of a *Gr28b.c-Gal4(NT21E7);UAS-lacZ* fly is detected after X-Gal staining. (B) Antibody staining of dissected heads from *Gr28b.c-Gal4(NT21E7)/ UAS-mCD8::GFP* flies labels several neurons in the 2nd antennal segment, which are near or associated with the Johnston's organ. (C) Live GFP expression of a *Gr28b.d-Gal4(Gr28a4B27)/ UAS-nucGFP* fly reveals three neurons at the base of the arista, a location reminiscent of hygro/thermoreceptive neurons. (D and E) X-Gal staining of dissected wings from *Gr28b.d-Gal4(Gr28a4C13)/UAS-lacZ* flies shows staining of campaniform sensilla on the L1 vein (D) and the L3 vein (E). (F) X-Gal staining of a foreleg dissected from a *Gr28a-Gal4(SF36B1); UAS lacZ* male shows a single tarsal stretch receptor. Note that this cell is not associated with a chemosensory sensillum (upward curved bristle) and does not extend a dendrite into any bristle shaft. (G) X-gal staining of dissected forelegs of a *Gr28b.c-Gal4(NT21B1)/ UAS lacZ* fly reveals intense β -Gal activity in the femoral chordotonal organ of the femur (asterisks). These cells send axons into the thoracic-abdominal ganglion (white arrowhead). Individual neurons in the chordotonal organ can be identified when using a *UAS-nuclacZ* reporter (inset). (H) X-Gal staining of *Gr28a-Gal(SF36B1)4/UAS-lacZ* shows extensive dendritic arborizations of multidendritic (md) neurons of the abdomen.

Maxillary palps: Along with the third antennal segment of the fly, the maxillary palps comprise an olfactory organ covered with olfactory sensilla that contain two to four olfactory sensory neurons and express members of the *Or* gene family. Two of the *Gr28* genes, *Gr28b.b* and *Gr28b.c*, are expressed in a single, large cell at the base of the maxillary palp (Figure 22A). This cell, however, is not an olfactory sensory neuron, and its function is not known.

Antennae: One receptor – *Gr28b.c* - is expressed in a few neurons of (or near) the Johnston's organ (Figure 22B). The Johnston's organ senses air vibrations and functions as the fly's "ear" (Kamikouchi et al., 2006; Stocker, 1994). It is built as an internal sensory receptor and it is composed of approximately 480 neurons, which function as stretch receptors. The sensory units of the Johnston's organ are the approximately 200 scolopidia, which contain two to three sensory neurons and connect the second and third antennal segment. Air vibrations are sensed by the arista which extends off the third antennal segment, and the joint movement/rotation of these two structures with respect to the second antennal segment mechanically activate these neurons (Kamikouchi et al., 2006).

Aristae: *Gr28b.d* is expressed in three neurons in the base of the arista (Figure 22C). In many insects, the arista houses sensory neurons that are postulated to function as thermoreceptors and hygroreceptors (Foelix et al., 1989). However, in *Drosophila*, this appears controversial as surgical ablation studies suggest that the arista may contain hygroreceptive, but not thermoreceptive, neurons (Sayeed and Benzer, 1996). Nevertheless, the location and morphology of *Gr28b.d*-expressing neurons is reminiscent of neurons contained within a sensory ganglion and postulated to function as thermo- and/or hygroreceptors (Foelix et al., 1989).

Wings: Campaniform sensilla are mechanoreceptors that detect distortions of the wing cuticle and are thought to play a role in flight (Keil, 1997). Four of the six receptors

(*Gr28b.b*, *Gr28b.c*, *Gr28b.d*, and *Gr28a*) show expression in a single cell associated with the dome-like sensillum characteristic of campaniform sensilla (Figure 22D+E). The expression of the four *Gr28* genes largely overlap, as all are expressed in a single sensillum on the L3 vein, and three (*Gr28b.b, Gr28b.c*, and *Gr28b.d*) are expressed in a single sensillum on the L1 vein. None of the *Gr28* genes shows expression in the halteres, which also contain campaniform sensilla.

Legs: These same genes - *Gr28b.c*, *Gr28b.d*, and *Gr28a* – show atypical expression in the legs, in neurons described as stretch receptors or proprioceptors (Smith and Shepherd, 1996) (Figure 22F+G). These proprioceptors are mechanoreceptors of the scolopidial type which sense movement between leg segments and therefore play a crucial role in terrestial locomotion (Keil, 1997; Smith and Shepherd, 1996). Based on their location (deeper in the leg tissue; Figure 22F) and failure to label dendritic extensions into the bristle shaft, they are not chemosensory neurons. The axonal processes of the neurons expressing these *Gr28* genes likely terminate in the thoracic-abdominal ganglion, a well-described target of proprioceptors (Figure 22G) (Smith and Shepherd, 1996).

Gr28b.c and *Gr28b.d* also show expression in several neurons of the femoral chordotonal organ (fco) in all three sets of legs (Figure 22G). This internal sensory organ, located proximally in the femur, is also involved in proprioception, i.e. in

coordinating movement of the legs in insects (Hess and Buschges, 1999; Reddy et al., 1997).

Abdomen: Abdominal sensory neurons were found to express four of the six *Gr28* genes - *Gr28a, Gr28b.b, Gr28b.c,* and *Gr28b.d* (Figure 22H). These neurons, located dorsally on the abdomen, have extensively branched dendrites that form intricate arborizations below the cuticule. Most of the receptors are expressed in one or two of these neurons per abdominal hemisegment and show the morphology of multidendritic (md) neurons, described as the lateral multidendritic neuron by Smith and Shepherd (1996). These neurons are likely to be of the dendritic arborization (da)-type, which are proposed to be proprioceptive in function(Bodmer and Jan, 1987). At least a subset of these neurons express the gene painless, which encodes a TRP channel, suggesting that these neurons might also function as nociceptors (pain detectors) that sense noxious heat (Tracey et al., 2003). Interestingly, painless, which is required for sensing the compound isothiocyanate (the "bite" found in and characteristic of wasabi), is also expressed in taste neurons of the labellum, legs, and wings (as well as neurons of the Johnston's organ) (Al-Anzi et al., 2006).

3.2.3 Projection of sensory neurons to the CNS and expression in the brain

We performed a series of experiments to map the target areas in the brain of gustatory and other sensory neurons expressing a given *Gr28* gene. For this analysis, we

focused on neuronal projections that were likely to target the brain, and we did not investigate projections from leg and abdominal sensory neurons in detail. While performing this analysis, we discovered that *Gr28b.b* and *Gr28b.c* were also expressed in neurons in the brain, further complicating the analysis of these two genes. Thus, the interpretation presented in the following section is not conclusive for every *Gr28* gene, due to the nature of the complex expression profiles in many types of sensory neurons as well as neurons in the brain. Nevertheless, our anaylsis provides novel information of how peripheral sensory neurons expressing a given *Gr* gene are connected to the CNS, especially sensory neurons not located in the gustatory system.

Unless specifically mentioned in the text, the UAS-nsybEGFP reporter was used in the experiments described in this section. We have, however, performed tracing experiments with the UAS-mCD8::GFP reporter for some genes, which is specifically indicated in text and figure legends. Visualization was performed after whole-mount antibody staining using anti-GFP antibody (see Material and Methods).

We have organized this part of the paper by describing the neural projection of individual *Gr28* genes. For convenience, we start each section with a brief summary of the expression profile at the periphery to facilitate interpretation of the projection analysis.

3.2.3.1 Gr28.a and Gr28b.e

These two genes are the only *Gr28* genes with taste neuron–specific expression restricted to the labial palps, the internal taste organs in the pharynx, and the legs. Labellar expression largely overlaps with expression of *Gr66a* and *Gr22e* (Figure 21) (Thorne et al., 2004; Wang et al., 2004). Not surprisingly, the axonal projection patterns of both *Gr28b.a*- and *Gr28b.e*-expressing GRNs are restricted to the SOG in a manner characteristic for *Gr66a*-expressing neurons (Figure 23A+B).

3.2.3.2 Gr28a

In addition to expression in a large number of taste neurons, *Gr28a* is also expressed in a few non-chemosensory neurons, including the campaniform sensilla of the wing, leg stretch receptors, and md neurons in the abdomen. Taste neurons of the legs project to the SOG (Figure 23C) (Thorne et al., 2004; Wang et al., 2004). The projection pattern for GRNs expressing *Gr28a* is somewhat similar to that of *Gr66a* (i.e. *Gr28b.a* and *Gr28b.e*), except that there is broader innervation dorsally in the SOG, which is likely due to the expression of *Gr28a* in a greater number of labellar neurons compared to *Gr66a* (Figure 23C). One insertion line, out of the three examined, revealed axonal projection to a single glomerulus of the antennal lobe - VM3 (R. Stocker, pers. comm.; data not shown).

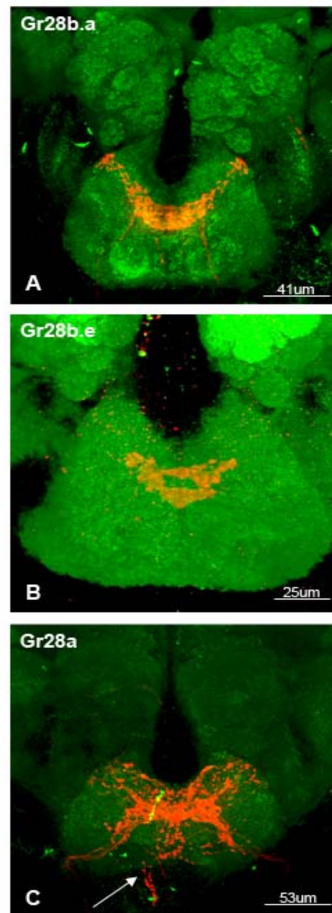


Figure 23: Axonal projection patterns in the CNS of gustatory receptor neurons (GRNs) expressing *Gr28a, Gr28b.a* and *Gr28b.e*. The axonal projection pattern of GRNs was visualized using the indicated *Gr-Gal4* drivers in combination with *UAS-nsybEGFP* reporter, after anti-GFP antibody staining (red) of whole brains. Neuropil (green) was visualized with anti-nc82 antibody. Driver lines used are NT42aC51 (*Gr28b.a*), Gr28a3AII (*Gr28b.e*), and SF36E1 (*Gr28a*). (A and B) Projections of GRNs expressing *Gr28b.a* and *Gr28b.e* is typical of GRNs expressing many previously

analyzed *Gr* genes, including *Gr66a*. (C) The projection pattern for *Gr28a* expressing GRNs is similar, but reveals a greater number of terminations anterodorsally. Arrow indicates afferents from tarsal taste neurons.

3.2.3.3 Gr28b.d

Gr28b.d is expressed in a small number of GRNs in the labial palps, the VCSO, and legs. Atypical expression is observed in three neurons of the arista, campaniform sensilla of the wing, stretch and femoral chordotonal organ receptors in the legs, and md neurons in the abdomen.

There are two striking features about the axonal projection pattern for neurons expressing this *Gr* gene: the tightly targeted innervation of a very discrete area in the tritocerebrum/SOG anterodorsally and innervation of a single glomerulus –either VP2 or VP3 - in the antennal lobe (Figure 24A+B). The staining restricted to the small area of the SOG derives from GRNs located in the labial palps, the legs, and possibly the VCSO.

To test whether the aristal neurons project to VP2/VP3, we surgically ablated the left arista, aged the animals to allow time for deafferentiation, and then performed immunohistochemistry on dissected brains to examine antennal lobe (AL) input. We found that AL staining is restricted to the side of the brain where the ipsilateral arista was left intact, indicating that these aristal neurons project only ipsilaterally to a single AL glomerulus (Figure 24C+D). It was previously reported that axons from aristal neurons project ipsilaterally and that VP2 and VP3 are target antennal lobe glomerulus for aristal neurons (Lienhard and Stocker, 1987; Stocker, 1994; Stocker et al., 1983). No

olfactory receptor neurons have been found to send afferents to VP2 or VP3 (Couto et al., 2005), and this glomerulus is not known to respond to any odors as characterized by Ca2+ imaging (Wang et al., 2003).

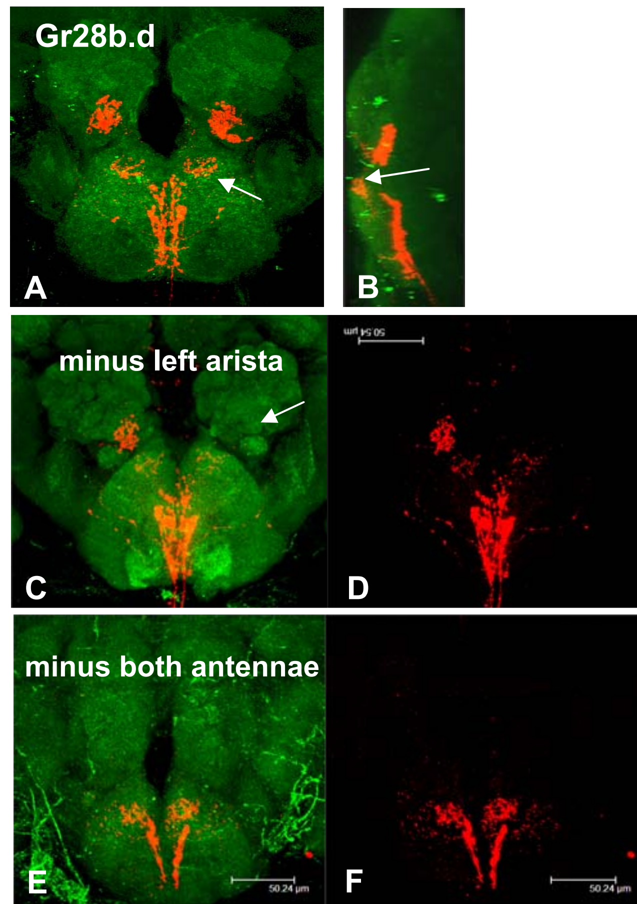


Figure 24: Axonal projection pattern of *Gr28b.d* expressing GRNs to the CNS. Whole brain of *Gr28b.d-Gal4(Gr28a4C13); UAS-nsybEGFP* fly stained with anti-GFP (red) and anti-nc82 (green). (A) The staining from afferents of neurons that express this receptor is localized to a discrete area located anterodorsally in the SOG (arrow). The staining of a single glomerulus, probably either VP2 or VP3, derives from afferents of three *Gr28b.d* neurons located in the arista (for details see text). (B) Image shown in A) rotated 90°, with anterior to the left. (C) When the left arista is removed, the staining of the glomerulus is gone. (D) If both the antennae are removed, then staining of both the ipsi- and contralateral glomerulus are removed, indicating that the afferents to the

antennal lobe are from the aristal neurons, and that these afferents project only to the ipsilateral glomerulus.

3.2.3.4 Gr28b.c

Gr28b.c is expressed by many GRNs in the labial palps, the pharyngeal taste clusters, and taste neurons in the legs. In addition, this gene is expressed in a single cell at the base of the maxillary palps (presumably the same cell expresses *Gr28b.b*), neurons in the Johnston's organ, campaniform sensilla of the wing, stretch receptors and the femoral chordortonal organ in the legs, and md neurons in the abdomen.

Staining of the brain using the UAS-nsybEGFP reporter reveal that this gene is also expressed in a few neurons in the brain, in a dorsally located single cluster in or near the pars intercerebralis (PI; Figure 25A-D).

The extensive staining in the SOG reflect to a large extent - if not exclusively – axonal input from GRNs located in the various taste organs and axons of the PI neurons (Figure 25A-D). We also observe staining of several glomeruli in the antennal lobe in two lines, one of which showed very intense labeling (Figure 25C+D).

Johnston's organ (JO) neurons project into the antennal mechanosensory and motor center (AMMC) (Kamikouchi et al., 2006), but we did not observe any staining in this region of the brain (Figure 25A-D). Instead, we wondered whether JO neurons project to the antennal lobe (Figure 25C+D). To investigate where the *Gr28b.c* neurons associated with the JO and the maxillary palp project, we conducted deafferentiation

experiments by surgical removal of various head appendages. The axonal projection pattern to the antennal lobes was not noticeably affected in animals with both maxillary palps removed (Figure 25E+F). On the other hand, when both antennae were surgically removed, antennal lobe staining was almost entirely abolished (Figure 25G+H), suggesting that antennal lobe input is derived from the neurons located in or near the JO. These afferents project both to the ipsi- and contralateral antennal lobes, since ablation of only one antenna did not affect antennal lobe staining significantly (Figure 25I+J). Taken together, this finding suggests that some neurons located in/or near the JO project to a small number of antennal lobe glomeruli.

It is well established that olfactory sensory neurons of the third antennal segment expressing a given *Or* gene project their axons to a specific glomerulus in the antennal lobes. We do not see expression of *Gr28b.c* in any olfactory sensory neurons with any of the reporter lines. We cannot, however, formally exclude the possibility that the observed axonal projection observed with the *Gr28b.c* Gal4 driver to the antennal lobe derives from olfactory sensory neurons expressing this driver at levels too weak to be detectable in the neuronal cell bodies.

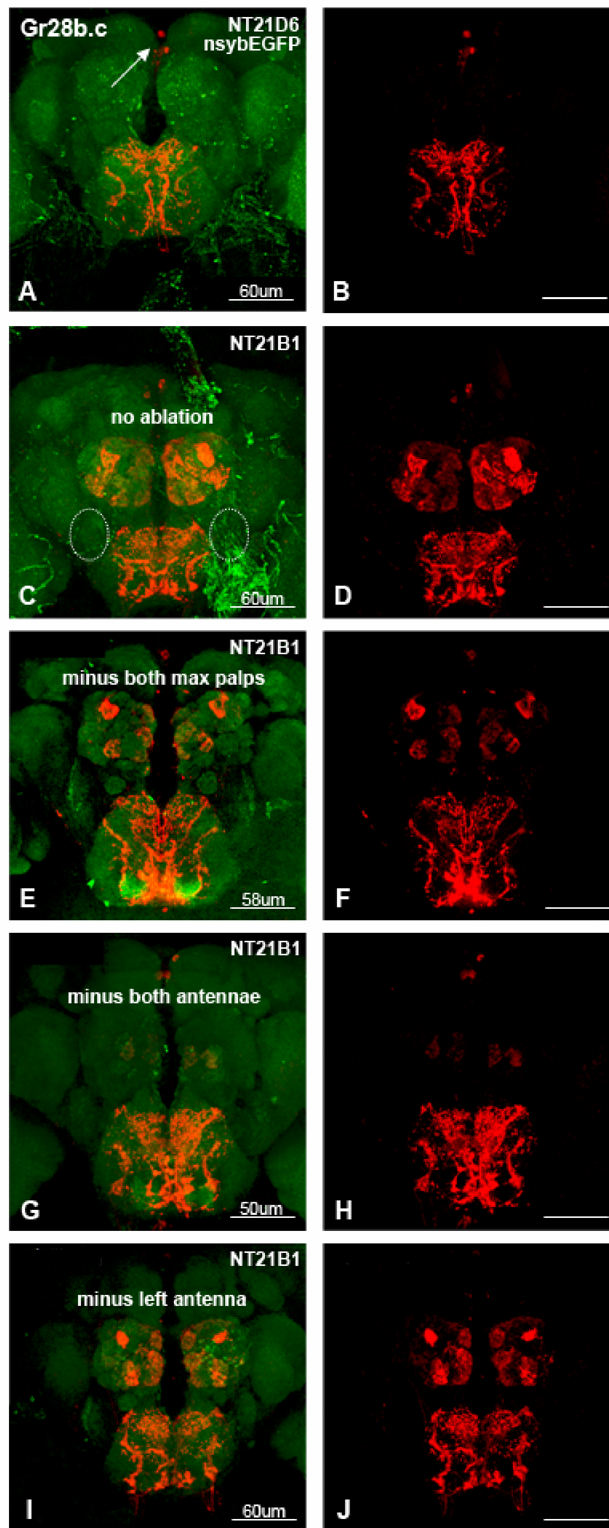


Figure 25: Axonal projection to the SOG and antennal lobes of peripheral sensory

neurons and neurons of the CNS expressing *Gr28b.c* Whole brain preparations of *Gr28b.c-Gal4; UAS-nsybEGFP* flies were stained with anti-GFP (red) and anti-nc82 (green). Note that (A and B) and (C-J) show staining from two different *Gr28b.c-Gal4* drivers. (A and B) Typical brain staining of *Gr28b.c-Gal4* lines (represented here by NT21D6) shows distinct pattern in the SOG, derived from both labellar/pharyngeal GRNs as well as the pars intercerebralis (PI) neurons in the brain (white arrow). (C and D) NT21B1, the only line which shows strong staining in the antennal lobe. Pattern in the SOG and expression in brain neurons of or close to the PI are the same as in the other lines (represented by NT21D6, see A and B). The antennal mechanosensory and motor center (AMMC) is denoted by a dashed white circle in (C). (E-J) Surgical ablation experiments show that afferents from maxillary palps do not significantly contribute to the staining seen in the antennal lobe or SOG (E+F). When both antennae are removed, however, the projection pattern to the antennal lobes is virtually eliminated (G+H). Afferents from the antennae project to both ipsi- and contralateral antennal lobes, as removal of the left antenna does not significantly reduce staining in the either antennal lobe (I+J).

3.2.3.5 Gr28b.b

Gr28b.b is the only receptor not expressed in GRNs in the labellum. We observe expression of this receptor in a single large cell at the base of each maxillary palp (similar to *Gr28b.c*), in campaniform sensilla of the wing, and md neurons in the abdomen.

When investigating the axonal projection pattern of neurons expressing *Gr28b.b* in the CNS, we observed staining throughout the CNS, especially in the antennal lobes and the SOG (Figure 26). Most interestingly, we find several clusters of neurons in the brain that express *Gr28b.b*, with most of the observed axon-dendritic staining derived from these neurons (see below). In the CNS, *Gr28b.b* is expressed in cells of the pars intercerebralis (PI) (Figure 26A), which very much resembles *Gr28b.c* expression (Figure 25). Additional neurons expressing *Gr28b.b* in the CNS are located posterioventral to the

SOG, frontal (anterior) to the SOG, on the dorsal and lateral sides of the antennal lobe, and scattered throughout the anterior and posterior surface of the CNS.

The neurons frontal to the SOG (Figure 26A; arrowhead) appear to be in a similar location as interneurons described in the SOG of the blowfly (Mitchell and Itagaki, 1992), which were found to have dendritic fields that cover part of the SOG. These apparently analogous neurons in *Drosophila* send dendrites into the tritocerebrum/SOG, into a region that largely overlaps with the projections of the PI neurons (seen using UAS-mCD8::GFP reporter; Figure 26C, asterisk). We note that this region of the SOG is also extensively innervated by axons from labellar and pharyngeal gustatory sensory neurons expressing many *Gr* genes, such as *Gr66a, Gr28a* and *Gr28b.c.* The PI neurons expressing *Gr28b.b* project axons to the anterodorsal region of the tritocerebrum/SOG, similar to that described by Rafjashekar and Singh (1994) (Figure 26C, arrows), and largely overlapping with the dendritic arborization of *Gr28b.b* – expressing interneurons (described above).

Finally, the neural staining observed as a loose net covering many antennal lobe glomeruli can be associated with cell bodies located in two clusters found dorsal and lateral to the antennal lobes (Figure 26B asterisks, Figure 26C arrowhead). It appears likely that these neurons correspond to local interneurons described by Ng et al. (2002) and Shang et al. (2007) whose dendrites form an interconnecting network between most, if not all antennal lobe glomeruli. The antennal lobe staining is clearly visible only with

two lines, yet a diffuse but similar staining can be observed in a third line as well (data not shown).

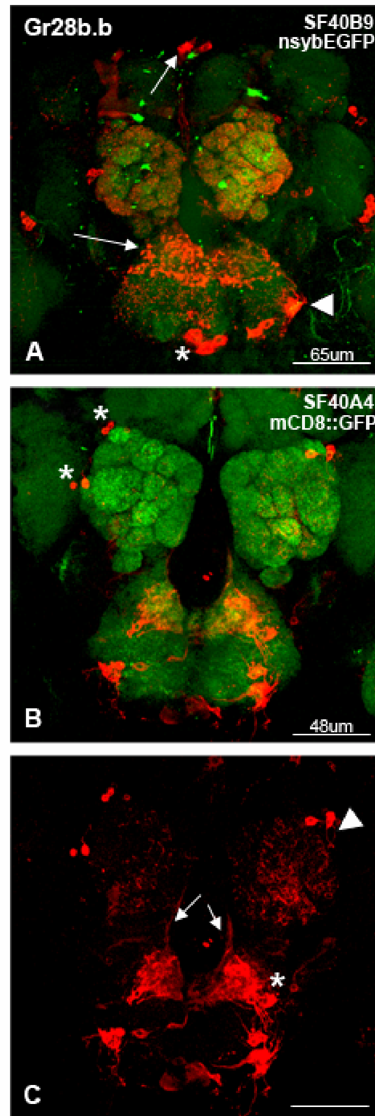


Figure 26: *Gr28b.b* is expressed in numerous neurons of the CNS that target the SOG and antennal lobes. Whole mount antibody staining of brains from a *Gr28b.b-Gal4; UAS-nsybEGFP* fly (A) and *Gr28b.b-Gal4/UAS-mCD8::GFP* fly (B and C) ,which allows visualization of dendritic arborizations, using anti-GFP (red) and anti-nc82

(green) antibodies. (A) PI neurons express *Gr28b.b* (top arrow), with axons that run ventrally to target the tritocerebrum/SOG (A, lower arrow). Neurons frontal/anterior to the SOG (arrowhead) have also dendrites in this region (A, lower arrow). *Gr28b.b* is also expressed in neurons posteroventral to the SOG (asterisk). (B and C) Two clusters of olfactory local interneurons are found dorsal and lateral to the antennal lobes (B, asterisks), with dendrites that cover most glomeruli (best seen in C, arrowhead). The axons from the PI extending to the tritocerebrum/ SOG (arrows) are easily visible here, innervating a dense dendritic network (asterisk).

3.2.4 Larval expression

The larval expression pattern of the *Gr28* genes is largely typical to that observed for previously analyzed Gr genes (Table 6) (Colomb et al., 2007; Scott et al., 2001). Four of the genes – *Gr28a, Gr28b.a, Gr28b.c*, and *Gr28b.e* - are expressed in one neuron of the terminal organ (TO), which houses neurons that function in taste (Colomb et al., 2007), olfaction (Oppliger et al., 2000; Stocker, 1994) and potentially thermoreception (Liu et al., 2003b). One of the receptors – *Gr28a* – is expressed in a single neuron per ventral pit. Only one other *Gr* gene – *Gr2a* – shows expression associated with the ventral pits (Scott et al., 2001). The ventral pits are also thought to have chemosensory function (Stocker, 1994). Two of the receptors – *Gr28b.b* and *Gr28b.c* - are expressed in larval md (da-type IV) neurons (D. Tracey, pers. comm.), in all abdominal segments (Figure 27A). The md neurons that express *Gr28b.b* and *Gr28b.c* exhibit a similar morphology as md neurons expressing *ppk* – a gene encoding a DEG/ENaC protein with a putative function as a salt receptor (Figure 27B) (Adams et al., 1998; Liu et al., 2003a). *Gr28b.b* is also expressed in one bipolar dendrite neuron per hemisegment (Figure 27C+D). Bipolar dendrite

neurons, located deeper in the body than md neurons, project two dendrites in opposite directions but lack extensive arborizations (Bodmer and Jan, 1987). The function of these neurons is not known.

Table 6: Expression of *Gr* genes in larvae. The numbers in each column indicate the number of neurons that were GFP-positive. "No" indicates no expression was observed. "Yes" indicates at least one neuron per structure was observed. Cell count was based on Gal4/UAS expression from multiple driver lines and a total of at least ten animals. Larval expression was determined by using the reporters UAS-mCD8::GFP and UAS-nucgfp with and without antibody staining.

	TO	DO	mouth	ventral pits	md neurons/hemisegment	other cells/hemisegment
Gr28a	1	No	No	Yes	No	No
Gr28b.a	1	No	No	No	No	No
Gr28b.b	No	No	No	No	2 dorsal	1 bd neuron
Gr28b.c	1	No	Yes	No	1 dorsal, 1 ventral	3 unidentified neurons
Gr28b.d	No	No	No	No	No	1 unidentified neurons
Gr28b.e	1	No	No	No	No	No

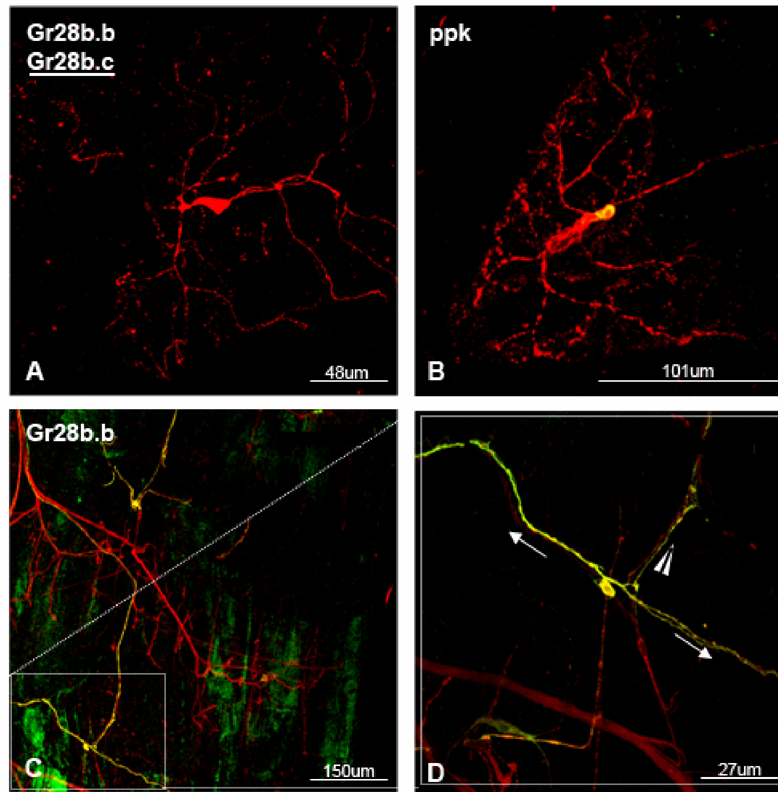


Figure 27: Both *Gr28b.b* and *Gr28b.c* are expressed in abdominal md neurons of larvae. *Gr28b.b-*, *Gr28b.c-* and *pickpocket (ppk)-* expressing md neurons were visualized in flies containing specific Gal4 drivers and *UAS-mCD8::GFP* reporters using whole mount antibody staining. Drivers used are NT21D6 (*Gr28b.c)* and SF40A4 (*Gr28b.b*). (A) A single, *Gr28b.c* expressing md neuron of the dendritic arborization (da)-type, with a centrally located cell body and an extensive dendritic network is shown. (B) A single, *ppk-* expressing neuron is shown, using a *pkk-Gal4* driver (Adams et al., 1998; Liu et al., 2003). Note the similarity in cell morphology of the cells shown in A and B. (C and D) *Gr28b.b* is also expressed in bipolar dendritic neurons whose dendrites project in opposite directions (arrows). The bipolar cell, located at the left bottom corner (C), is enlarged in (D); its dendrites and axon are indicated with arrows and an arrowhead, respectively.

