EXAMINING HOW PHARYNGEAL TASTE INPUT AND INTERNAL PHYSIOLOGICAL CONTEXT INFLUENCE FEEDING DECISIONS IN *DROSOPHILA*

by

Emily Elizabeth LeDue

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ABSTRACT

To survive animals must find and consume nutritive foods. The chemical composition of food sources is evaluated using gustation. Because of its importance to survival, feeding behaviors are tightly regulated. Changes in feeding occur in response to the external environment and the animal's internal physiological state. Expendable behaviours can be suppressed during starvation to prioritize feeding. This thesis examines the role of two factors in feeding decisions: the location of taste input and starvation.

Pharyngeal sense organs in *Drosophila* are the last evaluation point before food is ingested. It was previously unclear whether they served a unique function in feeding. To investigate this, we focused on appetitive gustatory pharyngeal neurons and showed they express nine gustatory receptors which respond to sweet compounds. Mutants lacking peripheral taste have functional pharyngeal sense organs. In the absence of peripheral taste cues, the pharyngeal sense organs can drive the choice and ingestion of sweet compounds by sustaining consumption. Knocking down pharyngeal neurons in these mutants allowed us to examine a sweet-blind fly in a short term feeding assay. Putatively sweet-blind flies do not show a preference for sweet compounds in a short-term feeding assay, suggesting that nutrient sensing is not operating in this context.

Starved flies have an increased tolerance for bitter foods, which is mediated by sensitization and desensitization of sweet and bitter taste, respectively. Mechanisms that cause the sensitization of sweet taste have been studied, but those that underlie

desensitization of bitter taste were unknown. We identify a pair of octopaminergic/tyraminergic modulatory neurons called the ventrolateral cluster of octopaminergic neurons (OA-VLs). Because OA-VLs exist in close physical proximity to bitter neuron axon terminals but are not postsynaptic to them, we examined their function as modulators of bitter neuron output. Tonic firing rate of OA-VLs decreases as a function of starvation. Octopamine and tyramine are sufficient to potentiate bitter neuron response in starved flies, suggesting that reduction in OA-VL activity during starvation depotentiates bitter neuron output. Silencing OA-VLs causes a reduction in bitter neuron output and increased acceptance of bitter compounds. OA-VLs may act directly on bitter sensory neurons through the Oct-Tyr receptor.

PREFACE

Chapter 2

Experiments discussed in this chapter were part of a collaborative effort with Dr. Anupama Dahanukar's group at University of California at Riverside in Riverside, California. This work produced a manuscript published as LeDue EE, Chen Y-C, Jung AY, Dahanukar A, Gordon MD (2015) Pharyngeal sense organs drive robust sugar consumption in *Drosophila*. *Nature Communications* doi: 10.1038/ncomms7667. For this publication, I performed proboscis extension experiments and the majority of the binary feeding assays. Aera Jung performed the initial binary feeding assays during the conception of the project. Michael Gordon and I performed experiments to examine the Gr-Gal4 expression of *poxn* mutants and the GCaMP imaging experiments. Yu-Chieh Chen and I performed temporal consumption assays. Yu-Chieh Chen performed the wildtype Gr-Gal4 expression analysis. Yu-Chieh Chen, Aera Jung, Anupama Dahanukar, Michael Gordon and I analyzed data. The projected was conceived and supervised by Michael Gordon and Anupama Dahanukar. Michael Gordon wrote the original manuscript with input from Yu-Chieh Chen, Anupama Dahanukar, and I.

Chapter 3

Work published in this chapter produced a manuscript published as LeDue EE, Mann K, Koch E, Chu B, Dakin R, Gordon MD (2016) Starvation-induced depotentiation of bitter taste in *Drosophila*. *Current Biology*. This work was carried out exclusively at the Life Sciences Institute at the University of British Columbia. This project was conceived and supervised by Michael Gordon. The anatomical

GRASP screen that initially identified our neurons of interest was carried out by Ellen Koch. I performed the anatomical analysis and immunohistochemistry screens. Cell attached recordings and their analyses were performed by Kevin Mann and I. All calcium imaging experiments in the body of the manuscript were done with sparse Gal4 lines not identified in the original screen and were performed by myself. The original silencing and calcium imaging of Gal4 lines, that included more general labeling, were carried out by Bonnie Chu and I and were ultimately included in the supplemental data of the manuscript. I performed all behavioural assays. Statistical analysis of binary behavioural data was done by Roslyn Dakin. The original manuscript was written by Michael Gordon and I, while review and editing was done by Michael Gordon and I, with input from Bonnie Chu and Roslyn Dakin.