3.2.5 Non-neuronal expression of *Gr28* genes

One of the *Gr28* genes, *Gr28.b.b*, is also expressed in a reiterated fashion in all seven abdominal segments in larvae and adults (Figure 28A+B). In the adult, this expression is reminiscent of the abdominal oenocyte staining pattern observed in several Gal4 lines described in Ferveur et al. (1997). Recent studies indicate that oenocytes have hepatocyte-like function, by regulating lipid metabolism (Gutierrez et al., 2007). Larval expression in oenocytes is weaker but clearly visible when larvae are filleted and subjected to antibody staining, where clusters of six large cells located laterally can be identified in each hemisegment (data not shown). We also observed similar expression in embryos for two *Gr28* genes – *Gr28b.b* and *Gr28b.c* - with expression in laterally located cells in seven segments (Figure 28A, arrows).

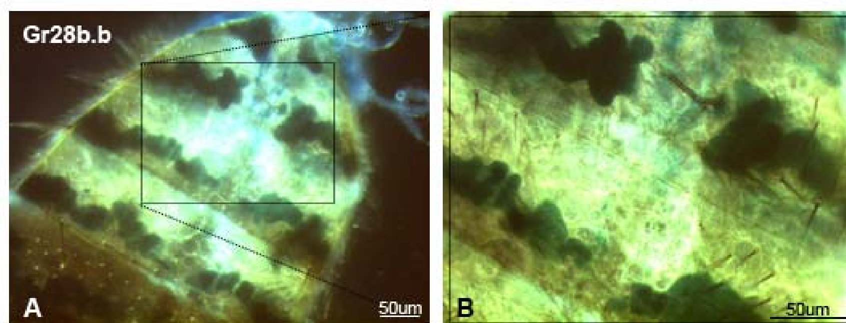


Figure 28: *Gr28b.b* is expressed in oenocytes in adults. (A) LacZ staining of an abdomen of an adult fly of the genotype *Gr28b.b-Gal4(SF40A4)/ UAS-lacZ*. The intensely blue-stained cells are reminiscent of oenocytes described by Ferveur et al. (1997). (B) shows an enlargement of the area indicated by the rectangle in (A).

3.2.6 Endogenous RNA expression of *Gr28* genes in non-chemosensory cells

The data presented thus far indicate that several of the *Gr28* genes exhibit atypical expression, most notably in several types of peripheral sensory neurons not associated with a function in chemosensory perception, but also in central neurons, at least some of which innervate chemosensory processing centers in the brain. In addition, we observed expression in non-neuronal cells in embryos, larvae and adults. The atypical expression reported here has been observed with several independent insertions of each of the Gal4 drivers, a generally accepted and usually reliable criteria for determining genuine, albeit possibly incomplete, endogenous gene expression. Nevertheless, we sought to show atypical expression for at least some of these genes more directly and performed RNA expression studies that allowed detection of endogenous mRNA. First, we carried out RT–PCR experiments using mRNA from dissected tissue samples that do not contain GRNs. Second, despite the notorious difficulties that investigators have faced in detecting Gr transcripts in chemosenosory cells, we performed RNA *in situ* hybridization experiments, with the assumption that transcript levels of the Gr28 genes would be high enough for detection by antisense RNA probes in at least some of the tissues tested.

For RT-PCR analysis we selected two tissue types –abdomen/terminalia and the femurs of legs - which show robust expression for one or more *Gr28-Gal4* drivers. We carefully removed and collected abdominal/terminalia tissue of adult flies and isolated RNA from this tissue. We initially carried out RT-PCR for 33 *Gr* genes in this tissue, eight of which produced amplification products, including *Gr28a* and *Gr28b* (Figure 29A and data not shown). When using gene-specific primers for the five *Gr28b* genes, we were able to amplify spliced mRNAs for *Gr28b.b* and *Gr28b.c* (Figure 29B), both of which are expressed in the abdomen by Gal4/UAS analysis. We were not able to amplify RNA products for *Gr28b.a, Gr28b.e* (which are not expressed in the abdomen by the Gal4/UAS system), or *Gr28b.d* (which actually is expressed in the abdomen by the Gal4/UAS system).

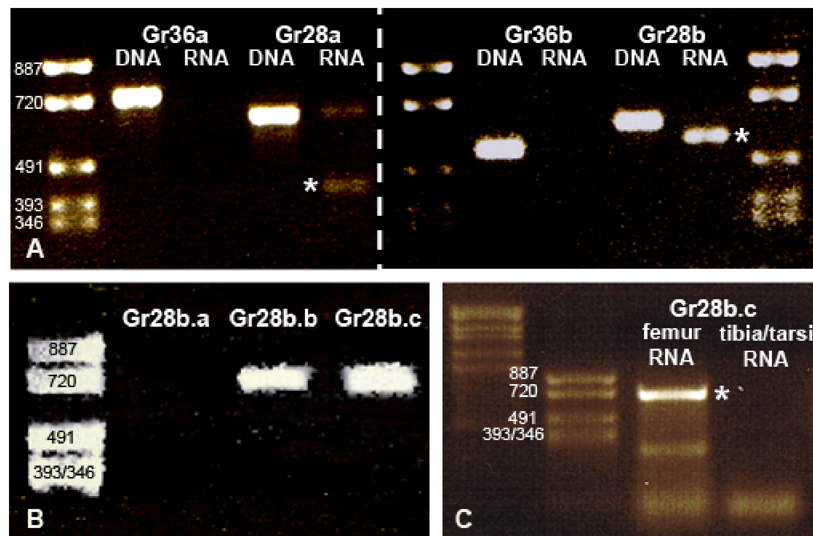


Figure 29: RT-PCR analysis indicates expression of *Gr28* genes in non-taste tissue. RT-PCR using RNA isolated from female terminalia (A and B) and dissected parts of the leg (C) with primers specific for various *Gr28* genes, as well as two control genes

(*Gr36a* and *Gr36b*). Genomic DNA or total RNA were used as templates (as indicated in the respective lanes). For details on PCR conditions and primers, see Material and Methods. (A) *Gr28a* gene-specific primers and primers specific to the common exons of *Gr28b* generate specific PCR products of the expected sizes (asterisks; 407bp for *Gr28a* and 573bp for *Gr28b*). Primers specific for most other *Gr* genes (*Gr36a* and *Gr36b* shown here) did not produce PCR products. Bands in DNA lanes correspond to amplified genomic DNA. (B) RT-PCR on total RNA isolated from terminalia using primers specific to the first exons of *Gr28b.a*, *Gr28b.b* or *Gr28b.c* and a primer to the common exon generate specific fragments for *Gr28b.b* (733bp product) and *Gr28b.c* (724bp product), but not *Gr28b.a* (674bp product). (C) RT-PCR on total RNA isolated from femur and tarsi/tibia RNA shows that *Gr28b.c* is expressed in the femur (724bp product; asterisk), a tissue not known to have taste bristles.

To further demonstrate that the *Gr28* genes are expressed in a tissue not known to contain GRNs, we separated femurs from tibia/tarsi and made RNA from these tissues. The femur harbors the femoral chordotonal organ in which we observed robust *Gr28b.c-Gal4* and *Gr28b.d-Gal4* expression (Figure 22G). In femur, but not tibia/tarsal RNA, a PCR product corresponding to a spliced *Gr28b.c* mRNA was amplified (Figure 29C). This result is consistent with the finding that the *Gr28b.c-Gal4* driver is only weakly expressed in non-taste neurons in the tibia and tarsi, but robustly in the femoral chordotonal organs (Figure 22F and 22G).

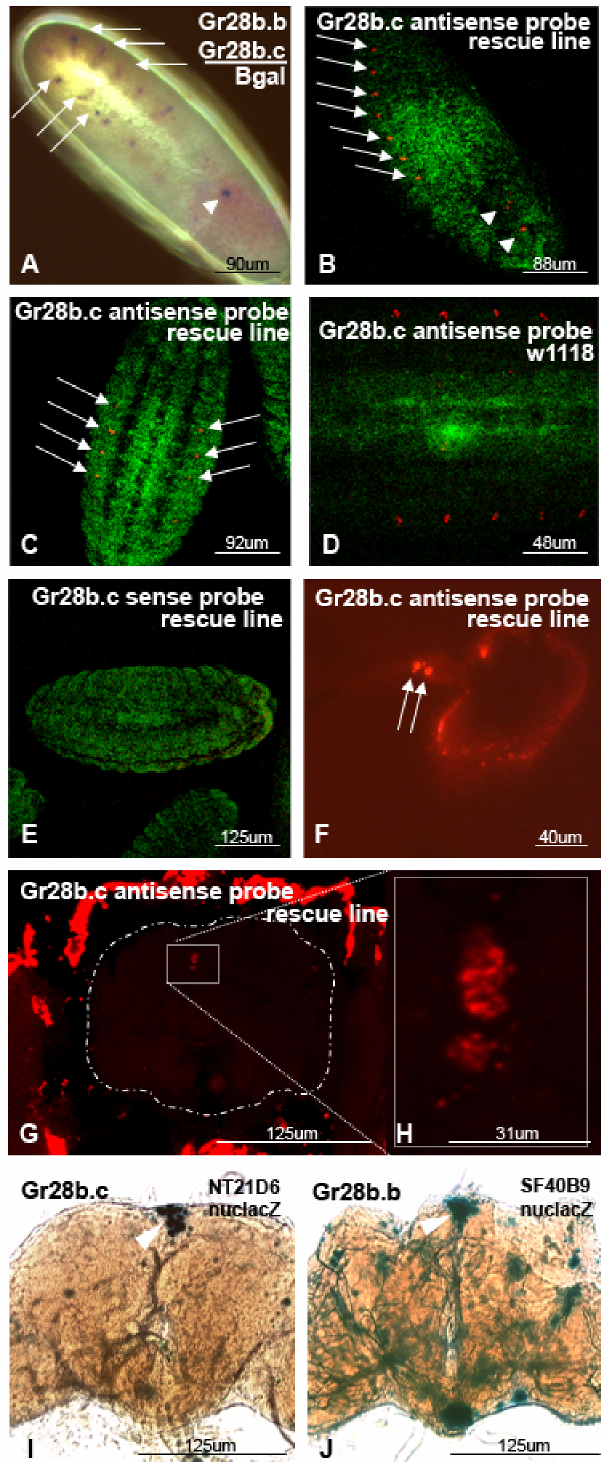
For RNA *in situ* hybridization, we used a full-length antisense probe for the *Gr28b.c* gene, which contains the common two exons and hence should detect all five *Gr28b* RNAs. We wanted to investigate expression in the adult brain and the embryo, tissues that are quite amenable to in situ hybridization methods. In stage 13 to 16 w^{1118} embryos with four extra copies of a genomic construct containing the entire *Gr28* gene

cluster (rescue line; see Materials and Methods), we detected hybridization signals reminiscent of the seven, laterally located cell clusters observed with the *Gr28b.b-* and *Gr28b.c -Gal4* drivers (in Figure 30, compare panel A with panels B+C). In addition, we also observed three to four punctate, internally located signals in the anterior part of the embryo, which correlate well with the cells found in the head region using the Gal4/UAS system (Figure 30A+B, arrowhead). We performed similar *in situ* hybridization experiments on w^{1118} embryos, and detected the same pattern of hybridization in seven, laterally located cell clusters. However, we found that the signal was significantly weaker (Figure 30D). We also produced a an antisense probe that was directed against the two common exons only. We found that transgenic rescue embryos had identical staining to that seen for the full-length *Gr28b.c* probe, but that the signal was weaker. In order to confirm that the staining in embryonic abdominal segments was real, and not an artifact of the probe, we applied the full-length *Gr28b.c* antisense probe to embryos that lacked the *Gr28* genes (see Materials and Methods, this chapter). We did not see any staining in these embryos (Figure 31), further indicating that the staining that we see in abdominal hemisegments of embryos is real *Gr28* expression. Control experiments using the corresponding sense probe did not produce any detectable hybridization signals (Figure 30E).

When we hybridized the antisense *Gr28b.c* probe to sections of adult heads from w1118 flies containing four extra copies of the entire *Gr28* gene cluster, we observed

signals in cells located dorsomedially in the brain (Figure 30G+H). The location of these cells corresponds well with that of β-Gal activity observed in *Gr28b.b-Gal4; UAS-nuclacZ* and *Gr28b.c-Gal4; UAS-nuclacZ* brains (Figure 30I+J) and are the neurons of the pars intercerebralis (see also Figures 25+26). We also searched the preparations from these experiments for hybridization signals in antennal appendages and found two instances of robust signals at the base of the aristae (Figure 30F). Location of these signals is reminiscent to that of the three GFP –positive cells in *Gr28b.d-Gal4/UAS-nucGFP* flies (Figure 22C). When we performed in situ hybridization on heads of w^{1118} flies lacking the genomic transgene construct, we did not detect expression in the CNS, suggesting that the expression of the two endogenous gene copies may be too low for consistent detection by this method.

Taken together, the *Gr28* expression of endogenous RNA in non-chemosensory cells supports the findings observed with the *Gr28-Gal4* drivers, and confirms atypical expression of *Gr* genes in non-chemosensory and brain neurons, suggesting novel functions for these genes.



Gr28b.b
Gr28b.c
Bgal
90um
A
Gr28b.c antisense probe
rescue line
88um
B
Gr28b.c antisense probe
rescue line
92um
C
Gr28b.c antisense probe
w1118
48um
D
Gr28b.c sense probe
rescue line
125um
E
Gr28b.c antisense probe
rescue line
40um
F
Gr28b.c antisense probe
rescue line
125um
G
31um
H
Gr28b.c
NT21D6
nuclacZ
125um
I
Gr28b.b
SF40B9
nuclacZ
125um
J

Figure 30: RNA *in situ* hybridization using antisense RNA complementary to *Gr28b* genes reveals expression in the CNS and embryo. Hybridization experiments were carried out with a full-length antisense probe derived from a *Gr28b.c* cDNA clone and hybridized to whole embryos (stages 13-16) (B to E) or cryostat sections of adult heads (F to H). Embryos and heads were derived from a fly strain with four extra copies of the *Gr28* locus in all *in situ* hybridization panels except (D). For RNA *in situ* panels (B to H), the DIG-labeled antisense probe was detected by a primary anti-DIG antibody and signal was visualized with TSA-Cy3 (red). For more detail, see Material and Methods. For embryonic *in situs* only (B to E), green signal was generated by anti-nc82 (neuropil marker) or anti-ELAV (neuronal marker) followed by ALEXA 488. Anti-nc82 and anti-ELAV staining is somewhat non-specific, but still provides morphological context. (A) X-Gal staining of a *Gr28b.c-Gal4(NT21E7)/ UAS-lacZ* embryo shows punctate staining in abdominal hemisegments (arrows) and staining in the anterior part of of stage 13-16 embryos (arrowhead). (B and C) *In situ* hybridization using the antisense *Gr28b.c* probe recapitulates staining observed with the Gal4 drivers, with punctate positive signal located laterally for each hemisegment (arrows), seen in both lateral (B) and dorsal (C) views in stage 13-16 embryos. In addition, two strong signals are observed in the anterior protion of the embryo (arrowheads), which appears to correspond to the X-Gal staining in (A) (arrowhead). (D) Punctate staining in abdominal hemisegments is also seen in w^{1118} embryos, but the staining is weaker, and only clearly visible at higher magnification (as shown). (E) The *Gr28b.c* sense probe did not produce any positive signal in embryos. (F) Hybridization signals of the antisense *Gr28b.c* probe were observed at the base of the arista (arrows).(G and H) Antisense *Gr28b.c* probe also labels cells in the adult CNS, in a location that corresponds to the pars intercerebralis. The CNS is outlined by a white-dashed line. Outside of the CNS is the head capsule, which shows non-specific staining. The area of the signal (rectangle in G) is shown enlarged in (H). (I and J) Pars intercerebralis expression revealed by X-Gal staining of whole brains from *Gr28b.c-Gal4/ UAS-nuclacZ* and *Gr28b.b-Gal4/ UAS-nuclacZ* flies.

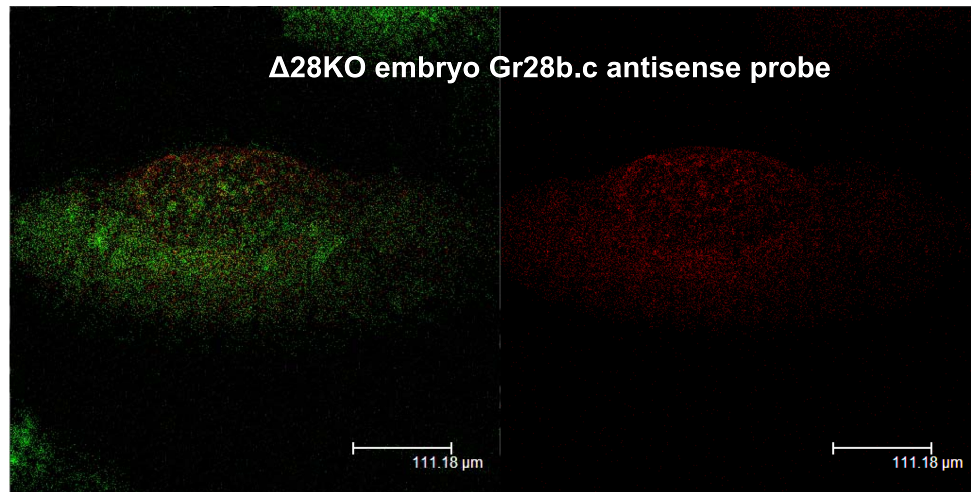


Figure 31: No signal is seen when the full-length *Gr28b.c* antisense probe is hybridized to Δ28 knock-out lines (produced from ends-out homolgous recombination), indicating that the signal seen in wild-type and rescue embryos using the same probe is not an artifact.

3.3 Discussion

Gr genes are thought to encode seven transmembrane, cell surface receptors expressed in chemosensory neurons necessary for the detection of external chemical compounds. Indeed, most *Gr* genes analyzed prior to this study were found to be expressed in the gustatory system of the fly and proposed to detect sugars, bitter and other noxious chemicals, and non-volatile pheromones (long chain hydrocarbons). The only exceptions thus far were *Gr21a* and *Gr63a*, which are expressed in small sets of olfactory sensory neurons and required for carbon dioxide detection (Jones et al., 2007; Suh et al., 2004). Thus, our finding that the expression of four *Gr* genes, all members of a small *Gr* subfamily, in the CNS and in non-chemosensory neurons provides a precedent

of insect chemosensory receptors with likely functions other than the detection of external chemosensory cues.

It is certainly possible that the expression analysis with the Gal4 system does not reveal the entire expression profile of each of the six genes described here. However, because we analyzed the expression profiles of at least three independent lines per driver, and confirmed expression in key tissues using RT-PCR and in situ hybridization, we are confident that the expression profiles reported here are comprehensive and offer a genuine reflection of endogenous *Gr28* gene expression.

In mammals, which employ different types of receptors for the detection of odors and tastants, both olfactory and taste receptors are known to be expressed in tissues other than the olfactory and gustatory sensory cells (Branscomb et al., 2000; Dyer et al., 2005; Finger et al., 2003; Gaillard et al., 2004; Goto et al., 2001; Parmentier et al., 1992; Sbarbati and Osculati, 2005; Vanderhaeghen et al., 1997; Wu et al., 2002). Many olfactory receptors have been found to be expressed in sperm, and it has been proposed that these receptor might play a chemosensory roles during fertilization (Branscomb et al., 2000; Goto et al., 2001; Parmentier et al., 1992; Vanderhaeghen et al., 1997). Moreover, olfactory receptor proteins are also necessary for targeting the axon to the appropriate region in the olfactory bulb during development, a process that requires expression of the receptor at the growth cone (Chen and Flanagan, 2006; Imai et al., 2006; Mombaerts et al., 1996a; Mombaerts et al., 1996b; Serizawa et al., 2006). Taste receptors were also

found to be expressed outside taste cells of the tongue, namely in the gastrointestinal tract (Dyer et al., 2005; Finger et al., 2003; Wu et al., 2002). Whereas a role in chemosensation can easily be deduced in all these cases, the atypical expression of Drosophila Gr genes described here does not provide a straightforward link to chemosensory modalities. Nevertheless, since five of the six genes are clearly expressed in GRNs of various taste organs, the GR28 proteins are likely to function as receptors for external soluble ligands, in addition to other, non-traditional roles.

Three of the genes (*Gr28b.a*, *Gr28b.d*, and *Gr28b.e*) are solely expressed in labellar neurons that express *Gr22e*, neurons known to function as avoidance-type taste neurons (Marella et al., 2006; Thorne et al., 2004; Wang et al., 2004). Additionally, *Gr28b.a* and *Gr28b.e* expression is restricted to the taste system. These receptors, therefore, could be receptors for bitter compounds or other repulsive-type ligands. However, *Gr28a* and *Gr28b.c* are expressed, in addition to *Gr22e*-expressing bitter taste type neurons, in other labellar neurons that likely include sugar sensitive neurons. Neuronal ablation experiments will help to determine the function of these neurons as avoidance or attractive-type neurons, or perhaps both. It is also, of course, possible that *Gr28a* and *Gr28b.c* may act to modulate or modify the interaction of other Grs with tastants. Alternatively, some or all of the GR28 receptors may not be involved in interaction with ligands at all, but may paly a role that is similar to OR83b in the *Drosophila* olfactory system. Olfactory receptors (ORs), to which GRs are related, are proposed to function as

dimers or mulitmers, with the ubiquitously expressed OR83b required for cell surface expression of ligand-binding ORs in olfactory sensory neurons (Benton et al., 2006). No single GR has been identified with ubiquitous expression in taste neurons, but the broadly expressed GR28 receptors may play a similar role in the taste system to that of OR83b in the olfactory system, by facilitating cell surface expression of other GRs. Moreover, GR21a and GR63a are both required in a small group of olfactory neurons in the antennae and mediate CO2 detection, suggesting that this pair of GRs may also function as a multimeric receptor complex (Jones et al., 2007).

3.3.1 Expression of Gr28b.b and Gr28b.c in higher-order chemosensory neurons

We were surprised to find two GRs expressed in the brain; both *Gr28b.b* and *Gr28b.c* are expressed in cells near or associated with the pars intercerebralis (PI) (Figures 25, 26, and 30). Some PI neurons project their axons to the neuroendocrine ring gland in larvae and corpora cardiaca-corpora allata (cc-ca) complex in adults (Rajashekhar and Singh, 1994). Neurons in the PI, known as median-neurosecretory cells (de Velasco et al., 2007), release *Drosophila* insulin-like peptides (Ikeya et al., 2002), which act similar to mammalian insulin in regulating sugar levels in the body. Expression and secretion of the DILPs is dependent on the nutritional state of the fly – satiety or starvation (Ikeya et al., 2002), and ablation of the m-NCSs mimics a starvation phenotype (Ikeya et al., 2002). The PI neurons expressing *Gr28b.b* and *Gr28b.c* extend

afferents that arborize in the anterodorsal part of the tritocerebrum/SOG, the same region that also receives afferent input form GRNs located in the labellum and pharynx. We suggest that the PI cells expressing Gr28b.b and Gr28b.c define higher order neurons in the taste/feeding circuit, and may link activation of gustatory neurons with the endocrine signaling pathway that controls food intake.

Gr28b.b is also expressed in a number of neurons located anterior to the SOG, whose dendrites extend into the tritocerebrum/SOG. These neurons appear to correspond to gustatory interneurons (Mitchell and Itagaki, 1992).

Finally, *Gr28* expression in the CNS is not limited to higher-order taste neurons, as we find expression of *Gr28b.b* in two cell clusters located laterally and dorsally around each antennal lobe. The dendritic processes of these neurons cover most of the glomeruli in a net-like fashion and likely correspond to inhibitory (Ng et al., 2002; Wilson and Laurent, 2005) or excitatory (Olsen et al., 2007; Shang et al., 2007) local interneurons. It is thought that local interneurons facilitate higher order processing of olfactory information, by broadening or condensing olfactory neuron input prior to transmission to second-order olfactory neurons (the projection neurons) and higher-order brain centers. Taken together, our analysis has revealed expression of *Gr28* genes in higher-order neurons in multiple chemosensory circuits.

3.3.2 A role for GR proteins in the metabolism of nutrients?

It is interesting to point out that *Gr28b.b* is not only expressed in the PI where it may be involved in carbohydrate metabolism, but also in oenocytes of larvae and adults. Oenocytes play a critical role in lipid metabolism by regulating lipid stores in fat bodies (Gutierrez et al., 2007), which are the main fat storage tissue in *Drosophila* and considered the equivalent to adipose tissue in mammals. It has been observed that larval oenocytes accumulate large amounts of lipid droplets during nutrient deprivation (fasting/starvation), which parallels hepatocyte function during fasting in mammals (Gutierrez et al., 2007). Interestingly, when oenocytes are ablated by driving expression of the pro-apoptotic reaper gene under control of an oenocyte-specific driver, larvae move away from the normally attractive yeast paste food source and display reduced feeding activity (Gutierrez et al., 2007).

It is interesting to speculate that GR28b.b expression in the PI neurons and the abdominal oenocytes could indicate involvement of this receptor in nutrient signaling and metabolism. For example, this receptor might sense signals of the internal/humoral environment, such as peptides/amino acids or carbohydrates/sugars. Both types of ligands initiate a feeding-type response when encountered in the external environment, and when detected internally as nutrients, serve as a gauge in maintaining energy homeostasis. Although it is known that insulin (mammals) and DILP (*Drosophila*) expression and secretion is influenced by carbohydrate levels (Ikeya et al., 2002; Xu and

Rothenberg, 1998) - glucose in the blood of mammals or trehalose in the haemolymph of *Drosophila* – it is not known whether this is mediated directly by a nutrient sensor. In the yeast *Saccharomyces cerevisiae*, glucose receptors Snf3 and Rgt2 sense extracellular glucose and induce downstream intracellular signals that induce gene expression, without directly facilitating glucose transport into the cell (Ozcan et al., 1998).

Regulation of feeding is mediated by neuropeptides and their receptors. Two examples include the neuropeptides hugin and neuropeptide F, both of which are expressed in the CNS (Bader et al., 2007; Lee et al., 2004; Melcher et al., 2007; Wu et al., 2005). Interestingly, neuropeptide-F alters feeding behavior in response to noxious stimuli, with signaling through this pathway influenced by the insulin-like (DILP) pathway (Wu et al., 2005). Perhaps the GR28 receptors are part of a signaling pathway that plays a similar role in modulating feeding behavior.

3.3.3 Expression of *Gr28* genes in non-chemosensory neurons

A second, surprising observation of our study was to find expression of four *Gr* genes (*Gr28a, Gr28b.b, Gr28b.c* and *Gr28b.d*) in numerous types of sensory neurons, including proprioceptors, nociceptors, and hygro/thermoreceptors. To our knowledge, this finding sets a precedent in insects, as we are not aware of chemoreceptors expressed in non-chemosensory neurons in any other system. We note, however, that expression of membrane-associated signaling proteins in vastly different types of sensory neurons is not unique to *Drosophila* GRs. TRP ion channels, members of which are found in both

mammals and Drosophila, are involved in cellular activation in a variety of sensory processes - thermal detection, hearing, gustation, nociception, and photoreception (Minke and Parnas, 2006; Ramsey et al., 2006). Additionally, some TRP family members are expressed in cells with different sensory functions, for example the *Drosophila* TRP channel *painless* is expressed in nociceptors, taste neurons, and Johnston organ neurons (Al-Anzi et al., 2006). It is also fairly common for a single member of the TRP family to demonstrate activation by different types of sensory stimuli – such as heat and the "hot" chili pepper component capsaicin, in the case of mTRPV1 (Ramsey et al., 2006).

In conclusion, we have reported that members of the *Gr28* family are expressed in primary sensory neurons of the chemosensory, as well as other sensory systems, and in higher-order chemosensory neurons in the CNS. Functional, genetic analysis will ultimately reveal the specific role(s) of these receptors in taste sensation and in other sensory modalities. Moreover, it will be interesting to see whether other *Gr* genes might be expressed in an atypical fashion in non-chemosensory neurons, and hence, a more detailed and comprehensive analysis of this gene family is warranted.

3.4 Materials and Methods

3.4.1 Gene conservation/amino acid identity

In order to determine conservation of gene orientation and alternative first exon usage among different *Drosophila* species, we used blastn (nucleotide to nucleotide

query) found on the publicly available Flybase database (flybase.bio.indiana.edu). We also used blastp (amino acid to amino acid query) to search the GLEANR protein predictions database to find putative orthologs and confirmed results using tblastn (amino acid to nucleotide query). We determined amino acid identity between a GR28 and its putative ortholog using alignments generated by blastp or tblastn as well as by using ClustalW protein alignment from the MacVector software package. Putative orthologs of *Drosophila* GRs in *Anopheles gambiae* were found using the publicly available ensembl (www.ensembl.org) database.

3.4.2 Fly lines used

Most Gal4 driver lines used were generated by us and described below with the exceptions of *p[Gr28b.e]-Gal4* from the lab of K. Scott (Scott et al., 2001) and *p[ppk]-Gal4* from the lab of M. Welsh. UAS reporter lines were obtained from the Bloomington Drosophila Stock Center at Indiana University (flystocks.bio.indiana.edu).). Names of driver lines used for expression analysis of the *Gr28* genes are listed here: *Gr28b.a* – NT42aA48a, NT42aF15a, NT42aC51a, NT42aE52a; *Gr28b.b* – SF40A4, SF40C5, SF40B9, SF40E3; *Gr28b.c* - NT21B1, NT21D6, NT21E7, NT21C4; *Gr28b.d* – Gr28a4B27, Gr28a4E29, Gr28a4C13b3; *Gr28b.e* (from K. Scott) – Gr28a3B, Gr28a3AII; *Gr28a* – SF36B1, SF36G, SF36S, SF36E1.

3.4.3 Production of transgenic Gal4 lines

The transgenic Gal4-driver line for *Gr28b.e* was produced and provided by K. Scott (Scott et al., 2001). The other transgenic *p[Gr]-Gal4* driver lines were generated by PCR (targeting sequence immediate upstream of ATG) from genomic DNA of w1118

flies, with the resulting putative promoter fragment initially cloned directly into pGEMT, excised with Acc65I/Not1, and sub-cloned into the SM1 vector upstream of Gal4. Primers used to produce the putative promoters were: Gr28b.a (1400bp promoter fragment) 5′-GGGTACCATAACCCAGTGACTGACTTTG-3′ and 5′-GCTCGAATCCGAATAAATTAACGCTC-3′; Gr28b.b (1088bp promoter fragment): 5′-CGAGCTCCTGGCCATTTCCATCAC-3′ and 5′-GGCGAAATCGAACTAATGCACACCGG-3′; Gr28b.c (1326bp promoter fragment); 5′-CCGGCTGCTCGTCTCCCTGGATGT-3′ and 5′-CGCTTCGTTTGAGCTTCAACCGGC-3′; Gr28b.d (772bp promoter fragment): 5′-GGGTACCATGGCCCTGCACTTCACC-3′ and 5′-TCAACGTATTTTTAAAATATTTATAGTATGTAT-3′; Gr28a (1200bp promoter fragment): 5′-GGAGGCATAGTGCACTCCCTAC-3′and 5′-GATAAGACCGTTTGATATGG-3′. In all cases, the Acc65I cut site (GGTACC) was included/incorporated in the 5′ (upstream) primer to aid in the sub-cloning step.

3.4.4 RT-PCR

Tissues were collected and used to make RNA with Trizol Reagent from Life Technologies (#15596-926). We used Superscript Choice System, also from Life Technologies (#8090RT), for first-strand cDNA synthesis using random hexamer primers. We used the Elongase Enzyme Mix (Life Technologies; #10480-028) for subsequent PCR reactions. All cDNA was initially tested with primers against tubulin to check for quality. Primers used to detect Gr transcripts were directed towards

sequence flanking an intron, such that (contaminating) gemomic amplification products could be distinguished from amplification products that resulted from transcripts based on their difference in size. All amplification products were cloned (into pGEMT) and sequenced to confirm their identity. Primers used: Gr28a: 5′-GGGCATGAAGTTGGACTACCGCAGG-3′ and 5′-CGGGATTGAGGCGTATGACGGAGG-3′; Gr28b (common exon): 5′-CCCATCAATGGGACACCCGAAGCCT-3′ and 5′-GAGATAAGTGGTCAAGGCCCCGCTG-3′; Gr28b.b: 5′-CCGGCTGCTCGTCTCCCTGGATGT-3′ and 5′-GAGATAAGTGGTCAAGGCCCCGCTG-3′; Gr28b.c: 5′-CCTCGGAAGTGGCGCCCACCA-3′ and 5′-GAGATAAGTGGTCAAGGCCCCGCTG-3′.

3.4.5 RNA fluorescent *in situ* hybridization of embryos and adult CNS

Adults in cages were allowed to lay on apple juice plates with yeast for three hours before plates with embryos were aged at 18°C for 26 hours to give stage 13-16 embryos. Embryos were dechorionated with 50% bleach, fixed in heptane/formaldehyde/PBS, and devitellinated with methanol. Embryos were hybridized overnight at 55°C. For adult CNS in situs, six adults were placed in fly collars, the heads were embedded in OCT and then sectioned at 14μm. After fixation in

4% paraformaldehyde, the tissue was subjected to acetylation. Tissues were hybridized with the probe for 24 hours at 65°C.

The template used to make the probes was of a full-length 1414bp cDNA of *Gr28b.c* cloned into pGEMT. In order to make the antisense *Gr28b.c* probe, the construct was linearized with SpeI (in polylinker of pGEMT at 5′ end of transcript) and the T7 RNA polymerase site used to make the antisense transcript. In order to make the antisense *Gr28b* common exon probe, the construct was linearized with Eco47III, which cuts just after the beginning of the first common exon, to produce a probe of approximately 623nt using the T7 RNA polymerase site. The construct was linearized with NotI in order to make the sense *Gr28b.c* probe, and the SP6 RNA polymerase site was used for in vitro transcription. RNA probes were labeled with incorporated DIG-UTP using a kit from Roche Applied Biosciences (#1277073). Detection of DIG used anti-DIG-POD (Fab fragments; Roche Applied Biosciences, #11207733910) followed by tyramide signal amplification using Cy3-labeled tyramide (TSA-Cy3; Renaissance TSA Fluorescence Systems from PerkinElmer, #NEL704A).

3.4.6 Production of *Gr28* genomic rescue line

The 14.5kb genomic region containing the *Gr28* locus plus approximately 3.4kb 5′ and 1.5kb 3′ to the *Gr28* genes was cloned into the vector pCaSpeR4 in two steps. A BAC clone (BacR30I17; also RP98-30I17) containing the *Gr28* locus was obtained from the BACPAC Resource Center, and was used to isolate an approximately 13kb fragment

(containing all *Gr28* genes plus approximately 1.8kb 5′ sequence and 1.5kb 3′ sequence) via KpnI/StuI restriction endonuclease digestion. This fragment was cloned directly into pCaSpeR4. PCR was used to amplify a 1.5kb fragment that contained additional 5′ sequence. This fragment was initially cloned into pGEMT, and then sub-cloned into pCaSpeR4 using StuI and PstI cut sites. This 22.4kb construct was then injected into w1118 embryos to make transgenic flies.

Immunofluorescence: All reagents used, as well as procedures carried out, have been previously described in Thorne et al.(2004). Briefly, *p[Gr-promoter]-Gal4/ UAS-nucgfp* (or *UAS-mCD8::GFP* or *UAS-nsybEGFP*) flies were aged for at least four days before dissection. Primary antibodies used were rabbit anti-GFP (Molecular Probes; #A6455) at 1:1000 dilution, mouse anti-ELAV as a neuronal marker (provided by L. Vosshall) at a 1:10 dilution, mouse anti-nc82 (provided by R. Stocker) as a neuropil marker at 1:10 dilution, mouse anti-GFP (for larval fillet only – Figure 27) at 1:250 (Invitrogen; #A11120), and rabbit anti-HRP as a neuronal marker at 1:100 (Sigma; #P97899). Secondary antibodies used were goat anti-rabbit Cy3 (Jackson Immunoresearch Laboratories; #111165144) at a 1:500 dilution, goat anti-mouse ALEXA 488 (Invitrogen; #A11017) at a 1:100 dilution, and for larval fillet staining only goat anti-mouse ALEXA 488 (Invitrogen; #A11029) at 1:400 and goat anti-rabbit ALEXA 568 (Invitrogen; #A11036) at 1:400. Specimens were viewed with a confocal microscope (see below) or with a Zeiss Axioskop2 fluorescence microscope.

3.4.7 Confocal microscopy

A Leica TCS SL spectral confocal microscope (with a Leica DMIRE2 microscope) was used for immunofluorescence imaging. For all optical stacks produced, optical sections were taken at intervals of 0.2-0.5μM. Multicolored, depth-coded images from a single channel, which revealed only GFP-expressing neurons, were produced with the Leica LCS software. Adobe Photoshop CS2 was used to adjust contrast and brightness in images, when required.

3.4.8 X-Gal staining

Staining was carried out as previously described by Dunipace et al. (2001). In order to stain adult CNS, brains were dissected out of the head cavity after fixation and prior to exposure to staining solution. The amount of time tissues were stained for varied (depended on strength of Gal4 driver), but was generally between eight hours and overnight.

3.5 Acknowledgements

We would like to thank Drs. Reinhard Stocker, Dan Tracey, Steve Crews, and Nina Tang Sherwood for their advice and assistance in identifying cells and tissues that express the *Gr28* genes. We also would like to acknowledge the technical assistance and expertise of Lixian Zhong, Amaris Guardiola, and Elizabeth Graham. This work was supported by the National Institutes of Health (RO1 GMDC05606-01) to H.A.

3.6 Future directions

The expression pattern of the *Gr28* genes is interesting, and RNA *in situ* data indicates that at least one of the *Gr28b.b* genes is expressed in the CNS and non-taste neurons of the embryo abdominal segments. Confirmation as to the identity of the neurons/cells expressing these receptors is not known. It is interesting and essential to determine if *Gr28b.b*/*Gr28b.c* are actually expressed in DILP-producing cells of the pars intercerebralis. In addition, determining whether the identity of the embryonic abdominal cells that express these two receptors are indeed oenocytes would be additionally interesting.

Although the expression profiles are suggestive, based on this, functions for the *Gr28* genes can only be speculative. The only way to definitively determine functions for these genes is to produce animals that lack this gene cluster or members of this gene cluster. Because these *Gr*s are highly conserved, have a unique and suggestive expression profile, and are tightly located in a gene cluster with alternative splicing, they were considered good candidates for gene knock-out experiments. Production of *Gr28* knock-out animals, which could then be used for behavioral assays to determine the function of these genes, served as the final chapter of my graduate work.

4 Determining the function of the *Gr28* genes: putative function for cells expressing *Gr28*s and production of *Gr28* deletion animals and *Gr28* gene knock-out animals

4.1 Introduction

4.1.1 Cell function as a clue to gene function

Drosophila are attractive model organisms for numerous reasons, but their true value in the study of chemosensory systems owes a lot to the combination of the molecular genetic tools available and the ease of working with large numbers of animals in behavioral assays. A few of the technologies that we use to look at gene expression (Gal4/UAS system) and cell function (expressing cellular toxins via Gal4/UAS system) have been briefly discussed in previous chapters. In order to determine the function of a gene (reverse genetics), the most conclusive method is removal of that gene, but doing this is not always technically easy, and requires a large amount of time, regardless of the technique employed. For this reason, many groups have used the Gal4/UAS system to functionally ablate cells that express the gene of interest, like using the UAS-TNT reporter described previously, or to actually cause cell death, as with the UAS-Diptheria toxin reporter. While not offering an answer as to the specific function of the gene, these

experiments can provide insight into putative functions, especially if the gene of interest shows a restricted expression pattern that is limited to a relatively small number of cells that probably have the same function. This technique worked extremely well in identifying and characterizing the avoidance- and attractive-type taste neurons.

An alternative technique that works well in the chemosensory system, where many primary sensory neurons elicit a simple attraction or avoidance behavior, is to use the Gal4/UAS system to drive expression of a non-endogenous receptor for a ligand that normally produces no response in the fly (Tobin et al., 2002). The mammalian vanilloid receptor (VR1) is it not expressed in *Drosophila,* and its ligand, capsaicin, produces no behavioral responses (Marella et al., 2006). As previously described in Chapter 2, Marella and coworkers used this system to show that the neurons that express *Gr66a* mediate repulsive-type responses, whereas the neurons expressing *Gr5a* mediate attractive-type responses. This was simply determined by exposing the proboscis of adult transgenic animals (*p[Gr]-Gal4; UAS-VR1*) to capsaicin to see if the frequency of proboscis extension increased or was inhibited. Control flies have no change in frequency of proboscis extension, whereas *p[Gr66a]-Gal4* flies showed a decreased frequency and *p[Gr5a]-Gal4* flies show an increased frequency.

The Gal4/UAS system can also be used to visualize the activation of neurons expressing a gene of interest. Reporters in this system tend to be fluorescing proteins that contain a calmodulin motif, that, upon binding to intracellular Ca^{2+}, followed by

subsequent conformational change in the protein, cause a change in the wavelength and/or intensity of fluorescent emissions (Fiala et al., 2002; Miyawaki et al., 1999; Nakai et al., 2001; Wang et al., 2003). This technique has been used extensively in the *Drosophila* olfactory system (Fiala et al., 2002; Wang et al., 2003), and recently in the taste system (Marella et al., 2006). In the olfactory system, two reporters – cameleon 2.1 and G-CaMP – were selectively expressed in olfactory sensory neurons, whose axons, like those of taste receptors, project directly to the CNS. By removing a small piece of cuticle from the head capsule, the activation of live neurons can be microscopically visualized. Expression of cameleon 2.1 or G-CaMP during exposure of the fly to various odorants allows an odorant activity profile to be determined for neurons expressing a given receptor as well as characterizing which CNS targets receive specific odorant information (Fiala et al., 2002; Wang et al., 2003). As described in Chapter 2, G-CaMP was used to show activation of *Gr66a*-expressing neurons in response to bitter compounds (Marella et al., 2006).

In order to determine if the *Gr28* genes were expressed in taste neurons involved in avoidance- or attractive-type feeding behavior, we drove expression of the mammalian vanniloid receptor, VR1, in cells that express *Gr28b.c*. With expression in neurons of the larval TO and mouth, larval preference assays using 0.1mM capsaicin demonstrated that *Gr28b.c* is expressed in neurons that mediate avoidance-type feeding behavior.

Determining the function of the cells in which a gene of interest is expressed can provide important clues as to the function of the gene, however, ultimately in order to determine gene function, phenotypic analysis must be conducted on animals in which the gene is remove, mutated, or in some cases, overexpressed. In an effort to understand the function of the *Gr28* genes, animals were made in which the *Gr28* locus was removed, using two different methods – *piggyBac*-mediated deletions and ends-out homologous recombination.

4.1.2. P-elements and *piggy-Bac*-based deletions

P-elements are naturally occurring transposable elements – short sequences of DNA that can excise and insert within the genome – that have become genetic tools to explore gene function and regulation (Castro and Carareto, 2004; Venken and Bellen, 2005). One commonly used technique is that of excising a P-element in or near a gene of interest, with the hope of creating a deletion of the gene of interest due to the imprecise excision of the P-element. The usefulness of P-elements in reverse genetics has developed greatly in the last few years. There has been concerted effort by the Gene Disruption Project to place P-elements in or near every *Drosophila* gene, to be used as tools to explore gene function (Venken and Bellen, 2005). In addition, availability of the entire *Drosophila melanogaster* genome sequence has allowed researchers to determine the precise location of P-elements. Using traditional P-elements to knock-out or mutate

genes has its down-side, though. P-elements tend to be found clustered in "hot-spots", regions of the genome that for unknown reasons are more frequently targeted by P-elements (Venken and Bellen, 2005). Additionally, there are areas of the genome that seem recalcitrant to P-element insertion, so if one's gene of interest is in this area of the genome, P-elements cannot be used as tools for producing deletions.

Another type of transposable element – *piggyBac* – is now used to complement the use of traditional P-elements. *piggyBacs* do not have the insertional preferences that P-elements do, allowing wider genome coverage (Thibault et al., 2004; Venken and Bellen, 2005). Another feature of *piggyBacs* is that they excise precisely, which may not be helpful for disrupting a gene in which a *piggyBac* is found, but allows them to be used in *trans* to produce deletions (Parks et al., 2004; Thibault et al., 2004). The Exelixis group has generated almost 30,000 *piggyBac* insertion lines (lines that contain a single *piggyBac* element at a defined location), approximately 19,000 of which are available through the Bloomington Stock Center and Harvard University (Thibault et al., 2004).

The *piggyBacs* created for use by Exelixis contain a number of features that make them very amenable in their use to create deletions. Firstly, these elements contain a marker – the mini-*white* gene – which allows them to be tracked and mapped. Secondly, the *piggyBac*s made by Exelixis contain an FRT site, which, upon exposure to FLP recombinase, allows *trans*-recombination to occur between two *piggyBacs* located in *trans* on homologous chromosomes (Parks et al., 2004). Recombination between FRT sites

effectively removes sequence flanked by the two *piggyBacs* in *trans*. In this way, precise deletions of known size can be produced. The site where the deletion occurs remains marked by a single *piggyBac*, and depending upon the type of *piggyBacs* used, will allow screening for possible deletion lines via loss of the mini-*white* gene (due to recombination between sequences in the *piggyBac* elements themselves). Retention of a single *piggyBac* also allows for determination of a deletion via PCR using primers against the *piggyBac* and expected flanking sequence. Typically, there is a very high rate of producing a deletion of the desired region/gene of interest, with about 1/5 or 1/50 progeny with the deletion after the last cross (Parks et al., 2004).

We decided to use this technique of *piggyBac*-generated deletions due to its high rate of success, and produced seven lines that are excellent candidates for a Δ28 deletion. The trade-off in using this technique is that the deletions produced are relatively large, in this case, approximately 50kb, and require making transgenic rescue lines to replace genes flanking the *Gr28* locus. In order to produce deletions that were specifically targeted to the *Gr28* genes, we used ends-out homologous recombination.

4.1.3 Ends-out gene targeting as a method to produce gene deletions

Whereas producing deletions using the Exelixis *piggyBac* insertion lines produces desired deletions at a high frequency(Parks et al., 2004), with the downside of producing a large deletion that requires reintroduction of lost genes, gene targeting and replacement by homologous recombination produces desired deletions at a lower

frequency, occurring in only 1 in 30,000 gametes (Venken and Bellen, 2005), but the deletion produced is usually of a single gene.

Homologous recombination to produce deletions in *Drosophila* has only been around for a relatively short period of time, whereas homologous recombination techniques had been used successfully in mice and yeast for years to produce knock-out organisms. Significant problems in inducing homologous recombination between introduced DNA and homologous host DNA had prevented its use in *Drosophila* until recently(Rong and Golic, 2000). In 2000, however, Rong and Golic published their first paper describing successful use of this technique in *Drosophila*.

Homologous recombination describes the process in which exogenous DNA containing modified/mutated sequence replaces the homologous sequence in the host's genome, using the host's DNA repair machinery to recognize the double-stranded break in the introduced DNA (Gong and Golic, 2003; Rong and Golic, 2000). The introduced, or donor DNA, thus has to contain three elements to allow successful targeting: sequence homologous to the region targeted, a double-stranded break, and a marker gene (such as mini-*white*, to allow detection of integration) (Gong and Golic, 2003). Rong and Golic (2000) developed and optimized the technology in *Drosophila* using a donor construct that contained two FRT sites, that would allow excision of the donor construct from an untargeted region of the genome (flies are made that are transgenic for untargeted donor construct) upon exposure to FLP-recombinase. The donor construct

also contains an I-SceI endonuclease cut site (from yeast, and therefore not found in *Drosophila* genome), so that once the construct is excised (and circular), exposure to the expressed enodonuclease creates a recombinogenic double-stranded break. The donor construct is "exposed" to the FLP-recombinase and I-SceI endonuclease upon crossing transgenic lines containing these genes to the donor construct line. FLP-recombinase and I-SceI endonuclease expression are under the control of a heat-shock promoter (Rong and Golic, 2000).

Ends-out and ends-in gene targeting differ in the placement of the I-SceI cut site in the donor. If the cut site is placed at each end of the homolgous sequence, then the donor construct produces ends-out recombination, replacing the targeted gene with the mini-*white* gene (Figure 32). If the I-SceI cut site is located within the targeted gene itself, then upon integration of the donor construct, a duplication of the targeted gene occurs (Gong and Golic, 2003; Rong and Golic, 2000; Venken and Bellen, 2005).

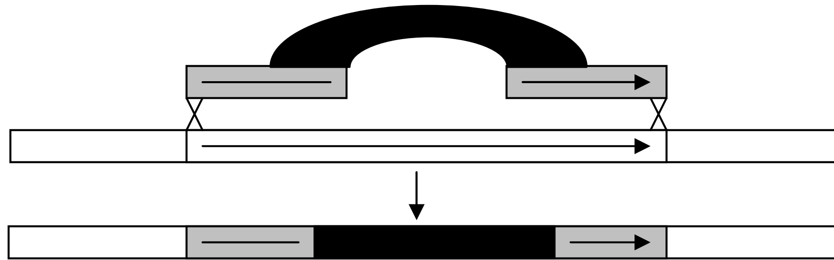


Figure 32: Schematic of ends-out gene targeting. The donor construct recombines with the endogenous locus to cause replacement with the mini-*white* gene (schematic adapted from (Gong and Rong, 2003))

We made a donor construct that would target and replace the entire *Gr28a* gene and the two common exons of *Gr28b* via ends-out gene targeting. Two separate rounds of homologous recombination produced two lines in which the *Gr28* genes were deleted – Δ28 30i and Δ28 54B3. These *Gr28* mutant flies could then be used in behavioral assays to determine the function of the *Gr28* genes.

4.2 Materials and methods

4.2.1 VR1 behavioral assays

Gr28b.c (NT21B1)-Gal4 flies were crossed to *GCaMP-56; UAS-VR1; TM2/TM6* (kindly provided by the lab of Dr. Richard Axel) to produce progeny that were *Gr28b.c-Gal4/UAS-VR1*. For controls: we used w^{1118} , the driver alone - *Gr28b.c (NT21B1)-Gal4* as a negative control, *Gr66a(KSA6A)-Gal4/UAS-VR1* as a positive control (this line should avoid capsaicin), and *Gr5a (Gr5a 2.6)-Gal4/UAS-VR1*. Wandering 3rd instar larvae were used for all behavioral assays. Larvae were collected from vials, briefly washed in PBS,

and put on wet Whatmann paper until the time of the assay. The assay was performed as soon after collection as possible, as we didn't want the larvae to become starved ("starvation" in larvae occurs occurs after only two hours). Larvae were then place in 60x15mm petri dishes, with one half of the dish filled with capsaicin (plus agarose) and the other half with just agarose. Plates were made directly before the assay was performed. Briefly, capsaicin was solubilized in 200 proof ethanol to make a stock solution. This capsaicin stock was then diluted in distilled water and added to 1% agarose solution before being poured into plates. The control solution was 200 proof ethanol of the same concentration as was added when adding the capsaicin stock, plus 1% agarose. Directly before the experiment, agarose from plates were cut out and replaced to make plates that were one-half capsaicin and one-half control. Larvae (n=35-40 per experiment) were then placed in the middle and allowed to wander to either side. The number of larvae on either side of the plate were counted at one hour intervals for four to five hours. It is noted that substrates may diffuse from one side of the plate to the other over the time period, but we didn't find this affected the results, ie results were consistent over time, the larvae either avoided one side or they didn't. The preference index (PI) for capsaicin is calculated as the number of larvae on the capsaicin side/total number larvae feeding (on both sides). Larvae on the sides or top of the petri dish were not counted. A PI of 0.5 indicates the larvae have no preference for either substrate (and are found equally on either side of the plate), a PI < 0.5 indicates preference for the

control (avoidance of capsaicin), and a PI>0.5 indicates a preference for capsaicin (attraction to capsaicin).

4.2.2 Producing *piggy-Bac* based deletions

In order to create *piggyBac*-based deletions, it is necessary to use *piggyBac* elements have FRT-sites that are in the same orientation, such that FRT-mediated recombination can occur between them. We found two sets of *piggyBacs* that contained FRT sites in the same orientation and could be used to produce deletions of approximately the same size – 50kb (Figure 33). One set was in the "plus" orientation, and the other set was in the "minus" orientation. It was decided to create deletions using both sets of *piggyBacs*, in order to increase the odds of successfully creating a deletion. The positive orientation elements used were WH(+)898 and WH(+)3909. The minus orientation elements used were WH(-)2768 and WH(-)3605.

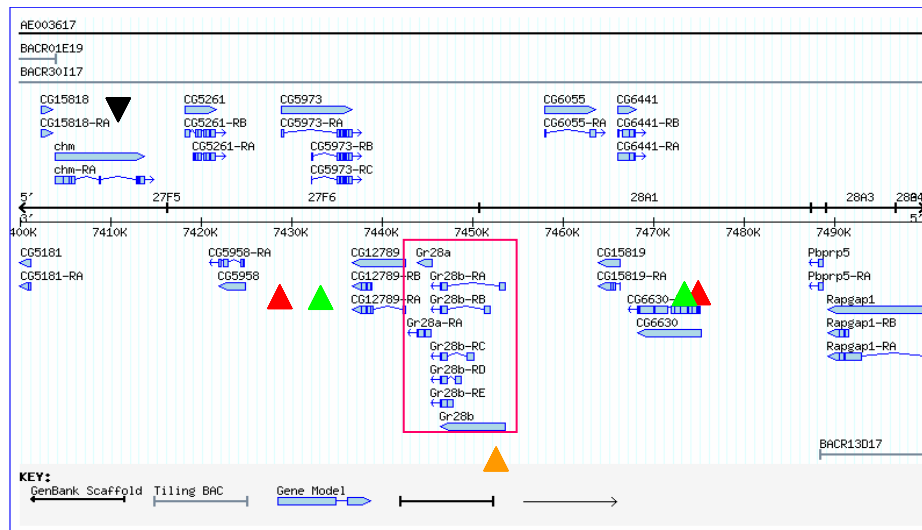


Figure 33: ***Gr28*** **locus (on chr. II) showing location of** ***piggyBac*** **elements used to create deletion. Red arrowheads indicate WH(+)** ***piggyBacs*** **and green arrowheads indicate WH(-)** ***piggyBacs*** **used to create deletion of** ***Gr28*** **locus. Black and orange arrowheads indicate other insertional elements (gene region copied from FlyBase website GBrowse: http://flybase.bio.indiana.edu).**

The following crosses were used to generate deletion lines as per Parks et al. (2004) (Figure 34).

1)♂isow/Y; WH(+/-)**1**/CyO X ♀$_{virgin}$ hs-FLP/hs-FLP; Adv^{1}/CyO 6 bottles; n= 80/bottle
2)♂hs-FLP/Y; WH(+/-)**1**/CyO X ♀$_{virgin}$ isow/isow; WH(+/-)**2**/CyO 10 bottles; n=80/bottle
3)Allow cross (2) to lay for 2 days in bottles, removed and allowed to lay for two more days in another bottle. As progeny from (2) cross have WH(+/-)**1** and WH(+/-)**2** in trans as well as a source of FLP-recombinase induced by heat shocking, parents and progeny were heat shocked at 37◦C for one hour in water bath. Progeny underwent heat shocks for four more days, for a total of five heat shocks.
4) ♀$_{virgin}$hs-FLP/isow; WH(+/-)**1**/WH(+/-)**2** X ♂isow/Y; wg^{Sp-1}/CyO; sens^{Ly-1}/TM6,Tb^{1} 4 bottles; n=30♀$_{virgin}$; 60♂
5) Red-eyed, CyO males from cross 4) were collected; these have the putative deletion
6)♂isow or hs-FLP/Y; putative Δ28(w^{+})/CyO X ♀$_{virgin}$ Exelixis balancer line 150 vials set up; n=1 ♂; 2 ♀$_{virgin}$

Figure 34: Crossing scheme for generating ***piggyBac*****-based deletions**

I obtained 300 individual lines with putative deletions of the *Gr28* locus, as 150 individual crosses had been set up for WH(+) *piggyBac* insertion lines and 150 individual crosses were set up for WH(-) *piggyBac* insertion lines. 50 lines from each were tested with PCR to determine if a deletion existed for that line. Because of the *piggyBac* elements used, it was not possible to screen for a deletion based on loss of the mini-*white* gene, thus a greater number of putative deletion lines had to generated and tested molecularly for the deletion.

In order to do PCR on genomic DNA from 100 lines (50 from each WH pair), it was necessary to use a technique that allowed high-throughput production of genomic DNA. This protocol only uses five flies to produce DNA and can be found in the Supplementary Methods online for Parks et al. (2004).

We weren't able to screen using loss of the mini-*white* gene as this marker was retained with the remaining recombinant *piggyBac* element, therefore, we conducted PCR analysis with six primer sets to determine whether the obtained strains contained the desired deletion. One member of each primer set was directed against the *piggyBac* element ("pWH" primers), and the other primer of the sets was directed against flanking sequence.

PCR reactions were all Primers called "pWH" are directed toward the *piggyBac* element and the other primers are directed toward flanking sequence. The following primer pairs are not expected to amplify a product if the line has a deletion for WH(+)

piggyBac: pWH2+pNTplus.f3, pWH2+p3909.2a, p898.1+pWH1, pNTplus.f4+pWH1. The following primer pairs should amplify a product if there is a deletion for WH(+)*piggyBac* (amplification product in parentheses): pNTplus.f1+pWH1 (132bp), p898.2+pWH2 (685bp). The sequences for these primers are: p3909.1a 5′-GCTATGAACTTCACCCGGTGGC-3′; pWH1 5′-TCCAAGCGGCGACTGAGATG-3′; pWH2 5′-CCTCGATATACAGACCGATAAAAC-3′; pNTplus.f3 5′-CCAGAGAGCCATAAGTTGCTTG-3′; p3909.2a 5′-CGACTTCACTTGCCACAGC-3′; p898.1 5′-GGGAGTGAGTGAGATGGCAATAAGC-3′; pNTplus.f4 5′-GAGAGACCAACACACGCAGCG-3′; p898.2 5′-GGACACGCCGATAAAGTTACC-3′; pNTplus.f2 5′-CCGAGGGTAGTTGCTCTGATGG-3′.

The following primer pairs are not expected to amplify a product if the line has a deletion for WH(-)*piggyBac*: pWH1+pNTminus.f3, pWH1+p2768.2, p3605.1+WH2, pNTminus.f4+WH2. The following primer pairs should amplify a product if there is a deletion for WH(-)*piggyBac* (fragment sizes in parentheses): pNTminus.f1+pWH2 (1916bp), pWH1+pNTminus.f2 (200bp). The sequences for these primers are: pNTminus.f3 5′-CAGCGCCACAAATGACGAAGC-3′;p2768.2 5′-GCGTTTCACTTACTGTCACTGGCAAG-3′; p3605.1 5′-CGAACACATGCCACTGCTCAG-3′; pNTminus.f4 5′-GCAGTAGATGTGGCAGTTACGAC-3′; pNTminus.f1 5′-

GCAAGGCACGAGAAATGCATTAGAC-3′; pNTminus.f2 5′-CCTATCTGAACTTCCAGCACTCATG-3′.

4.2.3 Production of 26kb genomic construct to rescue genes upstream of the *Gr28* locus

A deletion at the *Gr28* locus using *piggyBac*-based FRT-recombination produced deletions of approximately 50kb. In order for the mutant fly lines to be used for behavioral experiments to see if they had a taste-related phenotype due to loss of the *Gr28* genes, it is necessary to reintroduce the other genes. Reintroducing these other genes would also rescue the lethality produced in the homozygous deletion lines. Additionally, since this region of the chromosome is gene-rich, it was not possible to put back genes individually by making cDNA constructs, as this would require replacing nine genes. Instead, production of two larger genomic DNA constructs, one that included upstream genes, and another that included downstream genes, would be used to rescue the lethality. I was able to make one of these constructs, of approximately 23kb, that replaced upstream genomic DNA (Figure 35).