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LIST OF ABBREVIATIONS

AHL	adult hemolymph-like
AKH	adipokinetic hormone
AMMC	antennal mechanosensory and motor center
C	Celsius
CaM	calcium binding protein calmodulin
ChR2	Channelrhodopsin-2
cpGFP	circularly permuted GFP
DA	dopamine
DCSO	dorsal cibarial sense organs
DNA	deoxyribonucleic acid
dNPF	neuropeptide F
DopEcR	dopamine/ecdysteroid receptor
DTK	tachykinin
dTRPA1	*Drosophila* transient receptor potential channel A1
ENaC	epithelial sodium channel
Fdg	feeding neurons2
FLP	LexAop-Flippase
GABA	γ-Aminobutyric acid
GFP	green fluorescent protein
GPCR	G-protein coupled receptors
GRASP	GFP reconstitution across synaptic partners
Gr	gustatory receptor
GRN	gustatory receptor neuron
h	hours
I	intermediate
K^+	potassium

KCl	potassium chloride
KIR2.1	inwardly rectifying potassium channel 2.1
L	long
LSO	labral sense organ
M	molar
MIP	myoinhibitory peptide
mM	millimolar
ms	milliseconds
Na^+	sodium
NaChBac	voltage-gated bacterial sodium channel
NaCl	sodium chloride
ns	not significant
NST	nucleus of the solitary tract
OA	octopamine
OA-VL	ventrolateral cluster of octopaminergic neurons
OBP	odourant binding protein
Octβ1R	octopamine receptor beta 1R
Oct-TyrR	Octopamine-Tyramine receptor
ORK	open rectifier potassium channel
ORN	olfactory receptor neuron
PER	proboscis extension reflex
PI	preference index
PKD2L1	polycystic-kidney-disease-like ion channel
PLC	phospholipase C
poxn	pox-neuro
RASSL	receptor activated solely by a synthetic ligand
RNAi	ribonucleic acid (RNA) interference

s	seconds
S	short
SEM	standard error of the mean
SEZ	subesophageal zone
sGPNs	sweet gustatory projection neurons
sNPF	short neuropeptide F
sNPFR	short neuropeptide F receptor
Syt-GFP	Synaptotagmin-GFP
TA	tyramine
tdc2	tyrosine decarboxylase 2
TH-VUM	tyrosine hydroxylase positive ventral unpaired medial
TRC	taste receptor cell
TRP	transient receptor potential
TRPm5	transient receptor potential channel m5;
UAS	upstream activating sequence
μM	micromolar
VCSO	ventral cibarial sense organs
vnc	ventral nerve cord
VT	Vienna Tile

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CHAPTER 1   INTRODUCTION

1.1 MAMMALIAN TASTE

Across all species, gustation functions ubiquitously to evaluate food sources
and drive feeding decisions. Animals must be able to distinguish potentially toxic
food sources, from those that can provide nutritional value. In general, detection of a
bitter taste will provoke an animal to reject that food source to avoid harm. On the
other hand, sweetness is the universal indicator of caloric value in a food source and
sweet taste promotes the acceptance of a compound to maintain homeostasis and
reverse energy deficits.

In mammals, taste detection is mediated by specialized epithelial cells called
taste receptor cells (TRC). TRCs are located in taste buds on the tongue and mediate
the transduction of gustatory stimuli to primary sensory neurons. TRCs come into
contact with tastants through a terminal pore in the taste bud tip. When a tastant
contacts a TRC it causes a signal transduction cascade that generates graded
depolarization in the TRC. TRC depolarization is caused by action of voltage gated
ion channels, such as sodium (Na^+) and potassium (K^+) channels (Gao et al., 2009).
TRCs then activate gustatory nerves within different craniofacial nerve fibers,
depending on their location on the tongue. The chorda tympani and greater
superficial petrosal cranial nerves generally innervate TRCs found on the anterior
portion of the tongue, while TRCs found on the posterior portion of the tongue are
innervated by the glossopharyngeal nerve (Iguchi et al., 2011). Through the
craniofacial nerves, taste fibers first convey information to the nucleus of the

solitary tract (NST; Yarmolinsky, Zuker and Ryba, 2009). From the NST, information

then enters the parvocellular stream of the thalamus. Taste neurons in the thalamus

then send projections to the cerebral cortex through the parabrachial nucleus

(Spector and Travers, 2005).

Mammals can detect five distinct primary taste modalities: sweet, bitter, salt,

sour and umami. Sweet and umami are appetitive taste modalities, while sour and

bitter are considered aversive (Nelson et al., 2001; Zhao et al., 2003; Huang et al.,

2006; Mueller et al., 2005). Salt is unique in that high concentrations are aversive,

while low concentrations produce appetitive behavioural responses (Oka et al.,

2013). The membrane bound ion channels and G-protein coupled receptors (GPCRs)

responsible for detecting these tastants are located on the apical membrane of TRCs,

allowing for direct contact.

1.1.1 Appetitive Taste in Mammals

The mechanism of taste transduction depends on the nature of the taste

stimulus. The appetitive modalities include sweet, umami (L-amino acids) and low

concentrations of salt (10 – 150mM NaCl). Sweet and umami tastes activate

heterodimeric receptors composed of members of the T1R family of GPCRs: T1R2

and T1R3 for sweet, and T1R1 and T1R3 for umami (Zhao et al., 2003). Low

concentrations of salt activate the heterotrimeric amiloride-sensitive epithelial

sodium channel (ENaC) (Chandrashekar et al., 2010).

Appetitive taste modalities innately drive behavioural attraction to ensure

animals eat foods with nutritional value. Mammals prefer to consume sweet, umami,

and mildly salty substances over substances that lack these attractive tastes (Steiner et al., 2001; Nelson et al., 2001; Chandrashekar et al., 2010). Evidence linking the activation of the receptors responsible for detection of simple sugars and L-glutamic acid with behavioural attraction has come from studies in which the combinations of the T1R receptors (T1R2+T1R3 for sweet and T1R1+T1R3 for umami) have been knocked out and selectively rescued in mice (Damak et al., 2003; Zhao et al., 2003). Knocking out either T1R2 or T1R3 causes a severe defect in the animal's ability to physiologically respond to, and behaviourally select, compounds with sweet taste (Zhao et al., 2003). Similar methods were used to show that abolishing T1R1 or T1R3 leaves animals unable to detect the appetitive taste of umami, indicating that the two receptors form a complex responsible for the behavioural attraction to umami (Damak et al., 2003; Zhao et al., 2003). Interestingly, when the modified opioid receptor, RASSL, was misexpressed in sweet cells, mice developed a preference for RASSL indicating that the activation of sweet cells drives innate attraction (Zhao et al., 2003).

Salt drives behavioural attraction at low concentrations (10mM NaCl), especially in times of salt deprivation, and remains attractive to animals up until concentrations of around 300mM NaCl (Oka et al., 2003). This appetitive to aversive switch serves to maintain proper ionic balance without threatening water homeostasis. The attractive low salt pathway is unique from the high salt pathway in that it is activated only by the positively charged Na^+ ion of the NaCl ionic compound. High salt receptors are responsive to other positively charged ions of ionic salt compounds, such as KCl (Oka et al., 2003). In addition, the low salt

responsive ENaC receptors are also inhibited by the drug amiloride, whereas those receptors responding to high salt are not. A conditional knock-out of ENaC in mice caused the inability to respond to low concentrations of NaCl, while retaining responsiveness to other salts (Chandrashekar et al., 2010). In addition, ENaC knockout mice no longer show any amiloride sensitivity (Chandrashekar et al., 2010). Both of these pieces of evidence combined support that ENaC is the receptor for low concentrations of sodium.