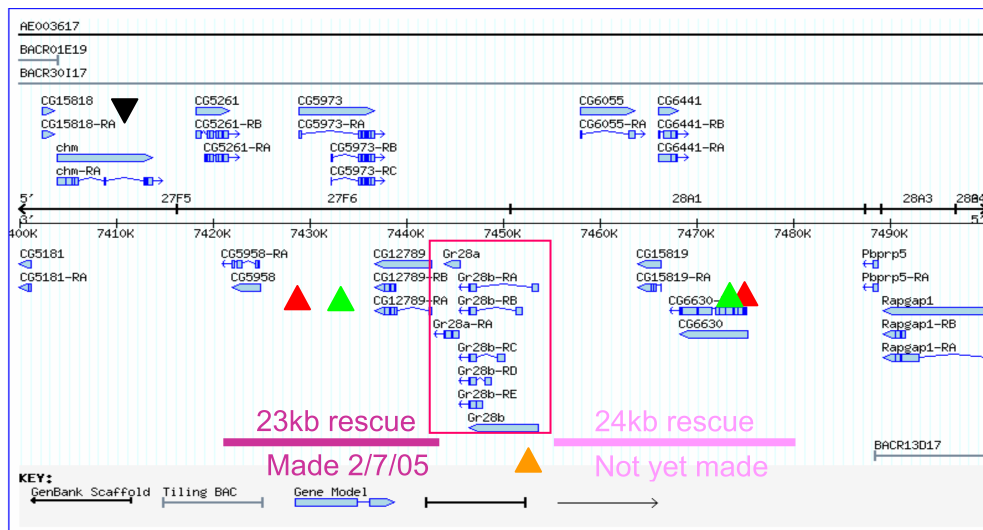


Figure 35: ***Gr28*** **locus showing upstream and downstream rescue constructs to replace genes lost in producing** ***piggyBac*****-based deletions (gene region copied from FlyBase website GBrowse: http://flybase.bio.indiana.edu).**

In order to clone a 23kb genomic fragment, we obtained a BAC clone from BACPAC Resources (http://bacpac.chori.org) that contained this portion of the second chromosome – RP98-30I17. The BAC clone was digested with XbaI and SbfI to release the 23kb fragment. The Xba1 cut site is located at the 5′ end of the construct, and was found between CG5261 and CG5958 on the second chromosome. The SbfI cut site at the 3′ end of the construct is located in the coding sequence of *Gr28a*. Gel purification and shotgun cloning of XbaI/SbfI fragments directly into the pCaSpeR4 vector (which was cut with XbaI and PstI; PstI and SbfI produce compatible cohesive ends) produced a 31kb construct termed "NT48B7f". This construct was sequenced and used to make transgenic flies called "NT48". The second genomic construct carrying genes downstream of the *Gr28* genes still needs to be made.

4.2.4 Ends-out homologous recombination

4.2.4.1 Donor construct production

A vector, CMC105, designed for ends-out insertion of the mini-*white* gene at the target locus, was generously provided by Dr. Gary Struhl. Approximately 5kb of both 5′ and 3′ homologous sequence was subcloned upstream and downstream of the mini-*white* gene to produce the donor construct (Figure 36).

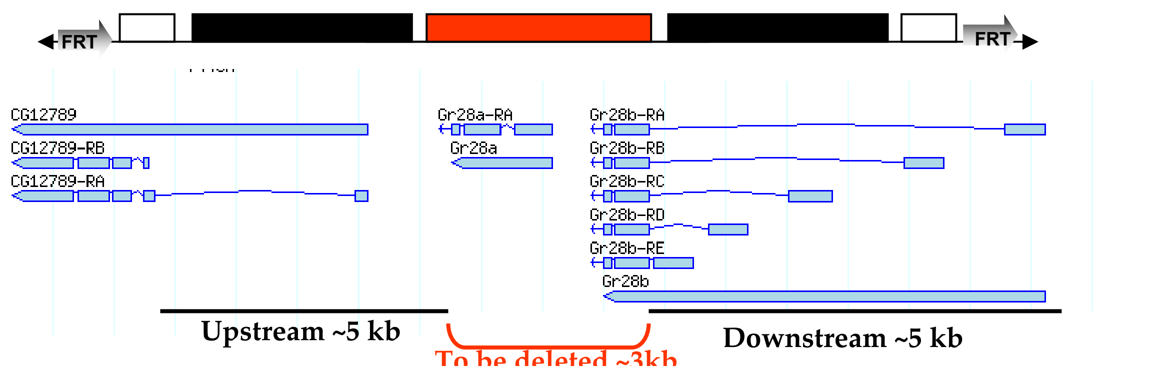


Figure 36: Five kilobases of homologous upstream and downstream sequence (black boxes) was cloned into the CMC105 vector to make the donor construct. The red box is the mini-*white* gene and the white boxes are the I-SceI sites (schematic adapted from (Gong and Rong, 2003)).

The upstream and downstream sequences were PCR amplified from BAC RP98-30I17 using LA Taq TaKaRa, fragments were gel purified, then cloned directly into the pCR-XL-TOPO vector (from Invitrogen). PCR primers used to clone the fragments contained restricton endonuclease sites that would allow the insert to be sub-cloned from the TOPO vector into the CMC105 vector. The primers used to clone the upstream

sequence were: “NThrUp28.1” 5′-GCCGTACGCTGGGCGATGGTGTGTGGATACC-3′ (with BsiWI site); “NThrUp28.2” 5′-CGCCTAGGTTGCTACGTCACTGACTGTGTTGCTGGC-3′ (with AvrII site). The primers to clone the downstream sequence were: “NThrDp28.3” 5′-GCGCGGCCGCGTTAAAATTTTAGAAATGCCTAGCC-3′ (contains NotI site); “NThrDp28.4” 5′-GGCCGCGGCTAGCTCACTTCGTTTGCATCCGTCG-3′ (contains NheI site). After cloning into TOPO, the fragments were checked by sequencing for mistakes. The upstream fragment used had one nucleotide mistake, but it was in intervening sequence between CG12789 and *Gr28a*. The downstream fragment contained no nucleotide mistakes from PCR. Once in the TOPO vectors, the downstream fragment was first sub-cloned into CMC105 by cutting with Not1 and NheI. The upstream fragment was then cloned into the CMC105 vector+downstream insert by cutting both constructs with BsiWI and AvrII followed by ligation. This construct, which is the donor construct, is called “NT47” and was injected to make transgenic flies also called “NT47”.

4.2.4.2 Crossing scheme to produce deletion lines via homologous recombination

Because the targeted gene (*Gr28*) is on the second chromosome, a fly line that had the donor construct integrated on the third chromosome was used for the homologous recombination fly crosses. This line is called “NT47G4”. Two separate rounds of homologous recombination were performed using this donor construct, to

generate two independent deletion lines, called “Δ28 30i” and “Δ28 54B3”, used in later experiments. The difference between the two rounds was the initial FLP-SceI fly line used (highlighted in bold in both crosses). The crossing scheme is for each is found in Figures 37 and 38.

1) ♂w/Y; +/+; NT47G4/NT47G4 X ♀$_{virgin}$ **yw; 70FLP, 701-SceI, noc^{Sco}/CyO** 30 bottles; n=80/bottle
2) parents from 1) cross allowed to lay for three days; parents transferred on day 4; larvae are heat-shocked for 1 hour at 38°C in water bath
3) ♀$_{virgin}$ non-CyO with mosaic or *w* eyes are collected – these females potentially have the donor construct excised
4) ♀$_{virgin}$ w/yw; 70FLP, 701-Sce1, noc^{Sco}, (targeted NT47G4)/(targeted NT47G4); +/+ X ♂w/Y; p[70FLP]/p[70FLP]; +/+ 91 bottles; n=80/bottle (30 females/bottle)
5) parents from 4) cross allowed to lay for three days; parents transferred on day 4; larvae are heat-shocked for 1 hour at 38°C in water bath (even though p[70FLP] line should be express FLP constitutively); this cross helps remove flies that have a non-targeted NT47G4 construct, as their progeny from this cross will be *w* – eyed due to loss of donor construct
6) select ♂ w^{+} non-mosaic progeny of genotype; set up individual crosses to ♀$_{virgin}$ w; CyO/+

Figure 37: Crossing scheme to produce Δ28 30i deletion line via homologous recombination.

The approximately 2730 females (cross 4) generated 26 independent w^+ lines. Of these 26 lines, 17 had the donor construct on the third chromosome (either not targeted or incorrectly targeted), five mapped to the X chromosome, and seven mapped to the second chromosome (lines labeled: 12, 17, 18, 27, 30, 48, and 69). Three of the seven lines were analyzed by PCR to test whether a deletion at the *Gr28* locus had occurred. One line – Δ28 30 – appeared to. These lines are listed in Table 7. Δ28 30i tested positive for the deletion via PCR (and later by Southern blotting), and was used for subsequent experiments. Details regarding the PCR reactions performed and the molecular determination of deletions is described later.

It was later found that many of the putative Δ28 deletion lines generated by these crosses retained the noc^{Sco} dominant marker (significant number of bristles missing on head and thorax, those remaining are scutellar), which is homozygous lethal, and which has defects in chemosensation in the heterozygous state. A few of the lines were made homozygous and did not appear to have this dominant marker. However, it was not ideal to have this marker on the second chromosome (as we are looking for defects in chemosensation caused by the Δ28 deletion), and for this reason, a second round of homologous recombination crosses were carried out that did not use the line carrying the noc^{Sco} dominant marker.

Table 7: Lines that have donor construct targeted to 2nd chromosome and may have *Gr28* deletion – first round of homologous recombination. Lines including the same number most likely come from the same female, and thus are not considered independent lines.

line	noc^{Sco} phenotype?	genomic DNA made?	Gr28 genes deleted according to PCR?
12A	N	Y	N
17H	N	Y	N
18L	Y	N	N/A
17/18	Y	N	N/A
27H	N	N	N/A
27O	N	N	N/A
27P	Y	N	N/A
27/28Z	N	N	N/A
30E3	N	Y	Y
30L2	N	N	N/A
30I	N	Y	Y
30J	Y	N	N/A
29/30V	N	Y	Y
29/30F3	Y	N	N/A
29/30G3	N	Y	not clear
48X	Y	N	N/A
69V2	N	N	N/A
80/81Z3	N	N	N/A

Figure 38 depicts the crosses that were set up for the second, independent round of homologous recombination. The crossing scheme is identical to the first round, except for the FLP/SceI line used in the first cross (and highlighted in bold) and the number of bottles (and thus number of flies screened) set up for each cross.

1) ♀virgin w/Y; +/+; NT47G4/NT47G4 X ♂**w; +/+; 70FLP, 701-SceI/TM6** 30 bottles; n=80/bottle

2) parents from 1) cross allowed to lay for three days; parents transferred on day 4; larvae are heat-shocked for 1 hour at 38°C in water bath

3) ♀virgin non-TM6 (dominant marker is Ubx) with mosaic or *w* eyes are collected – these females potentially have the donor construct excised

4) ♀virgin w/yw; (targeted NT47G4)/(targeted NT47G4); 70-FLP, 701-SceI/+ X ♂w/Y; p[70FLP]/p[70FLP]; +/+ 60 bottles; n=80/bottle (30 females/bottle)

5) parents from 4) cross allowed to lay for three days; parents transferred on day 4; larvae are heat-shocked for 1 hour at 38°C in water bath (even though p[70FLP] line should be express FLP constitutively); this cross helps remove flies that have a non-targeted NT47G4 construct, as their progeny from this cross will be *w* – eyed due to loss of donor construct

6) select ♂ w^+ non-mosaic progeny of genotype; set up individual crosses to ♀virgin w; CyO/+

Figure 38: Crossing scheme to produce Δ28 54B3 line via homologous recombination.

The approximately 1800 females (cross 4) generated 9 independent w^+ lines. Of these 9 lines, 3 had the donor construct on the third chromosome (either not targeted or incorrectly targeted), none mapped to the X chromosome, and six mapped to the second chromosome (lines labeled: 9/10B, 13B, 15/16B, 20/21B, 24B, and 54B) (Table 8). Lines listed with the same number (9/10B1 and 10B2, for example) originated from the same w^+ male, and therefore are most likely genotypically identical. Five of the six lines were

tested by PCR to see if they had the *Gr28* genes deleted. One line – Δ28 54B – appeared to. These lines are listed in Table 8. Details regarding the PCR reactions performed and the molecular determination of deletions is described later. The line Δ28 54B3 tested positive for the deletion by PCR (and later Southern blotting) and was used for further behavioral experiments.

Table 8: Lines that have donor construct targeted to 2nd chromosome and may have *Gr28* deletion – second round of homologous recombination

line	tested by PCR?	Gr28 genes deleted according to PCR?
9/10B1	Y	N
10B2	N (but see above)	N/A
13B1	Y	N
15/16B1	N	N/A
20/21B1	Y	N
24B1	N(but see below)	N/A
24/25B2	Y	N
54B1,B2,B4, B5,B6, B7, B8	N(but see below)	N/A
54B3	Y	Y

4.2.4.3 PCR reactions to determine presence or absence of Δ28 deletion

In order to determine whether or not the lines that mapped to the 2nd chromosome actually had undergone correct targeting and ends-out gene replacement with the mini-*white* gene, we performed four sets of PCR reactions. Genomic DNA was first made from each line by standard lab protocol (as found in the lab protocol binder). The first PCR reaction was with primers directed against *tubulin*, in order to confirm that the DNA was of decent quality. We then performed PCR reactions using primers downstream of the region that should be deleted as a control (Figure 39). This region

was not only not deleted, but not included as homologous sequence in the donor construct. The other two primer sets were against *Gr28a* and *Gr28b* common exons. We would expect the tubulin and upstream control primers to amplify a product, but not the primers against the *Gr28* genes, if the lines contained the expected Δ28 deletion.

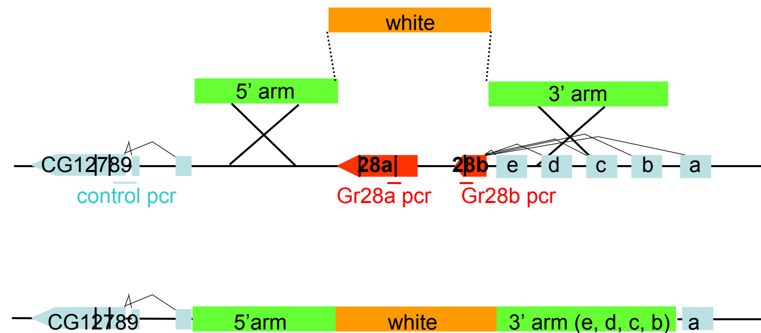


Figure 39: Schematic of donor construct (top) targeting and recombining with homologous sequence of endogenous locus (middle), resulting in endogenous gene replacement with the mini-*white* gene (bottom). PCR reactions used to determine if the *Gr28* genes are replaced are shown. Control PCR uses a primer set which produces an amplification product for a targeted and non-targeted locus. Primer sets against *Gr28a* and the *Gr28b* common exon should not amplify in the targeted locus (bottom) that contains a gene knock-out, but would produce an amplification product if the genes are not knocked-out (middle).

Following is information regarding the primers used and the expected sizes of the amplification products generated from a given primer set. Tubulin primers produce an amplification product of approximately 1100bp. Primers are "NT pTub 1.5" 5'-CCTTGTCGCGTGTGAAACACTTCC-3' and "NT pTub1.6" 5'-GATAGCCTCGTTGTCGACCATGAA-3'. Control primers to upstream sequence (aka "control pcr") produced a 940bp fragment. The sequences for these primers are: "pNThrcont.1" 5'-GGTAATTCCACGAGTGCATCTGAC-3' and "pNThrcont.2" 5'-

GGCATATTGTGCGGCATGTTC-3′. The primers directed to *Gr28a* produce a fragment that is approximately 625bp and are: “pTr28b.F” 5′-GGGCATGAAGTTGGACTACCGCAGG-3′ and “pTr28b.R′ 5′-CGGGATTGAGGCGTATGACGGAGG-3′. The primers directed toward the common exons of *Gr28b* produce an approximately 630bp fragment. The primers are: “pTr28a.F” 5′-CCCATCAATGGGACACCCGAAGCCT-3′ and “pTr28a.R” 5′-GAGATAAGTGGTCAAGGCCCCGCTG-3′.

The following lines (as shown in Tables 7 and 8) produced amplification products for the tubulin and control primer sets, but not for the *Gr28a* and *Gr28b* primer sets: 29/30v, 30e3, 30i (all “30” lines are probably molecularly identical, as having come from the same bottle thus probrably from the same female in which homologous recombination occurred), and 54B3. PCR reactions for “30i” are shown in Figure 40. PCR reactions for “54B3” are shown in Figure 41.

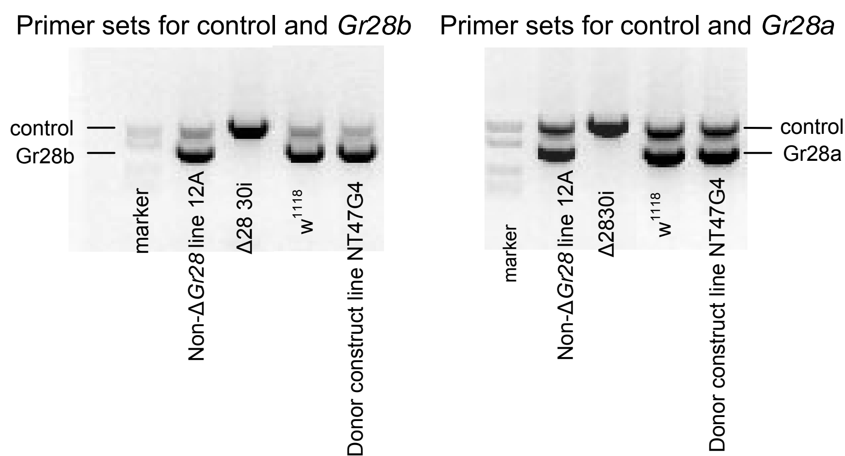


Figure 40: PCR reactions of the "30i" line that contains the Δ28 deletion. Primers for both the control PCR (against downstream sequence; Figure 40) were put in the same reaction as primers against *Gr28a* and *Gr28b*. Only the "30i" line that has the Δ28 knock-out does not amplify fragments for *Gr28a* or *Gr28b*.

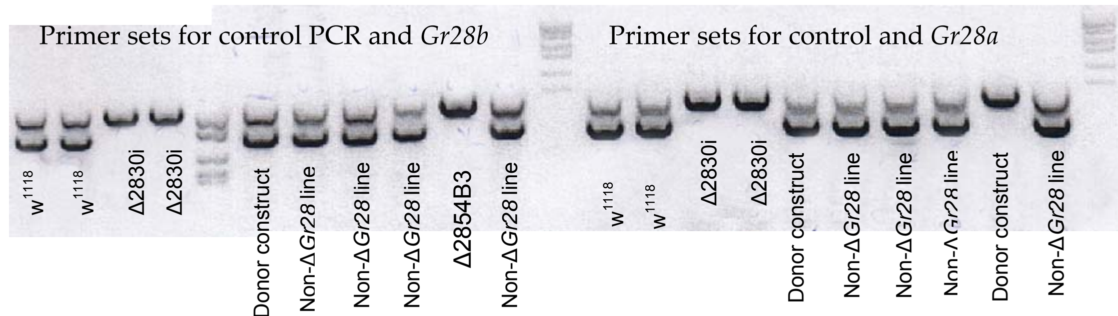


Figure 41: PCR reactions of the "54B3" line that contains the Δ28 deletion, plus other lines that targeted to the 2nd chromosome without the deletion. Top band is the 934bp amplification product from the control set of primers that amplify for all the control lines, and the bottom band is the 625bp or 630bp from *Gr28a* or *Gr28b* primers, respectively, that only amplify from lines without the deletion.

According to PCR, lines 29/30v, 30e3, 30i, and 54B3 should have *Gr28a* and the two common exons of *Gr28b* replaced with the mini-*white* gene. In order to molecularly confirm this, Southern blots were performed with genomic DNA from these lines.

4.2.4.4 Southern blots of genomic DNA from putative Δ28 deletion lines

Southern blots were performed according to lab protocols (found in lab protocol binder). Genomic DNA was digested to produce DNA fragments that should allow differentiation between a line that did not contain the expected deletion and one that did (Figure 42). Genomic DNA was made from the following fly lines: w^{1118}, original donor construct line (NT47G4), and putative deletion lines - 29/30v, 30e3, 30i, and 54B3.

Approximately 8μg of DNA was digested overnight at 37°C with the appropriate restriction endonuclease. Each DNA sample was tested with two digests, one that would provide molecular information regarding the 5′ end of the knock-out (BglII) and one that would provide information regarding the 3′ end of the knock-out (EcoRI). Radio-labeled probes were made (called the BglII/5′ probe or EcoRI/3′ probe) that were directed against this molecular region to allow differentiation between DNA with or without the expected deletion (Figure 42). Probes were directed to a region outside of the region targeted by the donor construct, so that it could be determined whether or not the construct had targeted to the precise location expected. BglII-digested DNA without a deletion should produce a fragment of approximately 12kb, whereas DNA with a deletion should produce a fragment of approximately 7.6kb. EcoRI-digested DNA without a deletion should produce a fragment of about 8.8kb and DNA with a deletion should produce a fragment of approximately 11.7kb. Probes directed against the *Gr28a* and the common exons of *Gr28b* were also made. Digests were run on a 1% gel at 45-60V for five hours (or until the blue band from the loading dye was at the bottom of the gel). The gel was stained with SYBR gold stain to visualize the markers, make sure the DNA was entirely digested, and take a picture of the gel. Transfer to nylon filter occurred overnight. The 5′ probe (934nt) was produced by PCR using the primers "pNThrcont.1" 5′-GGTAATTCCACGAGTGCATCTGAC-3′ and "pNThrcont.2" 5′-GGCATATTGTGCGGCATGTTC-3′. The 3′ probe (1020nt) was produced by PCR using

the primers "pNTecoprobe.1" 5′-GCCAGTTGTCAGAAGGACGGTATCC-3′ and "pNT ecoprobe.2" 5′-CGATGTTGGGTTGGAGTCGAGTGC-3′. ^{32}P-dCTP-radiolabeled probes were made the day of use, according to standard lab protocol. All probes made had a cpm/μL of 100,000 or greater, and probe was applied to filters such that there was at least 400,000cpm/filter. Hybridization occurred at 65°C overnight. Filters were exposed for 8 to 58 hours.

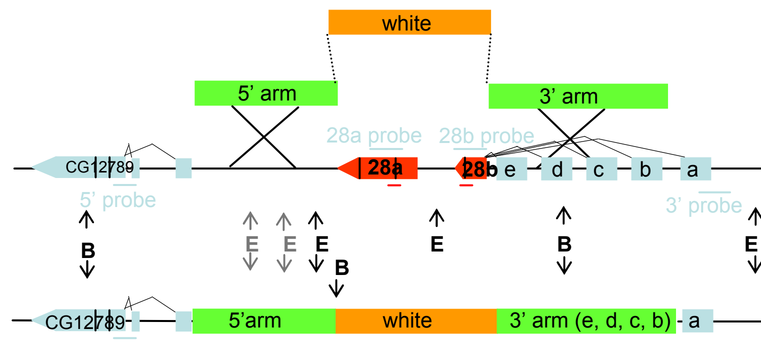


Figure 42: Schematic of the target donor construct and the *Gr28* locus. The BglII and EcoR1 restriction cut sites are shown for both, as are the 5′ and 3′ probes. The 5′ probe was used to probe Southern blots with BglII-digested DNA, and the 3′ probe was used to probe Southern blots with the EcoRI-digested DNA. The *Gr28a* and *Gr28b* common exon probes are also shown. These were used to determine the presence or absence of the *Gr28* genes.

Southern blot of the BglII digested DNA using the 5′ probe showed hybridization to a fragment of approximately 12.2kb for w^{1118} and the donor construct lines and a shorter fragment at about 7.6kb for 29/30v, 30e3, and 30i (Figure 43) - results expected for deletion lines. The EcoRI digest produced the expected fragment at approximately 8.9kb for for w^{1118} and the donor construct lines and a significantly larger

fragment for 29/30v, 30e3, and 30i (Figure 43), a fragment that was taken to be the expected 11.7kb fragment that indicated replacement at the *Gr28* locus.

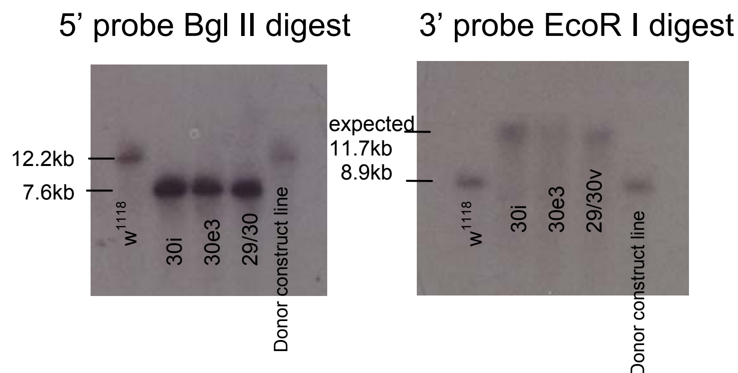


Figure 43: The lines (30i, 30e3, and 29/30v) that appeared to have *Gr28* deleted by PCR also appear to have the donor construct correctly targeted and the mini-*white* gene replaced at the locus according to Southern blots.

Using probes directed against *Gr28a* and the common exons of *Gr28b*, the lines also appeared to have the deletion, as there was no hybridization to any fragments seen for DNA from these lines, even after a four day exposure (96 hours) (Figure 44). The donor construct line and w^{1118} line should have a fragment at 12.2kb for the BglII digest and a fragment at 8.9kb for the EcoRI digest, as seen.

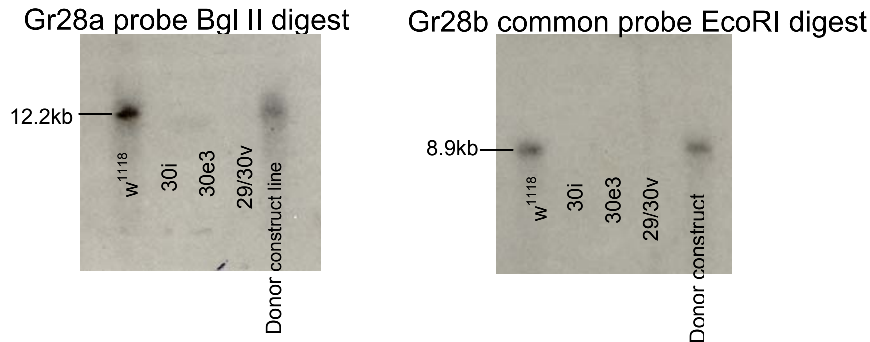


Figure 44: All three lines from the first round of homologous recombination ("30") lack *Gr28a* and the common exons of *Gr28b* according to Southern blots using probes directed against these genes.

Based on PCR and Southern blots, the lines 29/30v, 30e3, and 30i all appear to have replacement of the mini-*white* gene at the correct location. The line "30i" was chosen, arbitrarily, to be used for further experiments.

Southern blots were also done for 54B3, using the exact same protocol as was used for 30i. Genomic DNA samples from 30i were run alongside those of 54B3 in order to allow comparison between the samples (Figure 45). Bgl II digest of the DNA samples probed with the 5′ probe gave the same results for 30i and 54B3 lines, with hybridization of a fragment at approximately 7.6kb. The results from the EcoRI digest using the 3′ probe were less clear. It appears that the fragment that hybridizes for 30i is much larger than that for 54B3. Since both the BglII and EcoRI digests were run on the same gel and transferred onto the same blot (which was then cut directly before hybridization), the relative sizes of fragments can be compared between the two blots. From this, it looks

like the 30i fragment for the EcoRI digest is too large to be the 11.7kb fragment expected to hybridize with the probe (if this line contains a deletion). The fragment for 54B3 does look correct, however. Looking at the EcoRI sites at this locus (Figure 42), this larger fragment for 30i could be explained if a different upstream EcoRI cutsite is used. There are four EcoRI cutsites close to one another within the sequence that makes up the 5′ homologous region. It is possible than the EcoRI cut-site that would be used to make the 11.7kb fragment was altered during the homolgous recombination process, and one of the other EcoRI cut sites was used, which would produce a larger fragment. It is clear, though, that 30i does lack *Gr28a* and the common exons for *Gr28b* and that the 5′ region of the deletion is as expected, based on the results of the BglII digest and 5′ probe. Additional PCR reactions and Southern blots need to be done in order to molecularly characterize this locus more carefully.

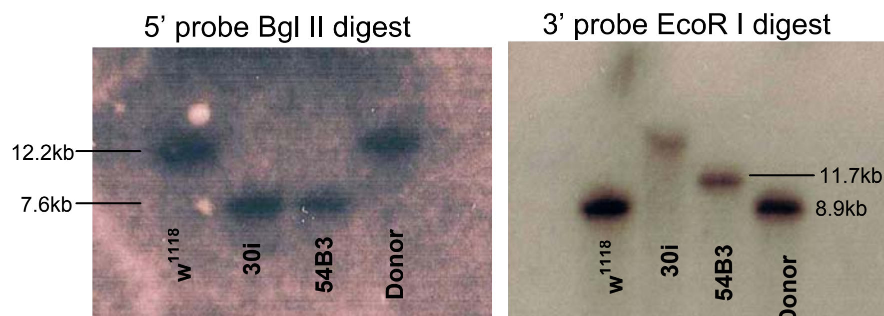


Figure 45: Southern blots using the 5′ and 3′ probe of line 54B3 run alongside deletion line 30i. The EcoRI digest hybridized to the 3′ probe indicates that line 30i, while definitely containing the expected deletion, differs molecularly in an undefined way from what is expected.

Southern blots were stripped, and then probed with probes against *Gr28a* and *Gr28b*. There is no hybridization signal for 54B3 for either probe even after exposure for 29 hours (BglII digest, *28a* probe) and 58 hours (EcoRI digest, *28b* probe) (Figure 46).

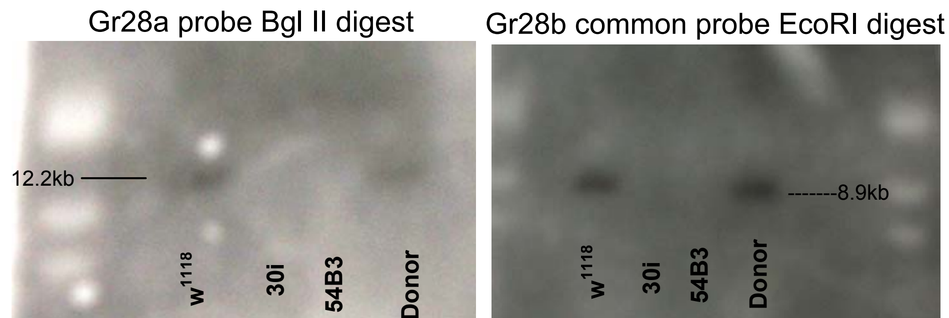


Figure 46: Southern blots using the probes against *Gr28a* and *Gr28b* common exons show that like 30i, the 54B3 line also has a deletion for these genes.