1.1.2 Aversive Taste in Mammals

Aversive tastes in mammals consist of bitter, sour and high concentrations of salt. Bitter chemicals act through the T2R family of GPCRs (Adler et al., 2000), and expressing the murine T2R specifically responsive to the bitter chemical cyclohexamide (mT2R5) in heterologous cells imparts cyclohexamide responsiveness, as measured by calcium imaging (Chandrashekar et al., 2000). TRCs containing the polycystic-kidney-disease-like ion channel (PKD2L1) have been identified as the candidate cells responsible for detecting acidic chemicals that evoke the taste of sour (Huang et al., 2006). Evidence for the role of PKD2L1 in acid sensing comes from studies in which the PKD2L1 expressing cells have been genetically ablated in TRCs. Mice lacking PKD2L1 in TRCs show reduced responses to multiple acids across varying pH ranges as measured by electrophysiological recordings of nerves coming from the tongue (Huang et al., 2006). Interestingly, it was shown that high concentrations of salt do not activate independent receptors, but activate both the bitter and sour TRCs to mediate behavioural aversion (Oka et

al., 2013). Calcium imaging of bitter sensing TRCs reveal responses to 500mM KCl and mice lacking bitter taste and sour sensing TRC function cannot detect high salt, suggesting that high salt recruits both the sour and bitter sensing pathways to promote aversion (Oka et al., 2013).

1.1.3 Transduction of Mammalian Taste into the Cortex

The sweet, bitter and umami signal transduction pathways all utilize common downstream signaling components, including TRPm5 and PLC (transient receptor potential channel m5; phospholipase C). Evidence for their downstream role came from studies using knockout mice in which TRPm5 and PLC expression was abolished. These animals displayed an inability to detect sweet, bitter or umami tastes (Zhang et al., 2003). This behavioural phenotype suggests that TRPm5 and PLC are common components of the taste transduction pathway, and play a role downstream of the GPCRs that interact with the chemical ligands that activate sweet and bitter pathways.

Taste modality is spatially segregated within the primary gustatory cortex of mice (Chen et al., 2011). A labeled line model of taste coding has been proposed due to the specificity of neuronal response dedicated to each modality at the periphery, which is maintained up into the primary gustatory cortex (Bradbury, 2004; de Brito Sanchez and Gruifa, 2011). Although, taste in both the mammalian and fly system does not conform truly to a labelled line model, as tastes such as high salt act through other aversive receptors such as the bitter pathway. However, tastes do remain separated in their valence, i.e., whether they are appetitive or aversive.

Overall, many similarities can be drawn between the mammalian taste system and that of the fly. Taste cells in both can be broadly categorized into those promoting or inhibiting feeding behaviour, although they are tuned to respond to a multitude of chemical ligands.

1.2 THE FLY GUSTATORY SYSTEM

Chemosensation is necessary for multiple evolutionarily relevant behaviours such as locating food sources, mate selection, and predator avoidance. Fruit flies (*Drosophila melanogaster*) are highly adept at detecting chemicals in their environment, through both olfaction and gustation. Although many mammals depend on vision as their primary sense, the gustatory system of the fly plays a critical role in its survival and propagation, making *Drosophila* an excellent model system for beginning to parse out the complex neural circuits that regulate perception of taste, and the integration of taste with internal physiological cues critical to making feeding decisions.

Another benefit of using *Drosophila* is that it provides a genetically tractable model in which to manipulate specific cell populations. This will ultimately aide in the dissection of circuits in the gustatory system that underlie taste perception and feeding behaviour. In contrast to other sensory systems, which have been studied extensively, relatively little is known about the gustatory systems of mammals and fruit flies. The fruit fly brain contains roughly 150000 neurons, of which about 4000 are contained in the taste center of the brain, formally called the subesophageal

zone (SEZ). The SEZ receives axonal projections from the gustatory sensory neurons that detect taste at the periphery.