4.2.5 Production of a a genomic *Gr28* rescue line

In order to determine the function of the *Gr28* genes, behavioral/phenotypic assays must be performed using the Δ28 lines alongside a line that has the same Δ28 background, but also contains a transgene that rescues the *Gr28* genes. Any differences between the Δ28 line and the rescue line can be attributed to the *Gr28* genes.

The 14.5kb genomic region containing the *Gr28* locus plus approximately 3.4kb 5′ and 1.5kb 3′ to the *Gr28* genes (Figure 47) was cloned into the vector pCaSpeR4 in two steps. A BAC clone (BacR30I17; also RP98-30I17) containing the *Gr28* locus was obtained from the BACPAC Resource Center, and was used to isolate an approximately 13kb fragment (containing all *Gr28* genes plus approximately 1.8kb 5′ sequence and

1.5kb 3′ sequence) via KpnI/StuI restriction endonuclease digestion. This fragment was cloned directly into pCaSpeR4. Restriction digests and sequencing confirmed the identity of the fragment. PCR was used to amplify a 1.5kb fragment that contained additional 5′ sequence. Primers used were: "pNTseq.4" 5′-GCATAGTAAGCACCTAGCAAATGGC-3′ and "pNTres28.B" 5′-CCCAGTAAGCAGCTCCAATGC-3′. This fragment was initially cloned into pGEMT, and then sub-cloned into pCaSpeR4 using StuI and PstI cut sites. This 22.4kb construct, called "NT51" was then injected into w^{1118} embryos to make transgenic flies. The transgenic flies are called "NT51E1" and "NT51A2". The difference in name (E1 vs A2) is because these constructs came from separate clones (colonies) for the last ligation step. Southern blot to confirm the presence of the *Gr28a* and *Gr28b* genes in these lines, once in the Δ28 genetic background, may be necessary. The lines made, and their chromosomal location, are found in Table 9.

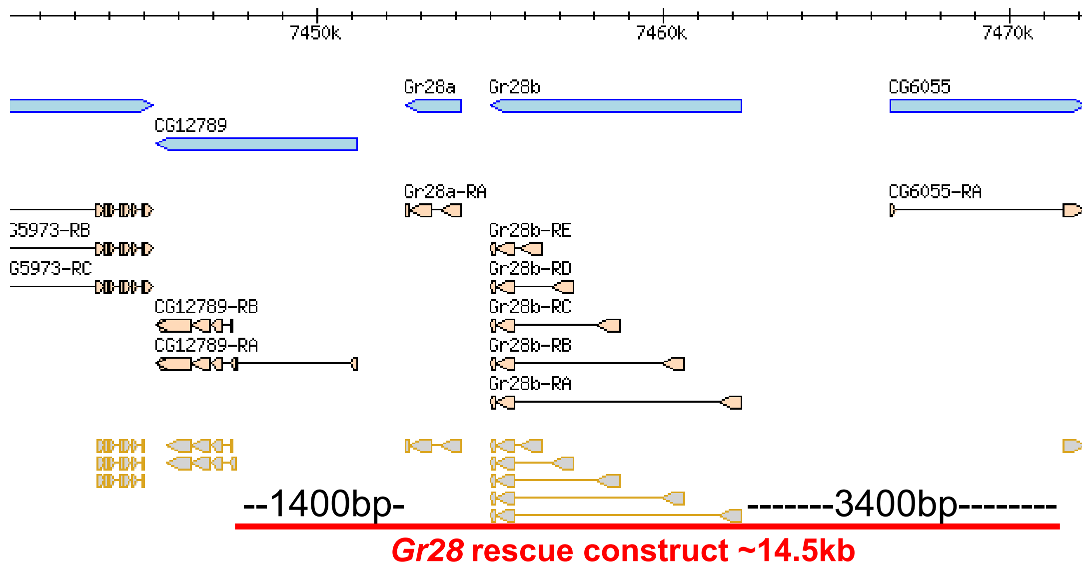


Figure 47: Schematic of coverage of the *Gr28* rescue construct that was made (NT51) including all the *Gr28* genes as well as upstream and downstream sequence.

Table 9: List of transgenic *Gr28* rescue insertion lines and their chromosomal location. * indicate that chromosomal location should be confirmed.

insertion line	chromosomal location
NT51E1 A4*	2
NT51E1 A6	2
NT51E1 A7*	2
NT51E1 B2*	3
NT51E1 C5	2
NT51E1 C14	2
NT51E1 D2*	2
NT51A2 A2	2
NT51A2 B1	2
NT51A2 C4	3
NT51A2 D13	2
NT51A2 E1	2
NT51A2 F1	N/D
NT51A2 I2*	3
NT51A2 I6	2
NT51A2 J1	3

Transgenic fly lines were made that have the rescue construct containing the genomic *Gr28* locus and are called "NT51". This rescue line is the same one that was used for RNA *in situ* experiments to confirm *Gr28* expression in embryos and sections of adult heads (Chaper 3). This *Gr28* rescue line still needs to be crossed into the Δ28 30i and Δ28 54B3 mutant background.

4.2.6 Δ28 30i, Δ28 54B3, and control lines are backcrossed into the same genetic background for behavioral experiments

Behavior is complex, involving sensory and motor systems, and cognitive processing. Not surprisingly, behavioral genetics, which involves the interaction of a great number of genes that influence behavior, from genes that code for cell signaling molecules, to transcription factors, to neurotransmitters, is also extremely complicated. Because any number of genes could influence behavior –from housekeeping genes to genes specific for sensory detection and perception – it is essential to analyze different alleles of the gene in the same genetic background.

The process of homologous recombination relies on the interaction of introduced DNA and endogenous DNA and results in the alteration of endogenous DNA. Although this interaction should be targeted, other molecular events can occur in other regions of the genome, introducing unwanted mutations. In order to remove any unwanted alterations that may exist in the genome of animals with the desired deletion (in this case, Δ28 30i and 54B3), it is necessary to backcross the lines for multiple generations,

thereby providing an isogenic background for the line. Additionally, since the phenotype of the deletion lines will be compared to control lines (such as the rescue line in the Δ28 background), it is necessary that the control lines share the same genetic background of the deletion lines. This assures that phenotypic differences between the mutant lines and control lines are due to the loss of the *Gr28* genes and not to a difference in genetic backgrounds or unknown abnormalities in the genome of the deletion line.

In order to obtain the Δ28 deletion lines 30i and 54B3, as well as control lines, with the same genetic background, five generations of backcrosses to w^{1118} were performed (see below). It should be noted that the w^{1118} line used was a recently isogenized line made by the Wharton lab. Virgin female progeny from the first cross were always used to cross to w^{1118} males to insure recombination occurred on the 2nd chromosome, allowing this chromosome to be made isogenic as well. Crosses performed as follows:

1. ♂w^{1118}/Y; +/+; +/+ X ♀^{virgin} w^{1118}/w^{1118}; $\Delta28^{mini\text{-}white}/\Delta28^{mini\text{-}white}$; +/+ (outcross to w^{1118})

2. ♀^{virgin} w^{1118}/w^{1118}; $\Delta28^{mini\text{-}white}$/+; +/+ X ♂$w^{1118}$/Y; +/+; +/+ (backcross to w^{1118} five times)

3. ♀^{virgin} w^{1118}/w^{1118}; $\Delta28^{mini\text{-}white}$/+; +/+ X ♂ w^{1118}/w^{1118}; $\Delta28^{mini\text{-}white}$/+; +/+ (make homozygous)

4. ♀^{virgin} w^{1118}/w^{1118}; $\Delta28^{mini\text{-}white}/\Delta28^{mini\text{-}white}$; +/+ X ♂$w^{1118}/w^{1118}$; $\Delta28^{mini\text{-}white}/\Delta28^{mini\text{-}white}$; +/+ (expand and use for experiments)

Deletion lines produced from homologous recombination (Δ2830i and 54B3), the donor construct line (NT47G4), and lines with the donor incorrectly targeted (17H-first HR round, 9/10B and 13B1 – second HR round) were all put in the same genetic background. The *Gr28* rescue line (described in previous section) was made with this genetic background (w^{1118} from Wharton lab), and has not been backcrossed.

4.3 Results

4.3.1 Driving expression of VR1 in *Gr28b.c*-expressing cells causes wandering 3rd instar larvae to avoid capsaicin

In order to get an idea as to what type of neurons the *Gr28* genes are expressed in, we decided to take advantage of the *Gr28-Gal4* driver lines that had already been made, and use these lines to try to characterize the cells as avoidance- or attractive-type. We decided to use the driver for *Gr28b.c*, as this receptor is expressed in a single neuron of the TO in larvae, the main gustatory tissue (Colomb et al., 2007), as well as neurons of the mouth (Table 6). *Gr28b.c* is also expressed in md-neurons of the abdomen, as well as unidentified abdominal neurons, and cells in the CNS (Table 6 and data not shown). It is not clear how VR1 expression in cells other than those involved in taste would effect the behavioral assay, but it was hoped that expression in the TO would produce an attractive or avoidance taste behavior that could be measured by this assay.

For behavioral assays involving avoidance or attraction to capsaicin, we decided to use wandering 3rd instar larvae instead of adults. Larvae were chosen over adults for several reasons: first, larvae are dedicated to searching for food and feeding, and, unlike adults, don't have to be starved to make food-choice preferences. This requires them to make food-choice decisions on a constant basis. Secondly, larvae could be visually counted at various time intervals during the assay, without having to use a video-capture apparatus, allowing data to be instantly collected and analyzed, and a quick assessment made as to whether the concentration of capsaicin was appropriate and whether the experiment was informative and worth pursuing.

One of the main difficulties of this taste assay, and any behavioral experiment in which animals must choose one substrate over the other, is to find a concentration of the test substrate that they are just able to detect and respond to. Concentrations too low yield random results, and concentrations too high prevent the animal from making a choice between the two substrates based on appropriate discrimination. For example, very high concentrations of some normally attractive substrates (like sucrose) can cause avoidance-type behavior (pers. observation). We therefore tried three concentrations of capsaicin – 4mM capsaicin, 1mM capsaicin, and 0.1mM capsaicin. It was found that 0.1mM capsaicin was high enough to elicit behavioral responses. The results for behavioral assays for all three concentrations of capsaicin are shown in Table 10. Results for the 0.1mM capsaicin preference assay are shown in Figure 48.

Table 10: Preference for capsaicin in larvae expressing VR1 under the control of *Gr* drivers.

concentration capsaicin	genotype	no. of experiments (35-40 larvae/exp)	PI capsaicin	preference
0.1mM capsaicin	w^{1118}	4	0.47	no preference
0.1mM capsaicin	Gr66a-->VR1	4	0.30	control
0.1mM capsaicin	Gr28b.c-->VR1	4	0.093	control
0.1mM capsaicin	Gr5a-->VR1	5	0.42	(slight) control
1mM capsaicin	Gr28b.c-Gal4	4	0.61	capsaicin
1mM capsaicin	Gr66a-->VR1	10	0.23	control
1mM capsaicin	Gr28b.c-->VR1	10	0.021	control
1mM capsaicin	Gr5a-->VR1	4	0.26	control
1mM capsaicin	Gr5a-Gal4	4	0.58	(slight) capsaicin
4mM capsaicin	w^{1118}	2	0.43	(slight) control
4mM capsaicin	Gr28b.c-Gal4	1	0.56	(slight) capsaicin
4mM capsaicin	Gr66a-->VR1	1	0.36	control
4mM capsaicin	Gr28b.c-->VR1	2	0.096	control
4mM capsaicin	Gr5a-->VR1	2	0.38	control

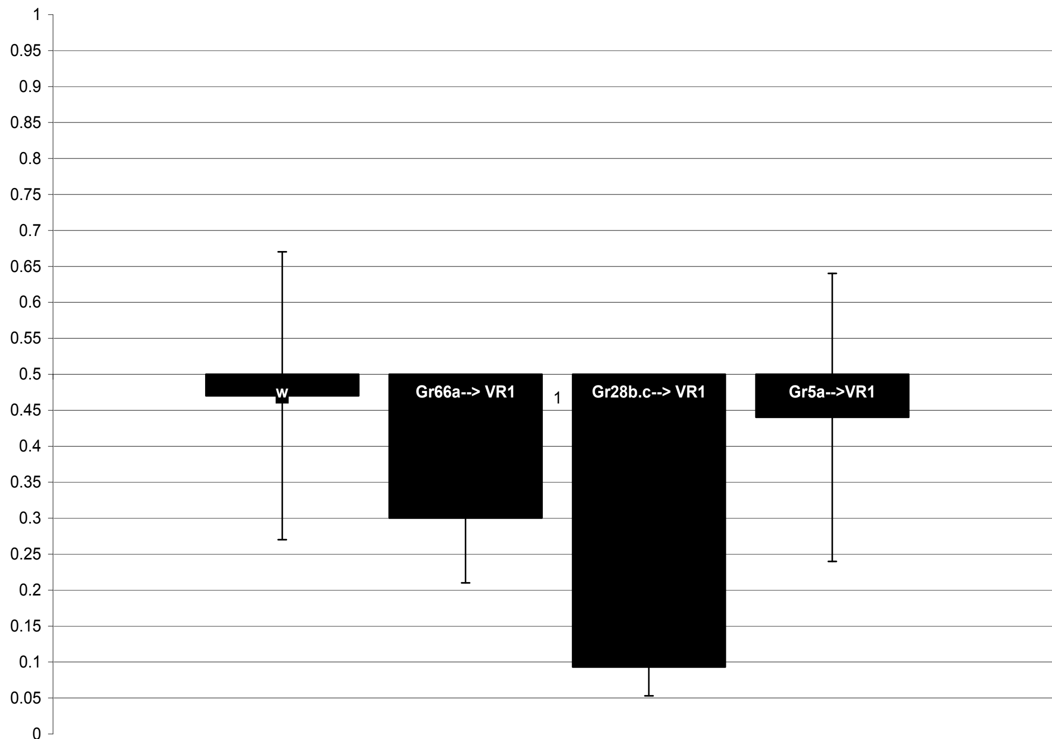


Figure 48: Preference for 0.1mM capsaicin in larvae expressing VR1. The level of aversion towards 0.1mM capsaicin for larvae expressing VR1 under the control of the *Gr28b.c* promoter (*Gr28b.c*-->VR1) is significantly different from that of *Gr66a*-->VR1 ($p<0.01$) and w^{1118} ($p<0.05$). Y-axis is PI, with a PI=0.5 as no preference, and a PI<0.5 as aversion to 0.1mM capsaicin.

When w^{1118} larvae have a choice between 0.1mM capaicin and a control substrate, they show no preference for either. This is expected, as wild-type larvae lack VR1 , which is necessary to detect capsaicin. As expected, when VR1 was expressed under the control of the *Gr66a* promoter, which is expressed in the TO and mouth of larvae (Scott et al., 2001) were repelled by capsaicin. Even more importantly, *Gr28b.c-Gal4/UAS-VR1* larvae were extremely repelled by 0.1mM capsaicin, and this avoidance was significantly different from that of both w^{1118} and *Gr66a-Gal4/UAS-VR1*. This result – extreme and

robust avoidance behavior of *Gr28b.c-Gal4/UAS-VR1* larvae was seen for all capsaicin concentrations tested. Avoidance is not seen with the *Gr28b.c-Gal4* driver alone at any of the concentrations tested. One unexpected result is the avoidance behavior displayed by *Gr5a-Gal4/UAS-VR1* larvae. These larvae never show attraction to capsaicin, at any concentration tested, as would be expected. A very recent publication by Colomb et al. (2007) has reported that *Gr5a* is not expressed in larval chemosensory tissues. This may explain why an attractive behavioral response was not seen for the *Gr5a-Gal4/UAS-VR1* larvae.

4.3.2 Seven homozygous lethal lines with 50kb deleted at the *Gr28* locus are produced using *piggy-Bac* transposable elements

We generated a total of 300 lines and tested a total of 100 lines with six sets of pcr primers (see Materials and Methods) to determine if these lines contained the expected deletion as produced using the *piggyBacs* in *trans*. Of the 100 tested, seven are excellent candidates for having the deletion as determined by PCR and an additional seventeen were possible candidates. "Excellent" candidates produce all expected amplification products and produce no amplification products from primer sets not expected to produce a product if there is a deletion (see Materials and Methods section for primer sets). "Possible" candidates fail to produce all expected amplification products, which may be because there is no deletion, or because the DNA prep was sub-satisfactory. The lines that were "excellent" or "possible" candidates for a deletion are maintained as

stocks, and are listed in Table 11. Putative deletions on the second chromosome are maintained over CyO, as these lines are not homozygous viable. Genomic Southern blots are necessary to confirm that these lines are knock-outs for the *Gr28* genes.

Table 11: *piggyBac*-based lines with putative 50kb deletion of *Gr28* locus

piggyBac line	excellent/possible candidate for deletion?
5(-)/CyO	excellent
8(-)/CyO	excellent
63(-)/CyO	excellent
24(+)/CyO	excellent
27(+)/CyO	excellent
31(+)/CyO	excellent
32(+)/CyO	excellent
2(-)/CyO	possible
10(-)/CyO	possible
11(-)/CyO	possible
13(-)/CyO	possible
24(-)/CyO	possible
26(-)/CyO	possible
30(-)/CyO	possible
37(-)/CyO	possible
40(-)/CyO	possible
42(-)/CyO	possible
48(-)/CyO	possible
54(-)/CyO	possible
6(+)/CyO	possible
17(+)/CyO	possible
19(+)/CyO	possible
36(+)/CyO	possible
37(+)/CyO	possible

4.3.3 Two Δ28 deletion lines are produced using homologous recombination

Two independent lines have been produced – Δ28 30i and Δ28 54B3 - that have a deletion of *Gr28a* and the common exons of *Gr28b*. The line 54B3, based on PCR and Southern blot, has replacement of the mini-*white* gene exactly as expected (Figures 45 and 46). The line 30i, while definitely without the *Gr28* genes (Figure 44), differs molecularly from 54B3 (Figure 46). Further PCRs and Southern blots will need to be performed to molecularly characterize this line in detail.

Fly lines that are transgenic for a 14.5kb genomic DNA construct containing the *Gr28* genes have also been made (see Materials and methods). This rescue can be put into the Δ28 mutant background and used as a control for future behavioral experiments. Additionally, the Δ28 30i, Δ28 54B3, the donor construct line, and a few other control lines have been backcrossed to w^{1118} to produce lines with the same genetic background, also to be used for future behavioral assays to determine the function of the *Gr28* genes (see Materials and methods).

4.4 Discussion

4.4.1 *Gr28* genes may play a role in detecting aversive tastants

We have shown that driving expression of the capsaicin receptor VR1 in *Gr28b.c*-expressing neurons elicits a strong repulsion to capsaicin. This repulsion is even

stronger than the avoidance seen by larvae that express VR1 in *Gr66a*-expressing neurons – neurons known to be involved in avoidance behavior. This indicates that *Gr28b.c* is expressed in at least some avoidance-type sensory neurons. The robustness of the result is a bit striking, as *Gr28b.c* is expressed in other neurons of the labellum in addition to avoidance-type taste neurons, and we would have predicted that some of these may be attractive-type taste neurons, and expression of VR1 in these neurons would have caused an attraction to capsaicin. We found that driving VR1 with the *Gr5a* driver line produced slight aversion to capsaicin. This is most likely due to the fact that *Gr5a* is not expressed in larval taste neurons (Colomb et al., 2007). Marella and coworkers (2006) found that when they drove VR1 expression by *Gr5a* in adult flies, these flies were attracted to capsaicin.

In conclusion, it appears that *Gr28b.c* is expressed in at least some sensory neurons that mediate avoidance behavior. Since other *Gr28* genes are expressed in chemosensory neurons of the larvae (*Gr28a, Gr28b.a,* and *Gr28b.e*) It will be interesting to see if driving VR1 expression with the other *Gr28* promoters produces a similar effect.

4.4.2 Homologous recombination proves to be a better method than *piggyBac*-mediated FRT-recombination at producing a Δ28 deletion

Producing deletions using *piggyBac*s was found to be straightforward. One drawback to this method, however, is not having a marker that allows one to initially screen for putative deletion lines. It should be mentioned that this is not the case for all

piggyBac- based deletions, and depends on the type of *piggyBacs* used to make the deletion. Some *piggyBac* elements, upon *trans*-recombination, result in loss of the mini-*white* gene (Parks et al., 2004), and thus not as many lines need to be initially tested with PCR.

The major drawback to this technique, however, is the large size of the deletion that is generated (50kb), requiring other genes removed in the deletion to be replaced in order to use the lines for phenotypic analysis. If only a few other genes are deleted, they could be rescued using transgenes with cDNAs for these genes. However, with such large deletions produced, especially in gene-rich regions, large genomic constructs most likely would need to be made. This could require the production of relatively large transgenes that must all be put in the mutant background. In this case, two large rescue constructs, of approximately 23kb and 24kb, would need to be made to cover upstream and downstream sequence. One of these constructs, covering upstream sequence, has been made and used to produce transgenic rescue lines. This is described in the next section.

It should be noted again that all the deletion lines produced with this technique are homozygous lethal, probably due to loss of essential gene(s) other than the *Gr28s*. In addition, lines must be tested by Southern blot to confirm the deletion.

Although the frequency of producing a deletion using homologous recombination is significantly less than producing a deletion using *piggy-Bac*

transposable elements (Venken and Bellen, 2005), the deletion produced is targeted and specific. However, one assumption should be noted. In producing the *Gr28* knock-out lines, we assumed that by removing the two common exons for *Gr28b* (and leaving the alternative first exons intact), it would not be possible to have functional *Gr28b* protein produced. It seems almost impossible that functional protein could be produced with the common exons removed, as well as the *Gr28a* gene completely removed, but lack of protein production has not been proven.

This is the first time a cluster of *Gr* genes has been deleted, and one of the few (if only) instances where homologous recombination has been used to do this. By removing all the genes for this locus, there is a greater likelihood that a phenotype will be revealed, and function for these genes determined. It is hoped that deletion of all these genes will eliminate possible redundancy of gene function that may be seen between individual members of this gene family. If a phenotype is seen using the *Gr28* knock-out lines, it will be necessary to rescue individual *Gr28* genes to determine to which gene(s) the phenotype can be attributed. This should be relatively easy, since a genomic rescue construct for the entire *Gr28* locus has been produced (see Materials and Methods) and can be manipulated to contain only the desired *Gr28* gene(s).

4.5 Acknowledgments

We would like to acknowledge the laboratory of Dr. Richard Axel for kindly providing the *UAS-VR1* flies used for these assays.

5 Determining the function of the *Gr28* genes: initial behavioral experiments

5.1 Introduction

With the production of fly lines that specifically lack the *Gr28* genes, as produced using ends-out homologous recombination, it was possible to do preliminary phenotypic analysis and initial behavioral experiments.

It was initially decided to determine if the Δ28 30i and Δ28 54B3 lines had a taste-related phenotype. This decision was made because the typical function of *Gr*s is in tastant detection, and based on the expression pattern for these genes in the taste system, both in adults and larvae, it is likely that the *Gr28* genes could have a taste function. It was also thought that even if a non-taste related phenotype was found, the taste behavior of these Δ28 flies would have to be tested, to rule out a possible taste-related function. Thus, preliminary experiments on the Δ28 lines mainly tested taste preference and detection, with a couple experiments to test larval growth and pain sensitivity. Additional behavioral experiments should address the possible function of these genes in metabolism, water/humidity sensing, thermoreception, and nociception.

It should be noted that the Δ28 30i and Δ28 54B3 animals do not have any obvious phenotypes; they appear physically normal, are healthy, and reproduce well.

5.1.1 Introduction to larval assays

Since a few of the *Gr28* genes are expressed in cells that influence metabolism (*Gr28b.b* and *Gr28b.c* may be expressed in DILP-producing neurosecretory cells, and *Gr28b.b* is expressed in oenocytes), an assay was performed to determine if Δ28 mutant larvae have a difference in larval lifespan. Ablation of oenocytes has been shown to cause larvae to grow more slowly (Gutierrez et al., 2007), and ablation of DILP-producing neurosecretory cells delays eclosion (Ikeya et al., 2002).

Larvae are constantly feeding and searching for food sources. For this reason, this stage is ideal to measure taste preference or feeding behavior. Unlike adult flies, which engage in a variety of behaviors and spend a good deal of time courting, mating, and laying eggs, larvae are dedicated to feeding. Larvae do not require an extensive starvation period to measure taste preferences, as adult flies do. Adults can take 28 hours or longer to starve, at which point a good percentage of animals still don't feed, and therefore do not provide data points as to their food preference. Larvae, if starvation is required, take only two hours to display starvation-like behavior (Wu et al., 2005). Under starvation conditions, the food choice preferences of larvae can change, with larvae actually feeding on contaminated food (Wu et al., 2005), a behavior not easily measured in adults. Larval position assays were used to determine if Δ28 larvae had a difference in sensitivity to different substrates compared to control animals.

Feeding behavior is not limited to tastant detection, however, but is quite complex; there is foraging behavior to find the food, detection of the food source, initiation of feeding, continuation of feeding, and cessation of feeding, to name a few (Melcher et al., 2007; Melcher and Pankratz, 2005). Generally it is assumed that the *Gr*s are involved in the detection of food (and toxins), and are not involved in other feeding behaviors. Several groups have found that neuropeptides, like huggin and neuropeptide-F, influence various feeding behaviors (Melcher and Pankratz, 2005; Wu et al., 2005). Based on the expression profile of the *Gr28* genes, in putative neurosecretory cells of the CNS as well as in sensory neurons not exposed to the external environment, it is possible that the GR28s could be receptors for internal ligands, like neurotransmitters, neuropeptides, or other signalling molecules.

Neuropeptides influence foraging behavior (Melcher and Pankratz, 2005; Wu et al., 2005). It has been found that if wild-type larvae are placed on a food source, they will generally stay at the food source for a given amount of time. Larvae that cannot detect food sources, or larvae that have other feeding behavior deficits, may leave the food source immediately or after a short period of time. A larval foraging assay (Wu et al., 2005) is a simple way to test whether the *Gr28* genes could be involved in the foraging behavior, in addition to testing the ability of larvae to detect a food source.

The unusual expression of the *Gr28* genes in multidendritic neurons led us to explore whether these genes could be involved in nociception. The multidendritic

neurons, specifically of the dendritic arborization (da) types III and IV, are thought to be involved in nociception (D. Tracey, pers. comm.). Four of the *Gr28* genes are expressed in adult md neurons, and at least two are expressed in larval md neurons of the da-type IV. Because of this pattern of expression, prelimary experiments to test the pain responsiveness of Δ28 lines were performed.

Whereas trial experiments did not show any difference in the time to eclosion between mutant and control animals and mutant animals did not demonstrate altered foraging behavior or thermal sensitivity compared to control larvae, we did find that Δ28 54B3 wandering 3rd instar larvae have slightly greater aversion to 10mM caffeine compared to the control line used.

5.1.2 Introduction to adult assays

Using the adult to test for feeding behavior phenotypes may be more difficult in the adult than in larvae, as prolonged periods of starvation are often required to induce feeding, and thus allow the determination of whether mutant animals have altered taste sensitivities. However, most of the expression analysis of the *Gr28* genes has been in the adult, and we have been relatively successful in examining feeding behavior in the adult stage using two-choice feeding assays. We attempted to measure adult taste sensitivity using residence assays (Marella et al., 2006), which is similar to the larval position assay. Like the larval assay, animals are given a choice to walk around on an agarose slab in a petri dish. The agarose slab is divided into half, with different substrates on each side.

Marella and coworkers (2006) found that adults would avoid standing on a bitter or repellant substrate, but would stay on a sweet substrate. Using this assay, we found that Δ28 54B3 mutant flies may have an enhanced avoidance response to allyl isothiocyanate (AITC) – which gives wasabi its "bite" – compared to control animals. Along with results from larval behavioral experiments, it appears that the *Gr28* genes may play a role in detecting aversive substrates, or modulating avoidance responses.

5.2 Materials and Methods

Initial behavioral experiments included here are of those in which both the deletion lines and control lines were put in the same genetic backgrounds. It should also be noted that at the time of these experiments, the *Gr28* rescue line (NT51) had not yet been made, so there was no optimal control line for the behavioral experiments.

Behavioral experiments, especially testing taste preference in adults and larvae, were also performed in the deletion lines before they were put in the same genetic background as the control. These experiments are not included in this Chapter in great detail. A summary of these experiments is presented as Tables later in the Chapter, however. Any behavioral experiments described here will have to be repeated, and will also have to be carried out with the appropriate controls (ie the rescue line in the Δ28 background).

5.2.1 Larval lifespan

Fly lines used in this assay have been backcrossed to w^{1118} for five generations, and are all in the same genetic background. In order to determine if larvae have increased larval lifespan (slower growth) or decreased larval lifespan, we set up the following crosses:

1. ♀^{virgin} Δ28 30i (or Δ28 54B3) X ♂w^{1118} in bottles n=30 ♀^{virgin}; n=50 ♂; parents left in bottles 2 days

2. ♀^{virgin} Δ28 30i (or Δ28 54B3)/+ X ♂ Δ28 30i (or Δ28 54B3)/+ cross sibs in vials; n=10 ♀^{virgin}; n=10 ♂; parents left in vial 1 day

3. expect ¼ +/+, ½ Δ28 30i/+, ¼ Δ28 30i/ Δ28 30i; collect progeny twice a day, noting date, time, and eye color (which will tell genotype)

By noting at what time larvae of different genotypes hatch out, it is possible to get an approximate time the individual spent as a larva. Eclosion time for experimental animals, ie those that are homozygous mutant (Δ28 30i/ Δ28 30i), could be compared to their siblings, wild-type (+/+) and heterozygous animals (Δ28 30i/+). Since environmental conditions affect time to eclosion, comparing the homozygous mutant larvae to their siblings serves as an internal control. Six vials were set up for both the Δ28 30i and Δ28 54B5 crosses. This experiment was only done once, and would need to be repeated for proper analysis of results.

5.2.2 Larval position assay

Fly lines used in this assay have been backcrossed to w^{1118} for five generations, and are all in the same genetic background. Approximately 40 3rd instar larvae were collected from food, washed briefly in 1XPBS, and collected on Whatmann paper wetted with water (Figure 49). The assay was performed as soon after collection as possible, as we didn't want the larvae to become starved ("starvation" in larvae occurs occurs after only two hours). Larvae were then place in 60x15mm petri dishes, with one half of the dish filled with one test substrate (plus agarose) and the other half with the other test substrate (plus agarose). Plates were made directly before the assay was performed. Briefly, a 100mM stock solution of caffeine or the test substrate was made. This stock was then diluted in distilled water and added to 1% agarose solution before being poured into plates. Directly before the experiment, agarose from plates were cut out and replaced to make plates that were one-half substrate 1 and one-half substrate 2. Larvae (n=35-40 per experiment)were then placed in the middle and allowed to wander to either side. Larvae were counted at various time intervals for approximately 180 minutes.

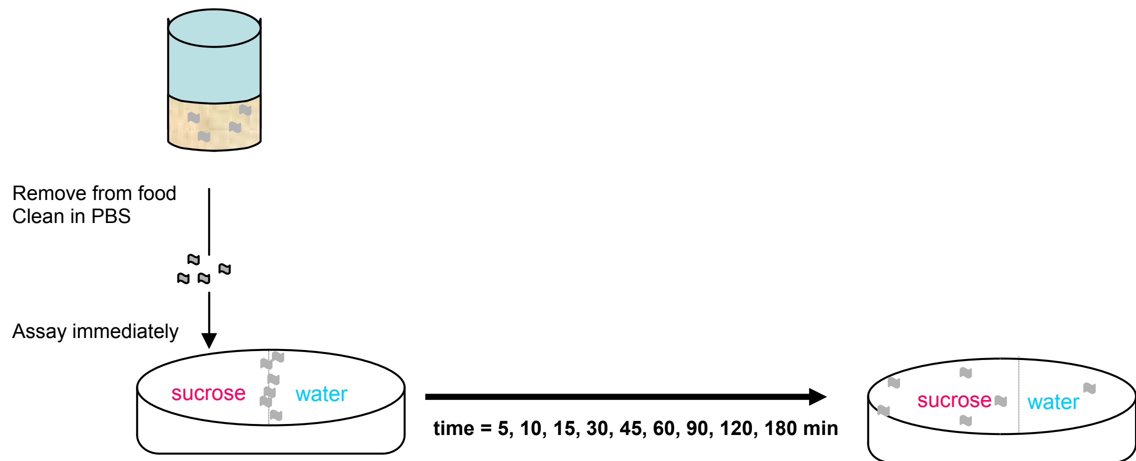


Figure 49: Schematic of set-up for larval position assay to test for larval preference.

The preference index (PI) for a test substrate is calculated as number larvae on the test substrate side/total number larvae feeding (on either side). Larvae on the sides or top of the petri dish were not counted. A PI of 0.5 indicates the larvae have no preference for either substrate (and are found equally on either side of the plate), a PI < 0.5 indicates preference for the control (avoidance of test substrate), and a PI>0.5 indicates a preference for the test substrate (attraction to test substrate).

5.2.3 Larval foraging assay

Fly lines used in this assay have been backcrossed to w^{1118} for five generations, and are all in the same genetic background. Wandering 3rd instar larvae are collected and washed in 1XPBS. Approximately 25 larvae are then placed in the center of a petri dish. In the center of the dish there is a disc of agarose that contains the test substrate, either sugar, which is a food source, or a repellant, like caffeine. The surrounding agarose is

generally neutral (Figure 50). The number of larvae that remain on the disc are counted every minute for 20 minutes. The experiment was repeated four times for the donor construct line (NT47G4) and Δ28 54B3, but only once for Δ28 30i.

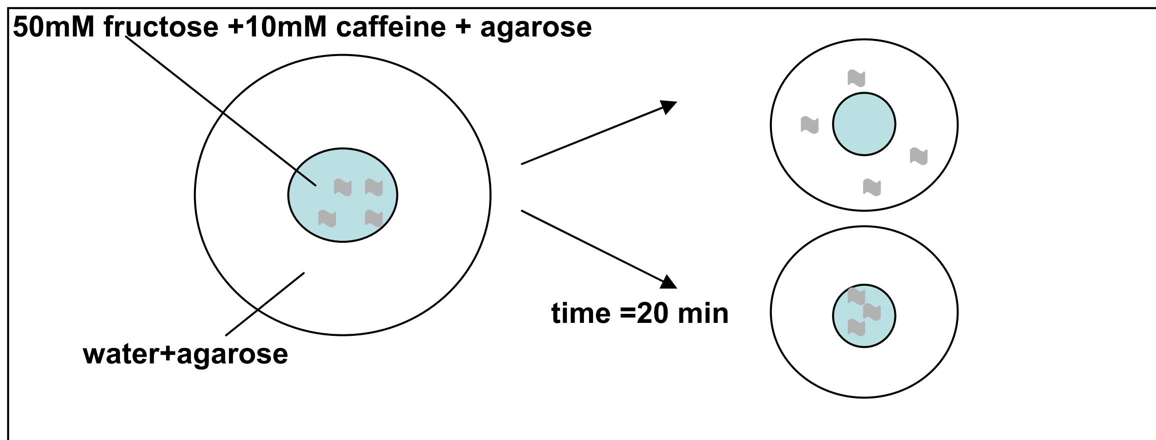


Figure 50: Schematic of larval foraging assay set-up.

5.2.4 Nociception assays

Larval nociception assays were carried out in the laboratory of Dr. Dan Tracey, by one of his graduate students, Lixian Zhong. Fly lines used in this assay have been backcrossed to w^{1118} for five generations, and are all in the same genetic background . Wandering 3rd instar larvae are collected and briefly rinsed in water. Approximately 30 larvae are tested per assay. The assay consists of placing a hot probe (47°C) against abdominal segments III to VI, and measuring the length of time it takes the larva to roll a full 180°. The hot probe is not removed until the larva has rolled. An individual larva is only tested once. It has been shown that larva that are have decreased sensitivity to thermal pain take a longer time to roll (Tracey et al., 2003). Hypersensitivity to thermal

pain is also possible, with the larvae responding more quickly to hot probe application, and responding at lower probe temperatures, not generally detected as painful by control larvae. This phenomenon is not tested by measuring length of time to response, as the response times are too quick. Instead, the temperature of the probe is decreased to 42°C, and then the length of time to response is measured. Most normal larvae do not sense this as pain, but hypersensitive larvae will quickly roll in response to probe application.

Thermal assays are taped with a digital video recorder. Recordings of the assays are played back, during which time a stop watch is used to determine time to response for each animal.

5.2.5 Adult residence assays

Male flies were collected and either starved for 28 hours (on wet Whatmann paper) ("starved"), or used immediately for an assay ("fed") . Agarose plates were made as described in the larval position preference assay (see section 5.2.3) the morning of the assay. Agarose plates were poured such that there would be just enough room for the flies to walk, but not fly, between the top of the agarose and the petri dish cover. All assays were conducted at 3PM. Directly before the assay, male flies were knock-out by placing their vial on ice for a short period of time, and then transferred to the assay plate. Six assay plates with 25 individuals were captured by video over the course of one hour. Snapshots of the plates with the flies were taken every 10 seconds (Figure 51).

These videos were converted to DVD quality (mpeg2 format) and then opened and saved as an "image sequence" in Quicktime Pro. This image sequence was then opened with Image J software (freely available to the public and found on-line) where the images were converted to black and white images, the size of a fly was defined (generally between 3 and 30 pixels), and the "Analyze Particles" tool was used to count the number of flies within a defined area (one-half of a given petri dish) for each 10-second frame. This data was copied into Excel, where it was then analyzed.

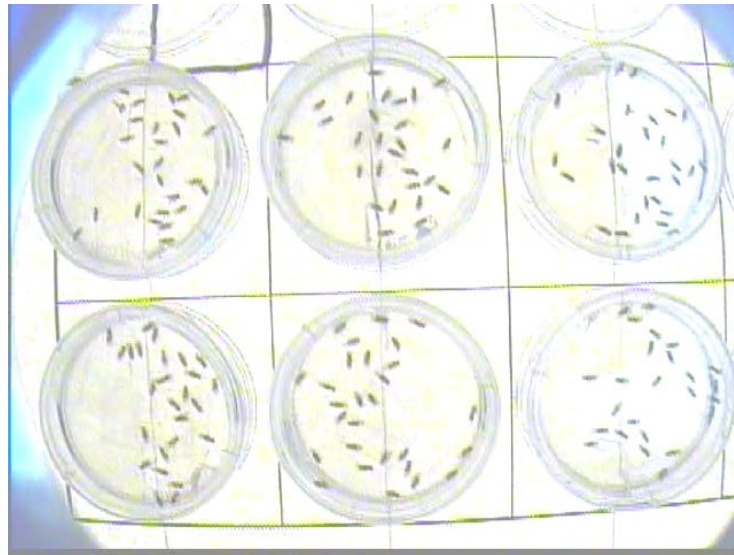


Figure 51: A single snapshot of all six residence assay plates was taken every 10 seconds by a video recorder. The number of flies on each side of the plate was counted using Image J to determine if adult flies of each genotype were similarly sensitive to a test substrate.

5.3 Results

5.3.1 Mutant larvae have normal development time

No noticeable differences were seen in the length of time it took for control larvae and mutant larvae to eclose after pupation (Figure 52 and 53). The ratio of larvae that hatched was also as expected, with 90:213:118 (+/+:Δ2830i het:Δ2830i hom) and 87:176:78 (+/+:Δ2854B3het: Δ2854B3hom).

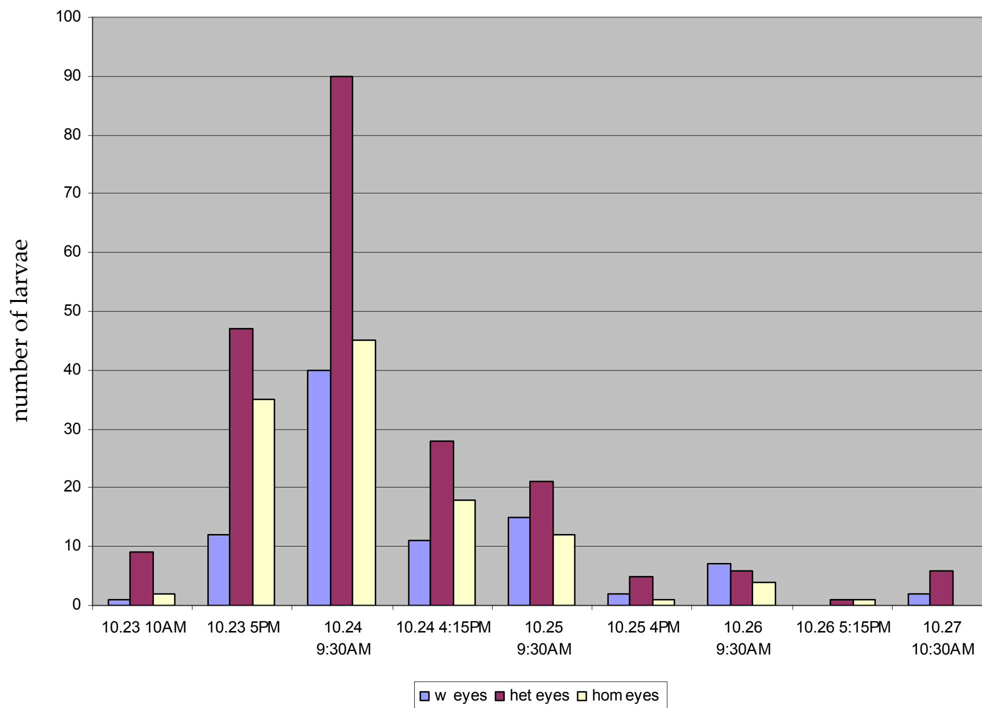


Figure 52: Eclosion time for Δ28 30i animals (hom eyes) is similar to that for animals that are heterozygous for the deletion (het eyes) and wild-type (w eyes). The x-axis shows the day and time of eclosion.

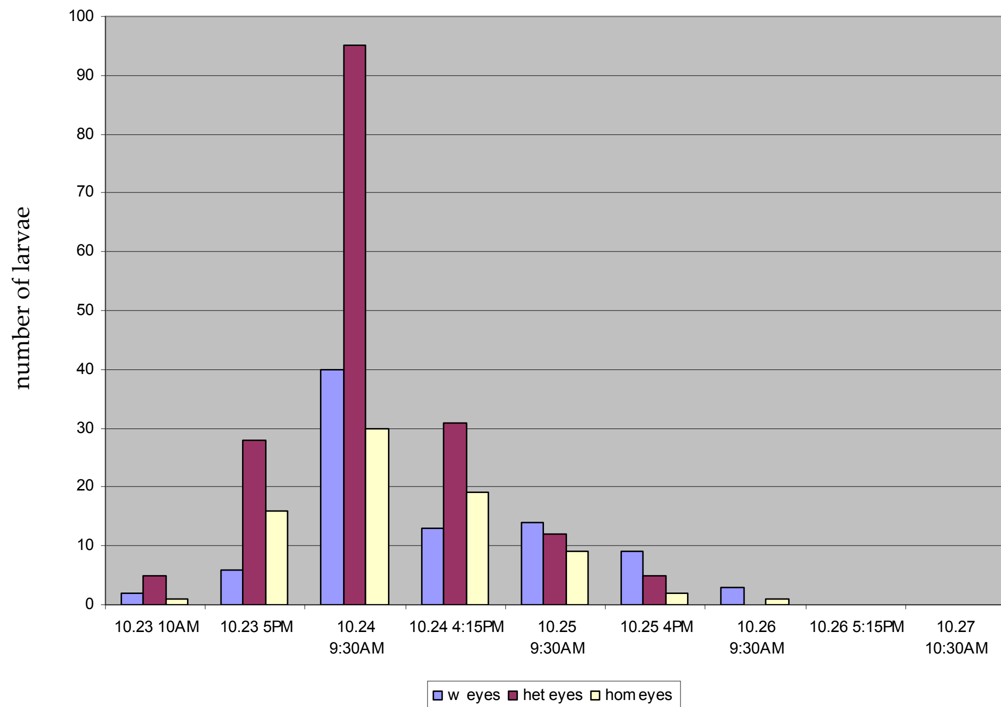


Figure 53: Eclosion time for Δ28 54B3 animals (hom eyes) is similar to that for animals that are heterozygous for the deletion (het eyes) and wild-type (w eyes). The x-axis shows the day and time of eclosion.

5.3.2 Δ28 mutant larvae may have enhanced sensitivity to caffeine

Larval position assays indicate that all the lines - control (donor construct line, NT47G4 in isogenic background), Δ28 30i, and Δ28 54B3 (both in same background) - showed aversion to 10mM caffeine over the time-frame tested. The Δ28 54B3 line did appear to show slightly greater aversion at most time points compared to the other lines (Figure 54), and had slightly greater aversion over total time (Figure 55). This experiment was only repeated two to three times for each genotype, however, so these

results are preliminary and need to be repeated. Additionally, the donor construct line (homozygous for donor construct) may not be the best control to use for this assay. A better control would be the *Gr28* genomic rescue line (NT51) in the Δ28 background. This line was not available at the time these assays were performed.

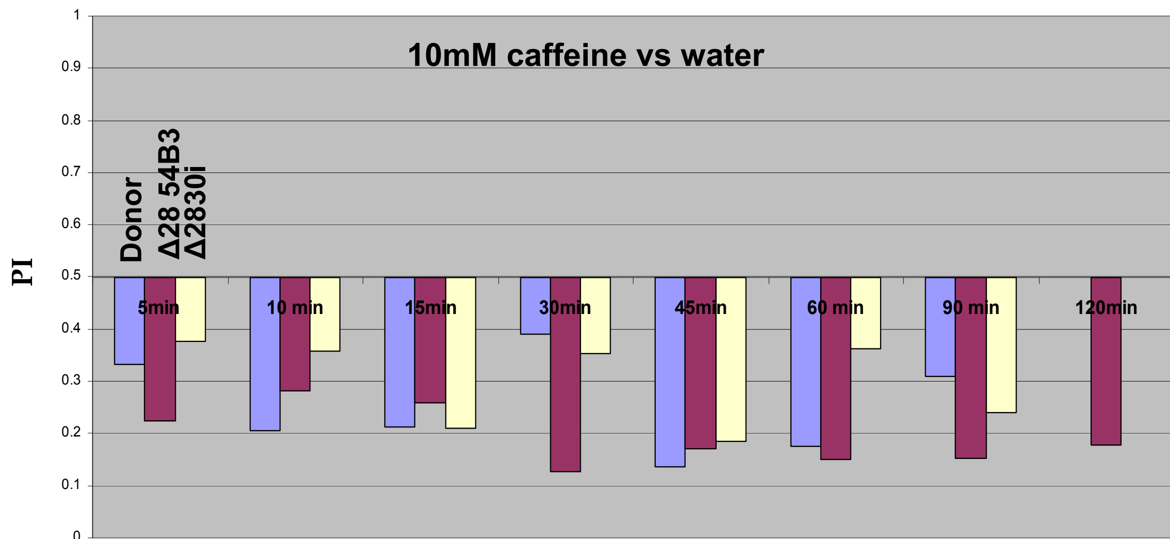


Figure 54: Larvae avoid 10mM caffeine over time. All larval lines are in the same genetic background. Results shown here are an average over two to three experiments. The donor construct was used as a control. Error bars were not added, as the number of experiments is too low for statistical analysis.

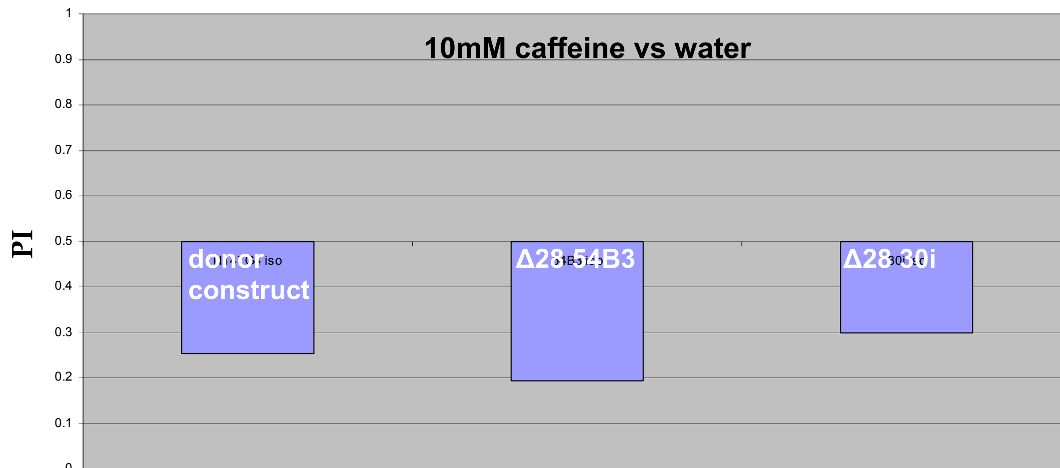


Figure 55: Average larval preference over time for each genotype. The Δ28 54B3 line may have slightly greater avoidance of caffeine compared to the control line, but this experiment would have to be repeated, and the appropriate control line used for comparison. Error bars were not added, as the number of experiments is too low for statistical analysis.

Though only one larval position preference assay (10mM caffeine vs water, shown above) was performed using lines of the same genetic background, many of these assays were performed on the Δ28 lines before they were outcrossed, with a variety of different substrates. Based on these assays, it appeared that the Δ28 lines could have a stronger aversion to bitter substrates than other lines. However, at the time not only were the Δ28 lines not in an isogenic background, but the control lines had a different genetic background. Additionally, the ideal control line – the *Gr28* rescue line – had not yet been made and was not available for these assays. The results of larval position

preference assays performed in lines that have not been backcrossed is summarized in Table 12.

Table 12: Summary of larval preference assays using lines that have not been backcrossed to w^{1118}. The PI listed is for the substrate other than water. If the PI<0.5, this indicates that larvae avoid the test substrate, preferring water.

Assay	Genotype	Number of plates (n=40 larvae/plate)	Average PI per plate
10mM aspartic acid vs water	w	4	0.76
	30i	4	0.73
30mM aspartic acid vs water	30i	4	0.74
	w	4	0.81
10mM glycine vs water	30i	4	0.48
	w	4	0.53
50mM glycine vs water	30i	4	0.5
	w	4	0.52
4mM sucrose vs water	w	4	0.6
	NT47G4	4	0.49
	30i	8	0.5
8mM sucrose vs water	w	4	0.48
	NT47G4	4	0.42
	30i	8	0.6
	yw;FLPSceI	2	0.64
20mM sucrose vs water	30i	12	0.61
	NT47G4	4	0.6
	w	8	0.65
35mM sucrose vs water	30i	4	0.44
	NT47G4	4	0.65
50mM fructose vs water	30i	4	0.68
	NT47G4	4	0.65
25mM trehalose vs water	w	8	0.45
	30i	4	0.23
	w;p[70FLP]	4	0.37
	yw;FLPSceI	4	0.49
	NT47G4	4	0.27
50mM trehalose vs water	w	8	0.41
	30i	4	0.43
	w;p[70FLP]	4	0.43
	yw;FLPSceI	4	0.46
	NT47G4	4	0.4
100mM trehalose vs water	30i	4	0.1
	NT47G4	4	0.53
	54B3	4	0.61
	w;p[70FLP]	4	0.65
1M trehalose vs water	30i	4	0.052
	NT47G4	4	0.61

Table 12 continued

	54B3	4	0.83
	w;p[70FLP]	4	0.54
10mM lobelineHCl vs water	30i	4	0.23
	NT47G4	4	0.34
1mM caffeine vs water	30i	4	0.4
	NT47G4	4	0.49
10mM caffeine vs water	w	4	0.25
	w;p[70FLP]	8	0.22
	30i	8	0.062
	54B3	4	0.085
	NT47G4	8	0.21
	yw;FLPSceI	4	0.4
100mM caffeine vs water	NT47G4	2	0.3
	30i	2	0.033
10mMcaff+4mMsuc vs 4mMsuc	30i	2	0.15
	w;p[70FLP]	4	0.44
	yw;FLPSceI	4	0.26
	w	4	0.16
10mMcaff+20mMsuc vs 20mMsuc	30i	4	0.13
	NT47G4	4	0.26
10mM NaCl vs water	w;p[70FLP]	4	0.81
	30i	4	0.59
	w	4	0.70
	yw;FLPSceI	4	0.5
	NT47G4	4	0.62
100mM NaCl vs water	w;p[70FLP]	4	0.7
	30i	4	0.5
	w	4	0.59
	yw;FLPSceI	4	0.68
	NT47G4	4	0.55
250mM NaCl vs water	30i	4	0.1
	NT47G4	4	0.26
	w;p[70FLP]	4	0.24
	54B3	4	0.19
1M NaCl vs water	30i	4	0.069
	NT47G4	4	0.15
2.5M NaCl vs water	30i	4	0.081
	NT47G4	4	0.13
10mM berberine-hemisulfate vs water	30i	4	0.11
	NT47G4	4	0.34
	w;p[70FLP]	4	0.11
	54B3	4	0.21
25mM berberine-hemisulfate vs water	30i	4	0.13
	NT47G4	4	0.14
10mM denatonium benz. vs meth control	30i	4	0.12

Table 12 continued

	NT47G4	4	0.32
	w;p[70FLP]	4	0.064
	54B3	4	0.097
25mM denatonium benz. vs meth control	NT47G4	4	0.28
	30i	4	0.22
10% NaOH soaked vs water	30i	4	0.21
	NT47G4	4	0.28
10%acetic acid vs water	30i	4	0.088
	NT47G4	4	0.24
	54B3	4	0.093
	w;p[70FLP]	4	0.14
10%HCl soaked vs water	30i	4	0.28
	NT47G4	4	0.35

5.3.3 Δ28 mutant larvae display typical foraging behavior

There did not appear to be any striking differences in the foraging behavior over time between the Δ28 lines and the control lines. The number of experiments done was very small, however; for example, the Δ28 30i line was tested only once. The center disc contained a food substance that is normally attractive to larvae (50mM fructose) plus a substrate that is repulsive to larvae (10mM caffeine). It would be expected that fed larvae would leave the disc, repelled by the caffeine and look for food sources elsewhere (forage). Starved larvae, or larvae insensitive to caffeine, may stay on the center disc to feed. We find that over the course of 20 minutes approximately half of the larvae leave the disc, and the approximate rate of emigration appears about the same for all lines tested (Figure 56). Based on this experiment, it doesn't appear that the Δ28 lines have abnormal foraging behavior, or lack sensitivity to, or are more highly sensitive to, 10mM

caffeine. This experiment would need to be repeated, with the appropriate controls. Since the larvae tested weren't deprived of food before the assay, it would also be interesting to see how starved larvae respond. In addition, different test substrates could be used for the center disc (only food source, only repellant substrate) to see if the Δ28 larvae display a difference in foraging behavior.

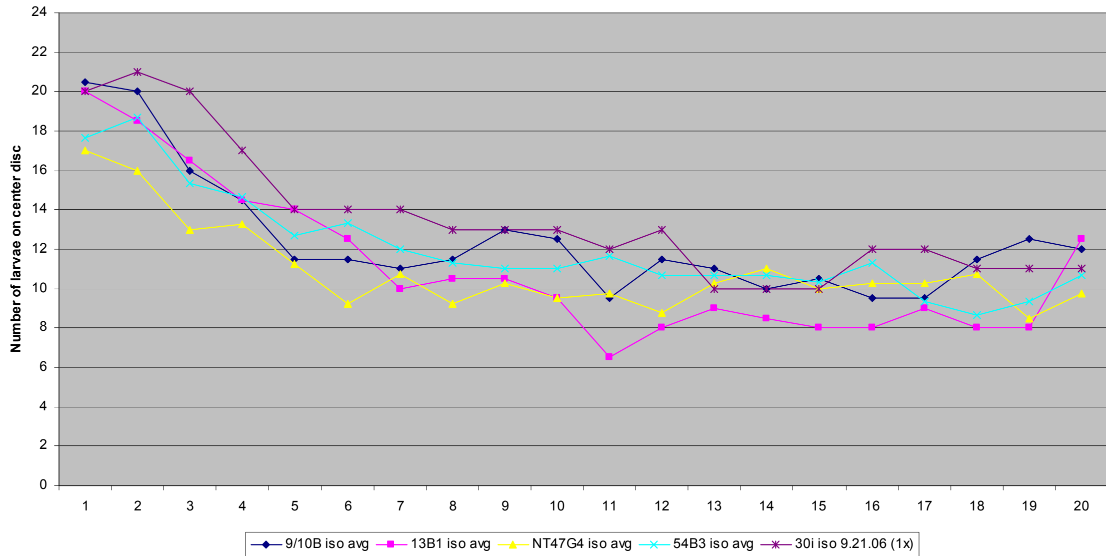


Figure 56: Larval foraging assay. The number of larvae on the center disc were counted every minute for 20 minutes. 9/10B and 13B1 are lines that had the donor construct not targeted to the correct location in the genome; they are homozygous for this incorrectly targeted donor construct. Error bars were not added, as the number of experiments is too low for statistical analysis.

5.3.4 Δ28 mutant larvae are not hyper- or hypo-sensitive to nociceptive thermal heat

The Δ28 30i and Δ28 54B3 larvae did not appear to have a nociception phenotype compared to the controls used – the donor construct control and w^{1118} (data not shown). The time to rolling response upon application of the 47°C probe did not differ significantly from the controls, and the mutant larvae did not appear to be hypersensitive (respond to 42°C probe) (data not shown). Experiments performed were very preliminary, and would need to be repeated, with the appropriate controls.

5.3.5 Δ28 mutant adults may have enhanced sensitivity to aversive substrates

Residence assays were used to test the sensitivity and preference of adult flies for certain substrates. It should be noted that only the results of lines that were backcrossed to w^{1118} are included in this section. Most of these experiments were only done once, or a few times, and again, without the ideal rescue control line, so all experiments should be repeated.

One residence assay performed was that of 50mM fructose plus 10mM caffeine versus water. The tested substrate contains both an attractive food source (fructose) plus a contaminant, that, alone, is repulsive. It would be expected that flies that did not undergo the 28 hour starvation period would avoid the fructose plus caffeine substrate, but that flies that were starved may stay on the contaminated food source. Not only does

this assay test whether flies can detect caffeine and fructose, but it also tests if flies are able to alter their choices based on satiety. We find, depending on the genotype, that generally flies either avoid the contaminated food substrate the entire time, or are initially are attracted to it, but about halfway through the time course, avoid it (Figure 57). The behavior of the Δ28 lines did not appear to differ dramatically from the behavior of the other genotypes.

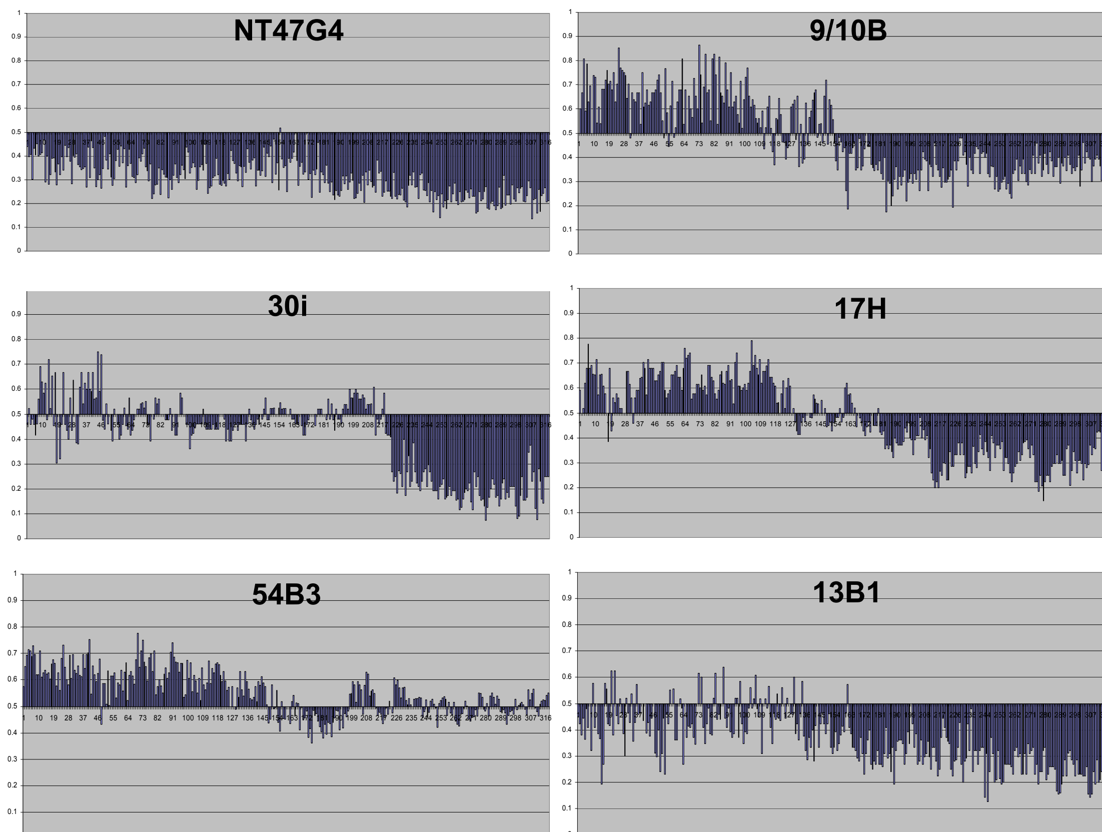


Figure 57: Adult residence assay using FED flies. Flies could choose to stand on 50mM fructose +10mM caffeine or water over a period of one hour, with data points taken every 10 seconds. The y-axis is the PI, and the intersection with the x-axis is at PI=0.5, which is no preference. PIs below 0.5 indicate avoidance of the fructose plus caffeine substrate, and a PI above 0.5 indicates attraction to this substrate. NT47G4 is the original donor construct line, and 9/10B, 13B1, and 17H are lines that have the donor

construct incorrectly targeted on the 2nd chromosome. The results shown are for an average of three assays for NT47G4 and Δ28 54B3. The other genotypes had only one assay performed.