The afferent projections and functions of gustatory sensory neurons in *Drosophila* have been well characterized and motor neurons controlling the behavioural output of the taste circuit have also been identified (Wang et al., 2004; Marella et al., 2006; Fischler et al., 2007; Gordon and Scott, 2009; Cameron et al., 2010; Manzo et al., 2012). Research has begun to focus on revealing interneurons in this circuit, but of those that have been described, few have been shown to make synaptic connections with gustatory sensory neurons. Thus, much work remains in characterizing the neural circuits underlying taste processing in the fly brain.

1.3 PERIPHERAL FLY TASTE

Adult *D. melanogaster* have multiple taste organs that are broadly distributed over the body, including taste sensilla located on the proboscis, pharynx, wing margins, and legs (Figure 1; Nayak and Singh, 1983; Lienhard and Stocker, 1987; Stocker, 1994). Each sensillum houses primary gustatory sensory neurons (GRNs), the dendrites of which come in contact with external taste stimuli through a single terminal pore. The proboscis is the primary taste organ of the fly, and taste sensilla on the labellum, which is the terminal segment of the proboscis, have been the focus of many studies of the gustatory system due to their function in feeding (Mitchell, Itagaki and Rivet, 1999). Each labellum contains about 31 sensilla (Falk, Bleiser-Avivi and Atidia, 1976; Vosshall and Stocker, 2007), which can be classified as short (S), intermediate (I) and long (L) type depending on their morphology and location
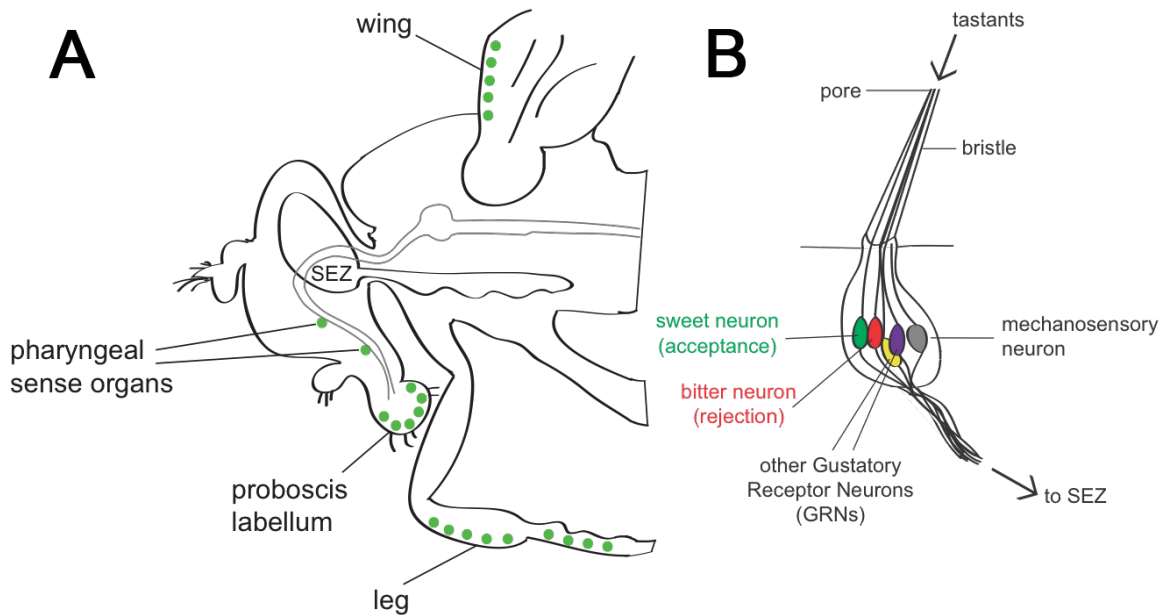
Figure 1. Schematic showing location of taste sensilla in the adult *Drosophila*, and structure of individual sensilla. Adult flies have taste sensilla on the legs, labellum of the proboscis, wing margin and within the pharynx (A). Generally, each taste sensilla contains one sweet and one bitter neuron that respond to sweet and bitter tastes and cause acceptance or rejection behaviour, respectively, along with one mechanosensory neuron (B). Depending on the type of sensilla, other GRNs housed within taste hairs can be responsive to compounds such as salt or water. GRN dendrites extend up into the bristle, coming into contact with tastants through an opening at the tip of the bristle called the pore. GRN axon terminals project to the taste center of the brain, formally known as the subesophageal zone (Adapted by Michael D Gordon from Chu, 2014).

(Montell, 2009; Benton and Dahanuker, 2011). L-type sensilla house four GRNs, with each responding to a particular stimulus: water, sugar, low salt or high salt (Hiroi, Marion-Poll and Tanimura, 2002). S-type sensilla also have four GRNs each, with neurons tuned to bitter and high salt, water, sugar, and pheromones (Hiroi et al., 2004; Thistle et al., 2012; Freeman and Dahanukar, 2015). I-type sensilla house only two GRNs each, one of which responds to low salt and sugar, while the other responds to high salt and bitter compounds (Hiroi et al., 2004). Additionally, the internal surface of the labellum is lined with a distinct class of taste structures, called taste pegs, which each house a single neuron that appears to sense carbon dioxide and polyamines (Fischler et al., 2007; Hussain et al., 2016). GRNs are also housed within the pharynx of the fly, which contains three internal taste organs: the labral sense organ (LSO), and the dorsal and ventral cibarial sense organs (DCSO and VCSO, respectively) (Mitchell, Itagaki and Rivet, 1999). The roles of and response profiles of GRNs in these taste organs were not well defined.

GRNs express a variety of receptors for taste ligands. The Gustatory Receptor (GR) family of genes was discovered based on predicted structural similarities to seven-transmembrane, G protein-coupled receptors (Clyne, Warr and Carlson, 2000). All 68 members of this receptor family share a common motif in the seventh transmembrane domain, and appear to be receptors for either sweet or bitter compounds (Clyne, Warr and Carlson, 2000; Scott et al., 2001; Dunipace et al., 2001; Robertson, Warr and Carlson, 2003). Another class of taste receptors is encoded by members of the pickpocket (ppk) ion channel family, which is a member of the Deg/ENaC family of ion channels (Cameron et al., 2010). Water GRNs express the

gene *ppk28* and are sensitive to low osmolarity. Other members of the ppk gene family, *ppk23*, *ppk25* and *ppk29*, act in GRNs to detect pheromones (Thistle et al., 2012).

Recently, members of the ionotropic receptor (IR) family, made up of 61 genes related to ionotropic glutamate receptors, have also been found to be expressed in GRNs of the fly (Benton et al., 2009). A specific member of this family, IR76B, was shown to respond to and mediate appetitive behaviour towards low concentrations of salt (Zhang, Ni and Montell, 2013). The appetitive effect mediated by this receptor is separate from the pathway that detects and assesses high concentrations of salt. Flies mutant for IR76B show a loss of attraction for low salt, but maintain the ability to perceive high salt concentrations as aversive. Since there are a number of other IR family members expressed in GRNs, it is likely that IRs act as receptors for additional taste modalities.

It is now well established that each sensillum houses a GRN that responds to sweet compounds. Sweet GRNs expresses members of a clade of sugar-sensitive GRs, which includes *Gr5a*, a receptor required for the response to trehalose (Dahanukar et al., 2001), and *Gr64a – f,* which respond to a wide array of sugars. Additionally, many sweet GRNs also express the fructose receptor Gr43a (Ling et al., 2014). In general, most sweet GRNs express Gr5a and Gr64f, in addition to a number of other sweet GRs. The majority of sensilla also contain a GRN that expresses the gene *Gr66a*, along with a suite of other GR family members, and respond to compounds that humans would perceive as bitter (Wang et al., 2004; Thorne et al., 2004).