When flies are starved for a period of 28 hours before the assay, we find that the avoidance for the caffeine-contaminated fructose is not as strong as when they were fed, for most genotypes (Figure 58). Two genotypes continue to show aversion over the entire length of time, and two genotypes show attraction over most of the length of the assay. The response profile over the time period for the Δ28 lines does look different from the resonse of the other lines, but more assays would be needed to confirm this difference. Overall, the results for this assay are expected for most genotypes tested, with a decrease in the avoidance of the caffeine-contaminated food source.

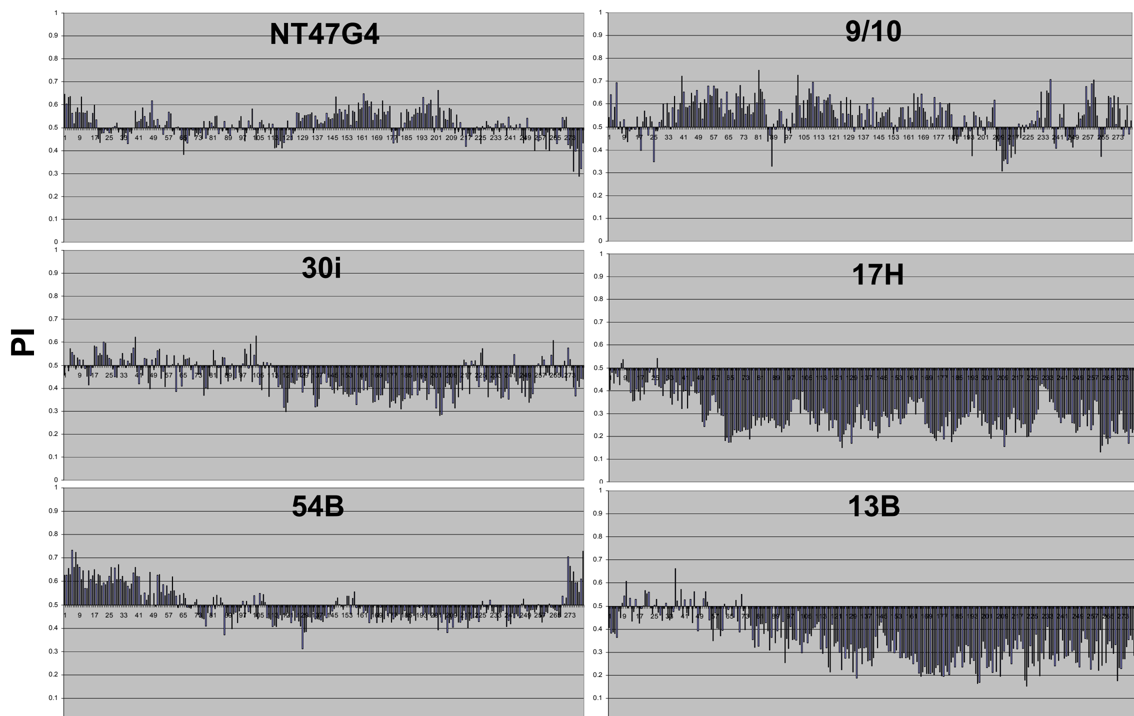


Figure 58: Adult residence assay using STARVED flies. Flies could choose to stand on 50mM fructose +10mM caffeine or water over a period of one hour, with data points taken every 10 seconds. The y-axis is the PI, and the intersection with the x-axis (time) is at PI=0.5, which is no preference. PIs below 0.5 indicate avoidance of the fructose plus caffeine substrate, and a PI above 0.5 indicates attraction to this substrate. NT47G4 is the original donor construct line, and 9/10B, 13B1, and 17H are lines that have the donor construct incorrectly targeted on the 2nd chromosome. The results shown are for an average of three assays for NT47G4 and four assays for Δ28 54B3. The other genotypes had two assays performed.

The second residence assay conducted was to determine if the Δ28 mutant flies could detect and were attracted to 1mM trehalose over water. Flies were not starved before the assay. It would be expected that starved flies would definitely be attracted to the 1mM trehalose over water, but it is not clear how fed flies would respond. We found that all genotypes preferred the 1mM trehalose over water throughout the time tested (Figure 59). The Δ28 lines didn't appear to behave differently from the other genotpyes, although the Δ28 54B3 line did not appear as attracted to the trehalose as the other

genotypes, especially at the beginning of the assay.

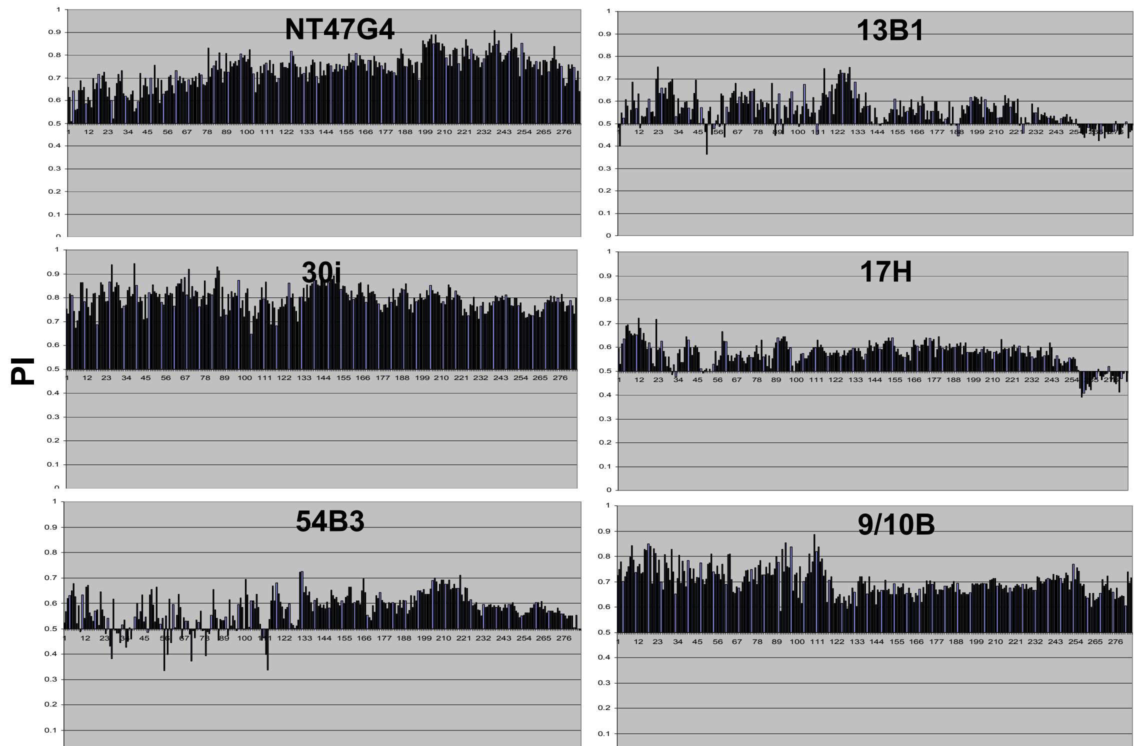


Figure 59: Adult residence assay using FED flies. Flies could choose to stand on 1M trehalose or water over a period of one hour, with data points taken every 10 seconds. The y-axis is the PI, and the intersection with the x-axis (time) is at PI=0.5, which is no preference. PIs below 0.5 indicate avoidance of the trehalose substrate, and a PI above 0.5 indicates attraction to this substrate. NT47G4 is the original donor construct line, and 9/10B, 13B1, and 17H are lines that have the donor construct incorrectly targeted on the 2nd chromosome. The results shown are for an average of one to two trials for each genotype.

The last adult residence assay performed was to test the ability of flies to detect alyll isothiocyanate (AITC), which is the “bite” found in wasabi, and is detected by neurons that express *painless* (Al-Anzi et al., 2006). This assay was performed because the *Gr28* genes may also be expressed in neurons of the labellum that express *painless*, as well as being expressed in abdominal md neurons that may mediate nociception. For

this assay, flies were given the choice of standing on a 2mM AITC substrate or plain agarose. It should be noted that we found that 4mM AITC is lethal to flies within 10 minutes.

All genotypes demonstrated aversion to the 2mM AITC, with the two Δ28 lines demonstrating greater avoidance behavior over time relative to the other lines (Figure 60). The Δ28 54B3 line showed dramatically increased avoidance to the AITC about halfway through the assay. Since the results shown are the average for only two assays, this sensitivity should be explored further.

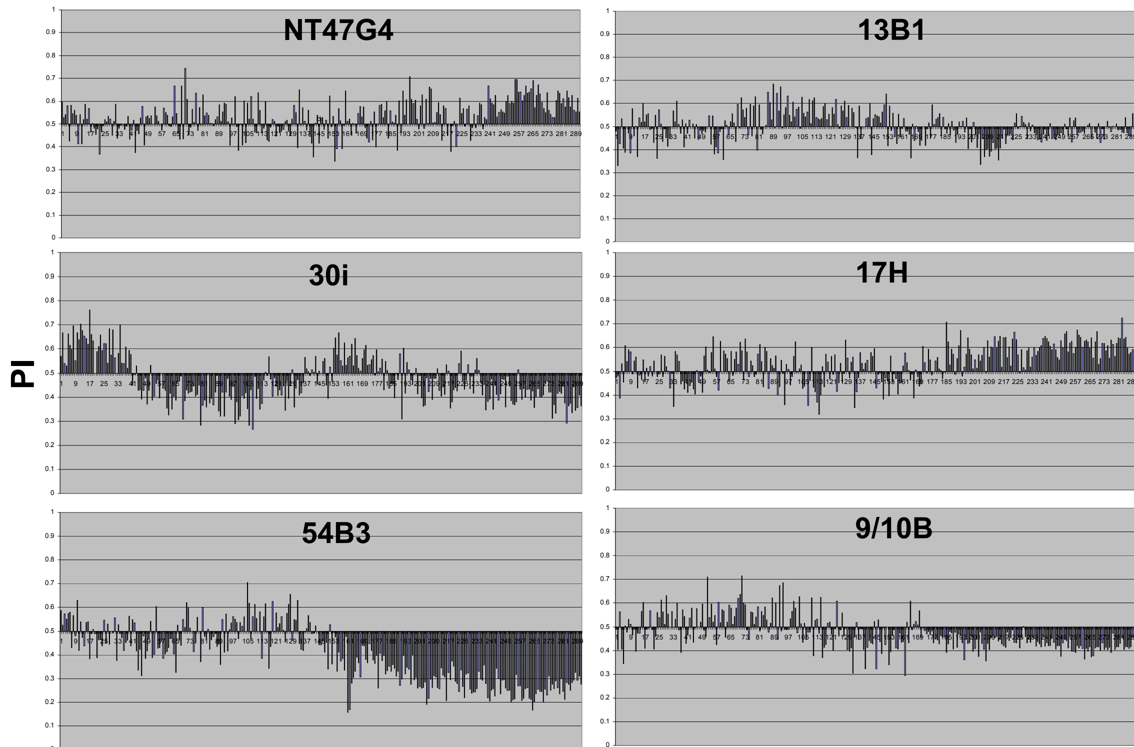


Figure 60: Adult residence assay using FED flies. Flies could choose to stand on 2mM AITC or water over a period of one hour, with data points taken every 10 seconds. The y-axis is the PI, and the intersection with the x-axis (time) is at PI=0.5, which is no preference. PIs below 0.5 indicate avoidance of the AITC substrate, and a PI above 0.5

indicates attraction to this substrate. NT47G4 is the original donor construct line, and 9/10B, 13B1, and 17H are lines that have the donor construct incorrectly targeted on the 2nd chromosome. The results shown are an average of two assays for each genotype.

Some adult behavioral assays were performed on fly lines that were not backcrossed to w^{1118}. The results of these assays are summarized in Table 13.

Table 13: Adult residence assay experiments on lines not put in same genetic background (not backcrossed to w^{1118}).

assay	genotype	number trials	fed or starved	average PI (for first substrate listed) over time
10mM caffeine vs water	NT47G4	4	fed	0.32
	30i	3	fed	0.30
	54B3	4	fed	0.40
10mM caffeine vs water	NT47G4	5	starved	0.34
	30i	4	starved	0.48
	54B3	6	starved	0.39
50mM fructose +10mMcaffeine vs 50mM fructose	NT47G4	4	fed	0.55
	30i	4	fed	0.67
	54B3	4	fed	0.61
50mM fructose +10mMcaffeine vs 50mM fructose	NT47G4	4	starved	0.54
	30i	3	starved	0.53
	54B3	3	starved	0.60

5.4 Conclusion and future directions for determining the function of the Gr28 genes

Based on the behavioral experiments that have been performed thusfar, it is still not clear what the function of the *Gr28* genes is. Experiments that address the function of the cells that express *Gr28b.c* suggest that at least a fraction of the cells that express these receptors are involved in avoidance-type behavior. These experiments would have to be

repeated to get a larger sample number, and driving the expression of VR1 using different *Gr28* drivers may prove informative. This type of experiment offers a promising approach to determining the function of cells that express the *Gr28* genes.

Many experiments were performed on Δ28 mutant larvae (30i and 54B3) before these lines were made isogenic. These experiments strikingly showed that the mutant animals may have enhanced sensitivity to aversive substrates, like bitter compounds (caffeine, denatonium benzoate), high-salt, and acetic acid (Table 12). This data looks promising, and needs to be repeated in the newly backcrossed Δ28 30i and Δ28 54B3 lines with the appropriate rescue line control. Based on these experiments, and expression of VR1 in *Gr28b.c*-expressing cells, it appears that the *Gr28* genes may play a role in detecting aversive substances or modulating the detection or response to aversive substances.

Behavioral experiments performed in adult flies generally did not offer clear or interesting results. One result that looks very promising, however, and should be followed up on, is the response of Δ28 54B3 adult flies to AITC. Although only two assays were performed, it appeared that these mutant animals had an enhanced aversive response to AITC (Figure 60). Al-Anzi and colleagues (2006) have found that *painless*, a TRP channel, also expressed in taste neurons of the labellum and md neurons of the abdomen, is required for the detection of AITC. It is interesting to speculate that perhaps the *Gr28* genes are involved in detection of AITC, considering these receptors are

probably expressed in the same labellar neurons and md neurons that express *painless.* Whereas loss of *painless* causes loss of AITC sensitivity, loss of *Gr28* appears to cause enhanced sensitivity to AITC (Figure 60). This may suggest a role for the *Gr28* genes in modulating avoidance resonses to aversive substrates.

Based on the limited number of behavioral experiments performed, it appears the *Gr28* genes may play a role in the detection of aversive substances or in modulating avoidance responses to aversive substrates. Both larval and adult residence assays suggest that Δ28 mutant animals may have enhanced responses to aversive substrates. Future experiments should focus on addressing this, by repeating experiments in newly backcrossed Δ28 lines with the rescue line as a control.

Preliminary exeperiments suggest a role for the *Gr28* genes in detecting aversive substrates and avoidance responses, however, members of this subfamily are expressed in cells that most likely would not carry out such a function, and that have interesting functions themselves. *Gr28b.b* and *Gr28b.c* are expressed in cells of the pars intercerebralis, which may be neurosecretory, and express the *Drosophila insulin-like peptides* (*DILP*s). It is possible that these GR28s are nutrient receptors, monitoring trehalose levels in the haemolymph, and thereby influencing DILP secretion. Future experiments should confirm whether *Gr28b.b* and *Gr28b.c* are coexpressed with the *DILP*s. If these receptors are coexpressed with DILPs, then experiments could also address whether Δ28 flies have difficulty monitoring humoral nutrient levels and

regulating DILP secretion. This could be measured by determining whether trehalose concentrations in the haemolymph during satiety and starvation are comparable to control flies. Other diabetic-type phenotypes, like growth and developmental delays, and reduced fecundity (Ikeya et al., 2002; Rulifson et al., 2002), have not been seen so far in Δ28 flies, but we have not yet rigorously tested for these phenotypes.

Gr28b.d is expressed in three neurons at the base of the arista, whose afferents project to the antennal lobe. Electrophysiological studies in *Drosophila* suggest that taste bristles contain a neuron sensitive to water (Fujishiro et al., 1984), and neurons of the arista have been proposed to be hygroreceptors (Foelix et al., 1989; Sayeed and Benzer, 1996). It is possible that *Gr28b.d* could function as a water receptor, as it has the expected expression pattern for such a receptor. Interestingly, it has recently been found that two *Gr*s function as CO_2 receptors (*Gr21a* and *Gr63a*) (Jones et al., 2007; Suh et al., 2004), so there is precedence for *Gr*s to interact with non-taste related ligands. The response of Δ28 flies to humidity and moisture should be explored.

Many of the *Gr28*s are expressed in md neurons of the abdomen. These neurons have been shown to have nociceptive functions, in addition to possibly functioning as sensors for touch and temperature (Smith and Shepherd, 1996; Tracey et al., 2003). Aristal neurons, three of which express *Gr28b.d*, are also thought to be involved in temperature detection (Foelix et al., 1989).The possibility that these receptors function in thermodetection should also be explored.

In conclusion, all the tools have been made to properly explore and determine the function of the *Gr28* genes. All that needs to be done, and in no way is this trivial, is to rigorously and methodically test the Δ28 mutant lines for phenotypes. The challenge will be to develop assays that are sensitive enough to determine the function of these genes, which may not act as traditional *Gr*s in the detection of tastants. In addition, since these genes are expressed in many cell types, it isn't inherently obvious which putative function – avoidance modulation, water receptor, nutrient receptor, thermoreceptor, to name a few – should be addressed. Hopefully the preliminary work presented here will offer ideas and provide a foundation for the future work of determining the function of the *Gr28* genes.

6 Summary and conclusions

A molecular understanding of taste - the detection of tastants and the perception of taste qualities – is a rapidly developing field. In just the last few years, the *Drosophila gustatory receptor* gene family has been discovered (Clyne et al., 2000; Dunipace et al., 2001; Scott et al., 2001), *Gr5a* has been defined as a trehalose receptor (Chyb et al., 2003; Dahanukar et al., 2001; Ueno et al., 2001), *Gr66a* has been characterized as a caffeine receptor (Moon et al., 2006), *Gr21a* and *Gr63a* have been found to function as CO_2 receptors (Jones et al., 2007; Suh et al., 2004), and *Gr68a* (Bray and Amrein, 2003) and *Gr32a* (Miyamoto and Amrein, unpubl.) are expressed in cells that may detect pheromones. It has been determined that at least two functionally defined sub-populations of taste neurons exist – those that mediate aversive taste behavior and those that mediate attraction to tastants (Marella et al., 2006; Thorne et al., 2004; Wang et al., 2004) – and that the activity of these sub-populations of taste neurons depends on the *Gr*s that they express (Marella et al., 2006).

Discoveries in the field, however, have brought up new questions. There are the obvious questions as to what the function is of the other *Gr*s analyzed so far (such as *Gr22e*, *Gr59b*, *Gr28*, etc) that are co-expressed with *Gr66a* , a caffeine receptor. It is very likely that they are receptors for other bitter tastants, but this has not yet been addressed. So far, the attractive-type taste neurons of the labellum have been found to

only express *Gr5a*, a trehalose receptor, (though the *Gr28*s may also be expressed in these neurons, it has not been confirmed), and so the search for other sweet taste receptors is ongoing. Members of the *Gr64* gene family are good candidates, as they have the greatest sequence similarity to *Gr5a* (Chyb et al., 2003; Dunipace et al., 2001; Robertson et al., 2003).

In contrast to bitter taste in mammals, all expression data argue that, in *Drosophila*, different avoidance neurons express distinct combinations of putative bitter taste receptors, which may indicate that discrimination between different bitter compounds may exist. However, experiments examining the activation profile of taste neurons using the calcium-indicator G-CaMP performed, by Marella and colleagues (2006), indicate that activation of these subsets of neurons may not allow discrimination. They found that *Gr32a* and *Gr47a*, receptors that are expressed in a subset of labellar neurons that express *Gr66a*, show largely similar activation profiles to each other, and to that generated by *Gr66a*-expressing neurons. This was shown for only a couple receptors, and against a limited panel of bitter substrates, and so the possibility of discrimination has not been fully explored. It also remains possible that these cells are responsive to repellent substrates other than bitter compounds, and this has not been adequately tested.

It is also not clear what the receptors are for other taste qualities, like umami (amino acid), sour, and salty taste. It is likely that the receptors for salty and sour taste

are channels, instead of GPCRs, and some studies have come up with possible candidates (Liu et al., 2003a; Nakamura et al., 2002). Salty and sour tastes are not only human perceptions, but also cause behavioral responses in insects and are especially relevant in fluid and electrolyte homeostasis. Whereas the perception of sour taste has not been explored in much detail in *Drosophila*, detection of salts, which regulates the uptake of Na+ and other cations, has been studied both at the electrophysiological and more recently at the molecular-genetic level. For example, the fly exhibits differentiated responses to salts and feeds on it at low to moderate concentrations (up to 100 mM), but avoids it at concentrations of more than 200 mM. Based on electrophysiological studies, these two opposing behaviors appear to be mediated by distinct neurons in the taste sensilla, the L1 and L2 cells, respectively, (Figure 4).

In mammals, salts (NaCl or KCl) are thought to be detected by epithelial-type sodium channels (ENaCs), several of which are expressed in taste cells and are thought to be involved in the detection of extracellular Na+ ions that directly activate taste cells (Lindemann, 1996). Liu and collaborators specifically addressed the potential role of these channels, also known as the *pickpocket (ppk)* gene family, in *Drosophila* taste (Liu et al., 2003a). They found that at least six *ppk* genes (of a total of ~ 25) are expressed in the larval and/or adult taste system, among other tissues. A role in salt perception for two of these, ppk11 and ppk19, was addressed by expressing dominant negative forms of these channels, which are thought to inhibit channel function through the creation of

non-functional multimers, as well as by RNA interference experiments to prevent expression of the specific channels altogether. These experiments revealed that both *ppk* genes are required for the detection of low concentration of salt in the larva. Moreover, aversion to high salt concentration was markedly reduced, especially in adult flie. These data indicate that epithelial-type Na+ channels of *Drosophila* are required for the detection of salts and that this taste modality appears to be mediated by the same molecular machinery in mammals and insects (Figure 61).

Another type of membrane-associated protein was recently shown to be involved in the detection of salts (Nakamura et al., 2002). Nakamura and co-workers mapped a mutation in the defective *proboscis extension responsive 1 (dpr1)* mutant strain to a member of the *DPR-Ig* family of genes, which are characterized by two Ig repeats and a single transmembrane segment. None of the other twenty or so *dpr* like genes have been characterized and it is not known whether any of these are involved in the detection of salts, nor is it known whether the mammalian counterparts have a role in taste sensation.

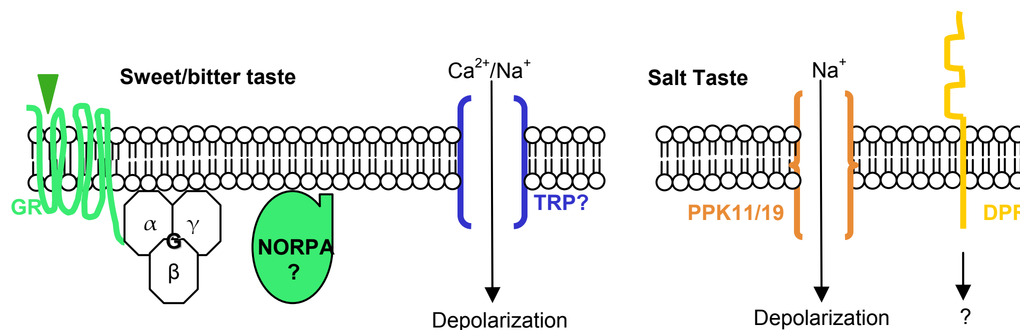


Figure 61: Taste signal transduction for sweet, bitter, and salt compounds. Shown are putative players in the signal transduction pathways for taste.

Although it has been assumed that the *Gr*s are GPCRs, based on their predicted membrane topology, and precendence for GPCR-signaling in the mammalian taste system, it has not been proven that the GRs are GPCRs. In fact, evidence for the *Drosophila* olfactory system suggests that the ORs, to which the GRs are related, may not have the topology initially predicted (Benton et al., 2006), may function as multimers (Benton et al., 2006; Jones et al., 2007; Larsson et al., 2004), and may not actually be GPCRs. Thus it is possible that the GRs also have a membrane topology other than what has been predicted, and do not function as GPCRs. It has also not been tested whether or not the GRs function as multimers. The discovery that both GR21a and GR63a are required for CO_2 detection suggests this may be the case.

Identifying downstream signaling components of the cell signaling pathway involved in tastant detection would help shed light on whether the GRs function as GPCRs. So far, only a couple downstream signaling components have been proposed, mainly based on their expression in taste neurons: NORPA - a phospholipase C protein (Koganezawa and Shimada, 2002)– and $G_{\alpha s}$ (Ueno et al., 2006) (Figure 9F and 59). Much work remains in determining the identities of downstream signaling components, and whether or not the *Drosophila* taste signal transduction is similar to that found in mammals, where taste receptors are GPCRs which signal through a phospholipase C and a TRP channel (Zhang et al., 2003).

Beyond the detection of taste, questions still remain as to how taste is perceived. We now know that avoidance- and attractive-type taste neurons exist, and that their axons project to discrete and non-overlapping areas of the SOG, the primary taste center in the brain. Presumably this will eventually lead to appropriate behavioral output – either feeding or avoidance. It is also not clear if, and how, activation of peripheral sensory neurons is integrated to produce the appropriate response. The identities of neurons that make up the taste circuit are also largely unknown. Some neurons that produce neuropeptides have been identified and found to impinge on the SOG (Bader et al., 2007; Melcher and Pankratz, 2005; Wu et al., 2005), and we find that the *Gr28* genes are expressed in cells of the pars intercerebralis that project afferents to the SOG. These neurons are likely candidates for being higher order neurons in the taste circuit.

There is no doubt that there is plenty of work to be done, and exciting discoveries to be made, in the rapidly evolving field of *Drosophila* taste perception.

References

Adams CM, Anderson MG, Motto DG, Price MP, Johnson WA, Welsh MJ. 1998. Ripped pocket and pickpocket, novel Drosophila DEG/ENaC subunits expressed in early development and in mechanosensory neurons. The Journal of cell biology 140(1):143-152.

Adler E, Hoon MA, Mueller KL, Chandrashekar J, Ryba NJ, Zuker CS. 2000. A novel family of mammalian taste receptors. Cell 100(6):693-702.

Al-Anzi B, Tracey WD, Jr., Benzer S. 2006. Response of Drosophila to wasabi is mediated by painless, the fly homolog of mammalian TRPA1/ANKTM1. Curr Biol 16(10):1034-1040.

Antle MC, Steen NM, Mistlberger RE. 2001. Adenosine and caffeine modulate circadian rhythms in the Syrian hamster. Neuroreport 12(13):2901-2905.

Arora K, Rodrigues V, Joshi S, Shanbhag S, Siddiqi O. 1987. A gene affecting the specificity of the chemosensory neurons of Drosophila. Nature 330(6143):62-63.

Bader R, Colomb J, Pankratz B, Schrock A, Stocker RF, Pankratz MJ. 2007. Genetic dissection of neural circuit anatomy underlying feeding behavior in Drosophila: Distinct classes of hugin-expressing neurons. The Journal of comparative neurology 502(5):848-856.

Bello B, Resendez-Perez D, Gehring WJ. 1998. Spatial and temporal targeting of gene expression in Drosophila by means of a tetracycline-dependent transactivator system. Development (Cambridge, England) 125(12):2193-2202.

Benton R, Sachse S, Michnick SW, Vosshall LB. 2006. Atypical membrane topology and heteromeric function of Drosophila odorant receptors in vivo. PLoS biology 4(2):e20.

Bodmer R, Jan YN. 1987. Morphological differentiation of the embryonic peripheral neurons in Drosophila. Roux's Archives of Developmental Biology 196:69-77.

Brand AH, Perrimon N. 1993. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development (Cambridge, England) 118(2):401-415.

Branscomb A, Seger J, White RL. 2000. Evolution of odorant receptors expressed in mammalian testes. Genetics 156(2):785-797.

Bray S, Amrein H. 2003. A putative Drosophila pheromone receptor expressed in male-specific taste neurons is required for efficient courtship. Neuron 39(6):1019-1029.

Buck L, Axel R. 1991. A novel multigene family may encode odorant receptors: a molecular basis for odor recognition. Cell 65(1):175-187.

Buck LB. 1996. Information coding in the vertebrate olfactory system. Annual review of neuroscience 19:517-544.

Caicedo A, Kim KN, Roper SD. 2002. Individual mouse taste cells respond to multiple chemical stimuli. The Journal of physiology 544(Pt 2):501-509.

Castro JP, Carareto CM. 2004. Drosophila melanogaster P transposable elements: mechanisms of transposition and regulation. Genetica 121(2):107-118.

Chandrashekar J, Hoon MA, Ryba NJ, Zuker CS. 2006. The receptors and cells for mammalian taste. Nature 444(7117):288-294.

Chandrashekar J, Mueller KL, Hoon MA, Adler E, Feng L, Guo W, Zuker CS, Ryba NJ. 2000. T2Rs function as bitter taste receptors. Cell 100(6):703-711.

Chen Y, Flanagan JG. 2006. Follow your nose: axon pathfinding in olfactory map formation. Cell 127(5):881-884.

Chyb S, Dahanukar A, Wickens A, Carlson JR. 2003. Drosophila Gr5a encodes a taste receptor tuned to trehalose. Proceedings of the National Academy of Sciences of the United States of America 100 Suppl 2:14526-14530.

Clyne PJ, Warr CG, Carlson JR. 2000. Candidate taste receptors in Drosophila. Science (New York, NY 287(5459):1830-1834.

Clyne PJ, Warr CG, Freeman MR, Lessing D, Kim J, Carlson JR. 1999. A novel family of divergent seven-transmembrane proteins: candidate odorant receptors in Drosophila. Neuron 22(2):327-338.