Neurons expressing Gr5a mediate appetitive behaviours, while those expressing Gr66a mediate avoidance. This is demonstrated by artificial activation of Gr5a or Gr66a neurons, which results in taste acceptance or avoidance behaviours, respectively (Marella et al., 2006). In response to sweet, palatable substances, flies initiate feeding by extending their proboscis towards food. This robust feeding behaviour, known as proboscis extension reflex (PER), is easily quantifiable and can be used to measure taste acceptance in the lab (Figure 2; Shiraiwa and Carlson, 2007). Upon stimulation of the labellum or legs with a bitter compound, there is a retraction of the proboscis due to inhibition of the motor programs coordinating feeding behaviour (Rajashekhar and Singh, 1994).

In *Drosophila,* many bitter compounds not only activate bitter GRNs directly, but also inhibit the firing of GRNs that are responsive to appetitive taste stimuli (Meunier et al., 2003). By using single sensillum recordings, Meunier et al. (2003) showed that bitter compounds influence the activity of GRNs responsive to sugar or both sugar and water at a latency similar to the response seen in GRNs directly activated by bitter compounds. In addition, a lower threshold was required to produce inhibition of the sugar cell by a particular bitter chemical than was necessary to cause an action potential in the bitter responsive GRN (Meunier et al., 2003).

More recently, it has been shown that the odorant binding protein (OBP) Obp49a is responsible for much of the suppression of the sugar response by bitter
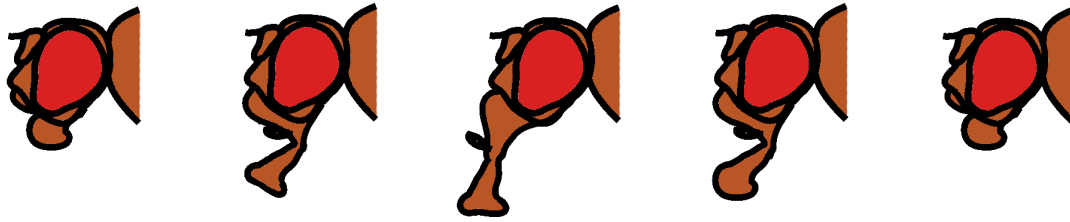
Figure 2. Proboscis Extension Reflex in *Drosophila melanogaster*. When an appetitive stimulus is presented to either the labellum of the proboscis or the legs flies will extend their proboscis in attempt to feed. This behaviour measures food acceptance and is easily quantifiable in the lab as a binary response: yes (full extension: middle panel) or no (partial extension or no extension: all other panels) behaviour (Adapted from Flood et al., 2013).

chemicals in sweet GRNs (Jeong et al., 2013). In wild-type flies, sugar solutions mixed with bitter compounds cause a blunted response in L-type sensilla, which is dependent on the amount of bitter tastant in the sugar solution. However, recordings from L-type sensilla in Obp49a mutants do not exhibit this inhibition by bitter compounds. In addition, this lack of inhibition occurs even though Obp49a mutants have normal electrophysiological responses to sugar and bitter stimuli, as recorded in the S and L-type sensilla. Importantly, this study also revealed a unique bitter stimulus, L-canavinine, that drives responses of bitter neurons in the fly without causing inhibition of the sugar response (Jeong et al., 2013). Use of L-canavanine is therefore ideal in studies that aim to parse out the response of sugar and bitter neurons, while eliminating crosstalk between the two pathways at the level of the receptors.