Colomb J, Grillenzoni N, Ramaekers A, Stocker RF. 2007. Architecture of the primary taste center of Drosophila melanogaster larvae. The Journal of comparative neurology 502(5):834-847.

Couto A, Alenius M, Dickson BJ. 2005. Molecular, anatomical, and functional organization of the Drosophila olfactory system. Curr Biol 15(17):1535-1547.

Dahanukar A, Foster K, van der Goes van Naters WM, Carlson JR. 2001. A Gr receptor is required for response to the sugar trehalose in taste neurons of Drosophila. Nature neuroscience 4(12):1182-1186.

Damak S, Rong M, Yasumatsu K, Kokrashvili Z, Varadarajan V, Zou S, Jiang P, Ninomiya Y, Margolskee RF. 2003. Detection of sweet and umami taste in the absence of taste receptor T1r3. Science (New York, NY 301(5634):850-853.

de Bruyne M, Foster K, Carlson JR. 2001. Odor coding in the Drosophila antenna. Neuron 30(2):537-552.

de Velasco B, Erclik T, Shy D, Sclafani J, Lipshitz H, McInnes R, Hartenstein V. 2007. Specification and development of the pars intercerebralis and pars lateralis, neuroendocrine command centers in the Drosophila brain. Developmental biology 302(1):309-323.

Dethier VG. 1976. The hungry fly : a physiological study of the behavior associated with feeding. Cambridge, Mass.: Harvard University Press.

Dethier VG, Goldrich-Rachman N. 1976. Anesthetic stimulation of insect water receptors. Proceedings of the National Academy of Sciences of the United States of America 73(9):3315-3319.

DiAntonio A, Parfitt KD, Schwarz TL. 1993. Synaptic transmission persists in synaptotagmin mutants of Drosophila. Cell 73(7):1281-1290.

Dunipace L, Meister S, McNealy C, Amrein H. 2001. Spatially restricted expression of candidate taste receptors in the Drosophila gustatory system. Curr Biol 11(11):822-835.

Dyer J, Salmon KS, Zibrik L, Shirazi-Beechey SP. 2005. Expression of sweet taste receptors of the T1R family in the intestinal tract and enteroendocrine cells. Biochemical Society transactions 33(Pt 1):302-305.

Edgecomb RS, Murdock LL. 1992. Central projections of axons from taste hairs on the labellum and tarsi of the blowfly, Phormia regina Meigen. The Journal of comparative neurology 315(4):431-444.

Estes PS, Ho GL, Narayanan R, Ramaswami M. 2000. Synaptic localization and restricted diffusion of a Drosophila neuronal synaptobrevin--green fluorescent protein chimera in vivo. Journal of neurogenetics 13(4):233-255.

Falk DR, Bleiser-Avivi N, Atidia J. 1976. Labellar taste organs of Drosophila melanogaster. J Morphol 150:327-342.

Ferveur JF, Savarit F, O'Kane CJ, Sureau G, Greenspan RJ, Jallon JM. 1997. Genetic feminization of pheromones and its behavioral consequences in Drosophila males. Science (New York, NY 276(5318):1555-1558.

Fiala A, Spall T, Diegelmann S, Eisermann B, Sachse S, Devaud JM, Buchner E, Galizia CG. 2002. Genetically expressed cameleon in Drosophila melanogaster is used to visualize olfactory information in projection neurons. Curr Biol 12(21):1877-1884.

Finger TE, Bottger B, Hansen A, Anderson KT, Alimohammadi H, Silver WL. 2003. Solitary chemoreceptor cells in the nasal cavity serve as sentinels of respiration. Proceedings of the National Academy of Sciences of the United States of America 100(15):8981-8986.

Foelix RF, Stocker RF, Steinbrecht RA. 1989. Fine structure of a sensory organ in the arista of Drosophila melanogaster and some other dipterans. Cell and tissue research 258(2):277-287.

Frank ME, Bouverat BP, MacKinnon BI, Hettinger TP. 2004. The distinctiveness of ionic and nonionic bitter stimuli. Physiology & behavior 80(4):421-431.

Fredholm BB. 1995. Adenosine, adenosine receptors, and the actions of caffeine. Pharmacol Toxicol 995:93-101.

Fredholm BB, Battig K, Holmen J, Nehlig A, Zvartau EE. 1999. Actions of caffeine in the brain with special reference to factors that contribute to its widespread use. Pharmacological reviews 51(1):83-133.

Fujishiro N, Kijima H, Morita H. 1984. Impulse frequency and action potential amplitude in labellar chemosensory neurons of Drosophila melanogaster. Journal of insect physiology 30:317-325.

Gaillard I, Rouquier S, Giorgi D. 2004. Olfactory receptors. Cell Mol Life Sci 61(4):456-469.

Galindo K, Smith DP. 2001. A large family of divergent Drosophila odorant-binding proteins expressed in gustatory and olfactory sensilla. Genetics 159(3):1059-1072.

Gao Q, Chess A. 1999. Identification of candidate Drosophila olfactory receptors from genomic DNA sequence. Genomics 60(1):31-39.

Gao Q, Yuan B, Chess A. 2000. Convergent projections of Drosophila olfactory neurons to specific glomeruli in the antennal lobe. Nature neuroscience 3(8):780-785.

Glendinning JI, Brown H, Capoor M, Davis A, Gbedemah A, Long E. 2001. A peripheral mechanism for behavioral adaptation to specific "bitter" taste stimuli in an insect. J Neurosci 21(10):3688-3696.

Glendinning JI, Davis A, Ramaswamy S. 2002. Contribution of different taste cells and signaling pathways to the discrimination of "bitter" taste stimuli by an insect. J Neurosci 22(16):7281-7287.

Gong M, Rong YS. 2003. Targeting multi-cellular organisms. Current opinion in genetics & development 13(2):215-220.

Gong WJ, Golic KG. 2003. Ends-out, or replacement, gene targeting in Drosophila. Proceedings of the National Academy of Sciences of the United States of America 100(5):2556-2561.

Goto T, Salpekar A, Monk M. 2001. Expression of a testis-specific member of the olfactory receptor gene family in human primordial germ cells. Molecular human reproduction 7(6):553-558.

Gutierrez E, Wiggins D, Fielding B, Gould AP. 2007. Specialized hepatocyte-like cells regulate Drosophila lipid metabolism. Nature 445(7125):275-280.

Hall JC. 1979. Control of male reproductive behavior by the central nervous system of Drosophila: dissection of a courtship pathway by genetic mosaics. Genetics 92(2):437-457.

Hall JC. 1994. The mating of a fly. Science (New York, NY 264(5166):1702-1714.

Hartenstein V. 1993. Atlas of Drosophila development. Plainview, N.Y.: Cold Spring Harbor Laboratory Press. v, 57 p. p.

Heimbeck G, Bugnon V, Gendre N, Haberlin C, Stocker RF. 1999. Smell and taste perception in Drosophila melanogaster larva: toxin expression studies in chemosensory neurons. J Neurosci 19(15):6599-6609.

Hess D, Buschges A. 1999. Role of proprioceptive signals from an insect femur-tibia joint in patterning motoneuronal activity of an adjacent leg joint. Journal of neurophysiology 81(4):1856-1865.

Hill CA, Fox AN, Pitts RJ, Kent LB, Tan PL, Chrystal MA, Cravchik A, Collins FH, Robertson HM, Zwiebel LJ. 2002. G protein-coupled receptors in Anopheles gambiae. Science (New York, NY 298(5591):176-178.

Hiroi M, Marion-Poll F, Tanimura T. 2002. Differentiated response to sugars among labellar chemosensilla in Drosophila. Zoological science 19(9):1009-1018.

Hiroi M, Meunier N, Marion-Poll F, Tanimura T. 2004. Two antagonistic gustatory receptor neurons responding to sweet-salty and bitter taste in Drosophila. Journal of neurobiology 61(3):333-342.

Hoon MA, Adler E, Lindemeier J, Battey JF, Ryba NJ, Zuker CS. 1999. Putative mammalian taste receptors: a class of taste-specific GPCRs with distinct topographic selectivity. Cell 96(4):541-551.

Huang AL, Chen X, Hoon MA, Chandrashekar J, Guo W, Trankner D, Ryba NJ, Zuker CS. 2006. The cells and logic for mammalian sour taste detection. Nature 442(7105):934-938.

Ikeya T, Galic M, Belawat P, Nairz K, Hafen E. 2002. Nutrient-dependent expression of insulin-like peptides from neuroendocrine cells in the CNS contributes to growth regulation in Drosophila. Curr Biol 12(15):1293-1300.

Imai T, Suzuki M, Sakano H. 2006. Odorant receptor-derived cAMP signals direct axonal targeting. Science (New York, NY 314(5799):657-661.

Ishimaru Y, Inada H, Kubota M, Zhuang H, Tominaga M, Matsunami H. 2006. Transient receptor potential family members PKD1L3 and PKD2L1 form a candidate sour taste receptor. Proceedings of the National Academy of Sciences of the United States of America 103(33):12569-12574.

Ishimoto H, Matsumoto A, Tanimura T. 2000. Molecular identification of a taste receptor gene for trehalose in Drosophila. Science (New York, NY 289(5476):116-119.

Isono K, Kikuchi T. 1974. Autosomal recessive mutation in sugar response of Drosophila. Nature 248(445):243-244.

Jones WD, Cayirlioglu P, Kadow IG, Vosshall LB. 2007. Two chemosensory receptors together mediate carbon dioxide detection in Drosophila. Nature 445(7123):86-90.

Jordan MJ, Tandon K, Shaw PE, Goodner KL. 2001. Aromatic profile of aqueous banana essence and banana fruit by gas chromatography-mass spectrometry (GC-MS) and gas chromatography-olfactometry (GC-O). Journal of agricultural and food chemistry 49(10):4813-4817.

Kamikouchi A, Shimada T, Ito K. 2006. Comprehensive classification of the auditory sensory projections in the brain of the fruit fly Drosophila melanogaster. The Journal of comparative neurology 499(3):317-356.

Keil TA. 1997. Functional morphology of insect mechanoreceptors. Microscopy research and technique 39(6):506-531.

Koganezawa M, Shimada I. 2002. Inositol 1,4,5-trisphosphate transduction cascade in taste reception of the fleshfly, Boettcherisca peregrina. Journal of neurobiology 51(1):66-83.

Kurtovic A, Widmer A, Dickson BJ. 2007. A single class of olfactory neurons mediates behavioural responses to a Drosophila sex pheromone. Nature 446(7135):542-546.

Laissue PP, Reiter C, Hiesinger PR, Halter S, Fischbach KF, Stocker RF. 1999. Three-dimensional reconstruction of the antennal lobe in Drosophila melanogaster. The Journal of comparative neurology 405(4):543-552.

Larsson MC, Domingos AI, Jones WD, Chiappe ME, Amrein H, Vosshall LB. 2004. Or83b encodes a broadly expressed odorant receptor essential for Drosophila olfaction. Neuron 43(5):703-714.

Lee KS, You KH, Choo JK, Han YM, Yu K. 2004. Drosophila short neuropeptide F regulates food intake and body size. The Journal of biological chemistry 279(49):50781-50789.

Lienhard M, Stocker RF. 1987. Sensory projection patterns of supernumerary legs and aristae in D. melanogaster. J Exp Zoology 244:187-201.

Lindemann B. 1996. Taste reception. Physiological reviews 76(3):718-766.

Liu L, Leonard AS, Motto DG, Feller MA, Price MP, Johnson WA, Welsh MJ. 2003a. Contribution of Drosophila DEG/ENaC genes to salt taste. Neuron 39(1):133-146.

Liu L, Yermolaieva O, Johnson WA, Abboud FM, Welsh MJ. 2003b. Identification and function of thermosensory neurons in Drosophila larvae. Nature neuroscience 6(3):267-273.

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

Marella S, Fischler W, Kong P, Asgarian S, Rueckert E, Scott K. 2006. Imaging taste responses in the fly brain reveals a functional map of taste category and behavior. Neuron 49(2):285-295.

Martin JR, Keller A, Sweeney ST. 2002. Targeted expression of tetanus toxin: a new tool to study the neurobiology of behavior. Advances in genetics 47:1-47.

Matsunami H, Amrein H. 2003. Taste and pheromone perception in mammals and flies. Genome biology 4(7):220.

Matsunami H, Montmayeur JP, Buck LB. 2000. A family of candidate taste receptors in human and mouse. Nature 404(6778):601-604.

Max M, Shanker YG, Huang L, Rong M, Liu Z, Campagne F, Weinstein H, Damak S, Margolskee RF. 2001. Tas1r3, encoding a new candidate taste receptor, is allelic to the sweet responsiveness locus Sac. Nature genetics 28(1):58-63.

McBride CS. 2007. Rapid evolution of smell and taste receptor genes during host specialization in Drosophila sechellia. Proceedings of the National Academy of Sciences of the United States of America 104(12):4996-5001.

Melcher C, Bader R, Pankratz MJ. 2007. Amino acids, taste circuits, and feeding behavior in Drosophila: towards understanding the psychology of feeding in flies and man. The Journal of endocrinology 192(3):467-472.

Melcher C, Pankratz MJ. 2005. Candidate gustatory interneurons modulating feeding behavior in the Drosophila brain. PLoS biology 3(9):e305.

Meunier N, Ferveur JF, Marion-Poll F. 2000. Sex-specific non-pheromonal taste receptors in Drosophila. Curr Biol 10(24):1583-1586.

Meunier N, Marion-Poll F, Rospars JP, Tanimura T. 2003. Peripheral coding of bitter taste in Drosophila. Journal of neurobiology 56(2):139-152.

Minke B, Parnas M. 2006. Insights on TRP channels from in vivo studies in Drosophila. Annual review of physiology 68:649-684.

Mitchell BK, Itagaki H. 1992. Interneurons of the subesophageal ganglion of Sarcophaga bullata responding to gustatory and mechanosensory stimuli. Journal of comparative physiology 171(2):213-230.

Mitchell BK, Itagaki H, Rivet MP. 1999. Peripheral and central structures involved in insect gustation. Microscopy research and technique 47(6):401-415.

Miyawaki A, Griesbeck O, Heim R, Tsien RY. 1999. Dynamic and quantitative Ca2+ measurements using improved cameleons. Proceedings of the National Academy of Sciences of the United States of America 96(5):2135-2140.

Mombaerts P, Wang F, Dulac C, Chao SK, Nemes A, Mendelsohn M, Edmondson J, Axel R. 1996a. Visualizing an olfactory sensory map. Cell 87(4):675-686.

Mombaerts P, Wang F, Dulac C, Vassar R, Chao SK, Nemes A, Mendelsohn M, Edmondson J, Axel R. 1996b. The molecular biology of olfactory perception. Cold Spring Harbor symposia on quantitative biology 61:135-145.

Montmayeur JP, Liberles SD, Matsunami H, Buck LB. 2001. A candidate taste receptor gene near a sweet taste locus. Nature neuroscience 4(5):492-498.

Moon SJ, Kottgen M, Jiao Y, Xu H, Montell C. 2006. A taste receptor required for the caffeine response in vivo. Curr Biol 16(18):1812-1817.

Morea M. 1985. Deletion mapping of a new gustatory mutant in Drosophila melanogaster. Experientia 41:1381-1384.

Morita H. 1992. Transduction process and impulse initiation in insect contact chemoreceptor. Zoological science 9:1-16.

Mueller KL, Hoon MA, Erlenbach I, Chandrashekar J, Zuker CS, Ryba NJ. 2005. The receptors and coding logic for bitter taste. Nature 434(7030):225-229.

Murphey RK, Possidente D, Pollack G, Merritt DJ. 1989. Modality-specific axonal projections in the CNS of the flies Phormia and Drosophila. The Journal of comparative neurology 290(2):185-200.

Nakai J, Ohkura M, Imoto K. 2001. A high signal-to-noise Ca(2+) probe composed of a single green fluorescent protein. Nature biotechnology 19(2):137-141.

Nakamura M, Baldwin D, Hannaford S, Palka J, Montell C. 2002. Defective proboscis extension response (DPR), a member of the Ig superfamily required for the gustatory response to salt. J Neurosci 22(9):3463-3472.

Nayak S, Singh RN. 1983. Sensilla on the tarsal segments and the mouthparts of adult Drosophila melanogaster. Int J Insect Morph Embryol 12:273-291.

Nayak S, Singh RN. 1985. Primary sensory projections from the labella to the brain of Drosophila melanogaster Meigen. Int J Insect Morph Embryol 14:115-129.

Nelson G, Chandrashekar J, Hoon MA, Feng L, Zhao G, Ryba NJ, Zuker CS. 2002. An amino-acid taste receptor. Nature 416(6877):199-202.

Nelson G, Hoon MA, Chandrashekar J, Zhang Y, Ryba NJ, Zuker CS. 2001. Mammalian sweet taste receptors. Cell 106(3):381-390.

Neuhaus EM, Gisselmann G, Zhang W, Dooley R, Stortkuhl K, Hatt H. 2005. Odorant receptor heterodimerization in the olfactory system of Drosophila melanogaster. Nature neuroscience 8(1):15-17.

Ng M, Roorda RD, Lima SQ, Zemelman BV, Morcillo P, Miesenbock G. 2002. Transmission of olfactory information between three populations of neurons in the antennal lobe of the fly. Neuron 36(3):463-474.

Olsen SR, Bhandawat V, Wilson RI. 2007. Excitatory interactions between olfactory processing channels in the Drosophila antennal lobe. Neuron 54(1):89-103.

Oppliger FY, P MG, Vlimant M. 2000. Neurophysiological and behavioural evidence for an olfactory function for the dorsal organ and a gustatory one for the terminal organ in Drosophila melanogaster larvae. Journal of insect physiology 46(2):135-144.

Ozcan S, Dover J, Johnston M. 1998. Glucose sensing and signaling by two glucose receptors in the yeast Saccharomyces cerevisiae. The EMBO journal 17(9):2566-2573.

Parks AL, Cook KR, Belvin M, Dompe NA, Fawcett R, Huppert K, Tan LR, Winter CG, Bogart KP, Deal JE, Deal-Herr ME, Grant D, Marcinko M, Miyazaki WY, Robertson S, Shaw KJ, Tabios M, Vysotskaia V, Zhao L, Andrade RS, Edgar KA, Howie E, Killpack K, Milash B, Norton A, Thao D, Whittaker K, Winner MA, Friedman L, Margolis J, Singer MA, Kopczynski C, Curtis D, Kaufman TC, Plowman GD, Duyk G, Francis-Lang HL. 2004. Systematic generation of high-resolution deletion coverage of the Drosophila melanogaster genome. Nature genetics 36(3):288-292.

Parmentier M, Libert F, Schurmans S, Schiffmann S, Lefort A, Eggerickx D, Ledent C, Mollereau C, Gerard C, Perret J, et al. 1992. Expression of members of the putative olfactory receptor gene family in mammalian germ cells. Nature 355(6359):453-455.

Pelosi P. 1998. Odorant-binding proteins: structural aspects. Annals of the New York Academy of Sciences 855:281-293.

Perez CA, Huang L, Rong M, Kozak JA, Preuss AK, Zhang H, Max M, Margolskee RF. 2002. A transient receptor potential channel expressed in taste receptor cells. Nature neuroscience 5(11):1169-1176.

Pikielny CW, Hasan G, Rouyer F, Rosbash M. 1994. Members of a family of Drosophila putative odorant-binding proteins are expressed in different subsets of olfactory hairs. Neuron 12(1):35-49.

Prasad BC, Reed RR. 1999. Chemosensation: molecular mechanisms in worms and mammals. Trends Genet 15(4):150-153.

Rajashekhar KP, Singh RN. 1994. Neuroarchitecture of the tritocerebrum of Drosophila melanogaster. The Journal of comparative neurology 349(4):633-645.

Ramsey IS, Delling M, Clapham DE. 2006. An introduction to TRP channels. Annual review of physiology 68:619-647.

Reddy S, Jin P, Trimarchi J, Caruccio P, Phillis R, Murphey RK. 1997. Mutant molecular motors disrupt neural circuits in Drosophila. Journal of neurobiology 33(6):711-723.

Robertson HM, Warr CG, Carlson JR. 2003. Molecular evolution of the insect chemoreceptor gene superfamily in Drosophila melanogaster. Proceedings of the National Academy of Sciences of the United States of America 100 Suppl 2:14537-14542.

Rodrigues V, Sathe S, Pinto L, Balakrishnan R, Siddiqi O. 1991. Closely linked lesions in a region of the X chromosome affect central and peripheral steps in gustatory processing in Drosophila. Mol Gen Genet 226(1-2):265-276.

Rodrigues V, Siddiqi O. 1981. A gustatory mutant of Drosophila defective in pyranose receptors. Mol Gen Genet 181(3):406-408.

Rong YS, Golic KG. 2000. Gene targeting by homologous recombination in Drosophila. Science (New York, NY 288(5473):2013-2018.

Rulifson EJ, Kim SK, Nusse R. 2002. Ablation of insulin-producing neurons in flies: growth and diabetic phenotypes. Science (New York, NY 296(5570):1118-1120.

Sayeed O, Benzer S. 1996. Behavioral genetics of thermosensation and hygrosensation in Drosophila. Proceedings of the National Academy of Sciences of the United States of America 93(12):6079-6084.

Sbarbati A, Osculati F. 2005. The taste cell-related diffuse chemosensory system. Progress in neurobiology 75(4):295-307.

Schroter U, Menzel R. 2003. A new ascending sensory tract to the calyces of the honeybee mushroom body, the subesophageal-calycal tract. The Journal of comparative neurology 465(2):168-178.

Scott K, Brady R, Jr., Cravchik A, Morozov P, Rzhetsky A, Zuker C, Axel R. 2001. A chemosensory gene family encoding candidate gustatory and olfactory receptors in Drosophila. Cell 104(5):661-673.

Serizawa S, Miyamichi K, Takeuchi H, Yamagishi Y, Suzuki M, Sakano H. 2006. A neuronal identity code for the odorant receptor-specific and activity-dependent axon sorting. Cell 127(5):1057-1069.

Shanbhag S, Singh RN. 1992. Functional implications of the pojections of neurons from individual labellar sensillum of Drosophila melanogaster as revealed by neuronal-marker horseradish peroxidase. Cell and tissue research 216:273-282.

Shanbhag SR, Park SK, Pikielny CW, Steinbrecht RA. 2001. Gustatory organs of Drosophila melanogaster: fine structure and expression of the putative odorant-binding protein PBPRP2. Cell and tissue research 304(3):423-437.

Shang Y, Claridge-Chang A, Sjulson L, Pypaert M, Miesenbock G. 2007. Excitatory local circuits and their implications for olfactory processing in the fly antennal lobe. Cell 128(3):601-612.

Singh RN. 1997. Neurobiology of the gustatory systems of Drosophila and some terrestrial insects. Microscopy research and technique 39(6):547-563.

Smith SA, Shepherd D. 1996. Central afferent projections of proprioceptive sensory neurons in Drosophila revealed with the enhancer-trap technique. The Journal of comparative neurology 364(2):311-323.

Steinbrecht RA. 1998. Odorant-binding proteins: expression and function. Annals of the New York Academy of Sciences 855:323-332.

Stocker RF. 1994. The organization of the chemosensory system in Drosophila melanogaster: a review. Cell and tissue research 275(1):3-26.

Stocker RF, Schorderet M. 1981. Cobalt filling of sensory projections from internal and external mouthparts in Drosophila. Cell and tissue research 216(3):513-523.

Stocker RF, Singh RN, Schorderet M, Siddiqi O. 1983. Projection patterns of different types of antennal sensilla in the antennal glomeruli of Drosophila melanogaster. Cell and tissue research 232(2):237-248.

Suh GS, Wong AM, Hergarden AC, Wang JW, Simon AF, Benzer S, Axel R, Anderson DJ. 2004. A single population of olfactory sensory neurons mediates an innate avoidance behaviour in Drosophila. Nature 431(7010):854-859.

Sweeney ST, Broadie K, Keane J, Niemann H, O'Kane CJ. 1995. Targeted expression of tetanus toxin light chain in Drosophila specifically eliminates synaptic transmission and causes behavioral defects. Neuron 14(2):341-351.

Tanimura T, Isono K, Takamura T, Shimada T. 1982. Genetic dimorphism in the taste sensitivity to trehalose in Drosophila melanogaster. Journal of comparative physiology 141:433-437.

Tanimura T, Shimada T. 1981. Multiple receptor proteins for sweet taste in Drosophila melanogaster. Journal of comparative physiology 141:265-269.

Thibault ST, Singer MA, Miyazaki WY, Milash B, Dompe NA, Singh CM, Buchholz R, Demsky M, Fawcett R, Francis-Lang HL, Ryner L, Cheung LM, Chong A, Erickson C, Fisher WW, Greer K, Hartouni SR, Howie E, Jakkula L, Joo D, Killpack K, Laufer A, Mazzotta J, Smith RD, Stevens LM, Stuber C, Tan LR, Ventura R, Woo A, Zakrajsek I, Zhao L, Chen F, Swimmer C, Kopczynski C, Duyk G, Winberg ML, Margolis J. 2004. A complementary transposon tool kit for Drosophila melanogaster using P and piggyBac. Nature genetics 36(3):283-287.

Thorne N, Chromey C, Bray S, Amrein H. 2004. Taste perception and coding in Drosophila. Curr Biol 14(12):1065-1079.

Tobin D, Madsen D, Kahn-Kirby A, Peckol E, Moulder G, Barstead R, Maricq A, Bargmann C. 2002. Combinatorial expression of TRPV channel proteins defines their sensory functions and subcellular localization in C. elegans neurons. Neuron 35(2):307-318.

Tompkins L, Cardosa MJ, White FV, Sanders TG. 1979. Isolation and analysis of chemosensory behavior mutants in Drosophila melanogaster. Proceedings of the National Academy of Sciences of the United States of America 76(2):884-887.

Tracey WD, Jr., Wilson RI, Laurent G, Benzer S. 2003. painless, a Drosophila gene essential for nociception. Cell 113(2):261-273.

Ueno K, Kohatsu S, Clay C, Forte M, Isono K, Kidokoro Y. 2006. Gsalpha is involved in sugar perception in Drosophila melanogaster. J Neurosci 26(23):6143-6152.

Ueno K, Ohta M, Morita H, Mikuni Y, Nakajima S, Yamamoto K, Isono K. 2001. Trehalose sensitivity in Drosophila correlates with mutations in and expression of the gustatory receptor gene Gr5a. Curr Biol 11(18):1451-1455.

Vanderhaeghen P, Schurmans S, Vassart G, Parmentier M. 1997. Specific repertoire of olfactory receptor genes in the male germ cells of several mammalian species. Genomics 39(3):239-246.

Venken KJ, Bellen HJ. 2005. Emerging technologies for gene manipulation in Drosophila melanogaster. Nature reviews 6(3):167-178.

Vosshall LB, Amrein H, Morozov PS, Rzhetsky A, Axel R. 1999. A spatial map of olfactory receptor expression in the Drosophila antenna. Cell 96(5):725-736.

Vosshall LB, Wong AM, Axel R. 2000. An olfactory sensory map in the fly brain. Cell 102(2):147-159.

Wang F, Nemes A, Mendelsohn M, Axel R. 1998. Odorant receptors govern the formation of a precise topographic map. Cell 93(1):47-60.

Wang JW, Wong AM, Flores J, Vosshall LB, Axel R. 2003. Two-photon calcium imaging reveals an odor-evoked map of activity in the fly brain. Cell 112(2):271-282.

Wang Z, Singhvi A, Kong P, Scott K. 2004. Taste representations in the Drosophila brain. Cell 117(7):981-991.

Wieczorek H, Wolff G. 1989. The labellar sugar receptor of Drosophila. Journal of comparative physiology 164:825-834.

Wilson RI, Laurent G. 2005. Role of GABAergic inhibition in shaping odor-evoked spatiotemporal patterns in the Drosophila antennal lobe. J Neurosci 25(40):9069-9079.

Wu Q, Zhao Z, Shen P. 2005. Regulation of aversion to noxious food by Drosophila neuropeptide Y- and insulin-like systems. Nature neuroscience 8(10):1350-1355.

Wu SV, Rozengurt N, Yang M, Young SH, Sinnett-Smith J, Rozengurt E. 2002. Expression of bitter taste receptors of the T2R family in the gastrointestinal tract and enteroendocrine STC-1 cells. Proceedings of the National Academy of Sciences of the United States of America 99(4):2392-2397.

Xu GG, Rothenberg PL. 1998. Insulin receptor signaling in the beta-cell influences insulin gene expression and insulin content: evidence for autocrine beta-cell regulation. Diabetes 47(8):1243-1252.

Xu H, Staszewski L, Tang H, Adler E, Zoller M, Li X. 2004. Different functional roles of T1R subunits in the heteromeric taste receptors. Proceedings of the National Academy of Sciences of the United States of America 101(39):14258-14263.

Yetman S, Pollack G. 1987. Proboscis extension in the blowfly: directional responses to stimulation of identified chemosensitive hairs. Journal of comparative physiology 160:367-374.

Zhang Y, Hoon MA, Chandrashekar J, Mueller KL, Cook B, Wu D, Zuker CS, Ryba NJ. 2003. Coding of sweet, bitter, and umami tastes: different receptor cells sharing similar signaling pathways. Cell 112(3):293-301.

Zhao GQ, Zhang Y, Hoon MA, Chandrashekar J, Erlenbach I, Ryba NJ, Zuker CS. 2003. The receptors for mammalian sweet and umami taste. Cell 115(3):255-266.

Biography

Natasha Thorne was born in Tampa, Florida, on December 21st, 1976. In 2000 she graduated *Summa Cum Laude* with University Honors from the University of Massachusetts at Lowell, with a Bachelor of Science Degree in the Biological Sciences. During her undergraduate studies, she was named a United States Air Force Designated Scholar, was awarded the Daniel Cole Undergraduate Research Award, and was elected as an Associate Member of Sigma Xi. She also received undergraduate training fellowships to study at the Rocky Mountain Biological Laboratory in Gothic, Colorado, and Cold Spring Harbor Laboratory in Cold Spring Harbor, New York. During her graduate work in the laboratory of Dr. Hubert Amrein at Duke University, she published two research papers titled " Taste perception and coding in *Drosophila*" and "Atypical expression of *Drosophila gustatory receptor* genes in sensory and central neurons", as well as three review articles titled " Gustatory perception and behavior in *Drosophila melanogaster*", "Function and expression of *Drosophila Gr* genes in the perception of sweet, bitter, and pheromone compounds", and "Vomeronasal organ: pheromone perception with a twist." Natasha received her Ph.D. from the Department of Molecular Genetics and Microbiology in the Summer of 2007. She has been awarded a NIH Postdoctoral Intramural Research Training Award Fellowhip to pursue postdoctoral studies with the NIH Chemical Genomics Center.