1.4 FLY TASTE CIRCUITS

Interestingly, it has been shown that taste projections in *Drosophila* segregate in the SEZ by modality (Figure 3; Thorne et al., 2004; Wang et al., 2004; Marella et al., 2006; Zhang et al., 2013). Projections from sugar/low salt (i.e., those that elicit acceptance behaviour when activated) GRNs terminate in more lateral regions of the SEZ, while projections from bitter/high salt GRNs terminate in the medial region of the SEZ. Projections of GRNs from the different taste organs are also segregated within the SEZ. From anterior to posterior, we first see projections of GRNs from the internal mouthparts, followed by the proboscis and finally the legs
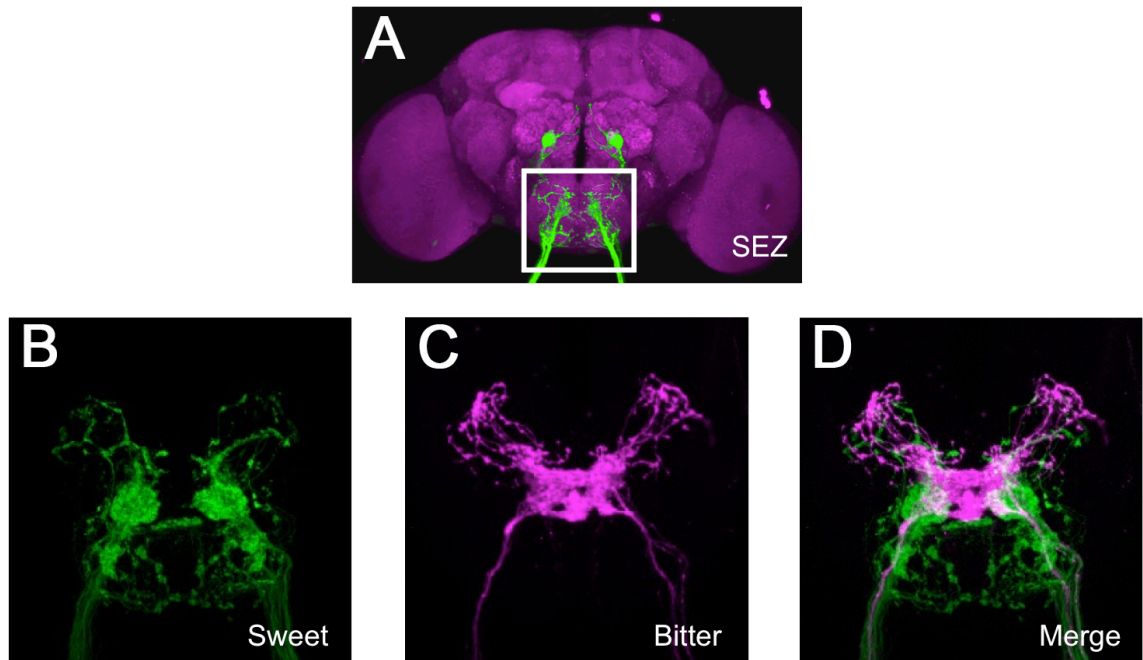
Figure 3. Peripheral gustatory sensory neurons send their axonal projections to the taste center of the fly brain, called the subesophageal zone (white box, A). Sweet (B) and bitter (C) axonal projections are non-overlapping (D) suggesting that taste modality is segregated in the fly brain.

(Wang et al., 2004). As mentioned previously, primary sensory neurons have been identified and well described in the fly (Marella et al., 2006; Wang et al., 2004; Thorne et al., 2004), and much recent work has focused on elucidating higher order neurons in the taste circuit.

A study by Flood et al. (2013) identified a pair of interneurons that cause full proboscis extension when artificially activated using UAS-dTRPA1. These feeding (Fdg) neurons show large calcium transients in response to sucrose stimulation in starved flies. It was suggested by Flood et al. (2013) that Fdg neurons are command neurons that control the feeding motor program, as laser ablation of the neurons decreased normal feeding in response to sucrose. However, no direct connections were found between Fdg neurons and Gr5a sensory neurons (Flood et al., 2013).

The first class of second-order gustatory neurons in the sweet pathway was described by Kain and Dahanukar (2015; Figure 4). These neurons, named the sweet gustatory projection neurons (sGPNs) project their axons to a higher brain region called the antennal mechanosensory and motor center (AMMC), which was not known to participate in higher order taste processing. When the sGPNs are silenced, there is a reduction in appetitive behaviours that promote food ingestion, such as PER. The converse is also true, in that artificial activation of these neurons promotes PER. The activity of these neurons is also modulated by satiety state. Starvation appears to increase the calcium activity in these neurons in response to stimulation of the proboscis with 25 and 50 mM of sucrose (Kain and Dahanukar, 2015). However, is it still unknown whether the sGPNs themselves relay
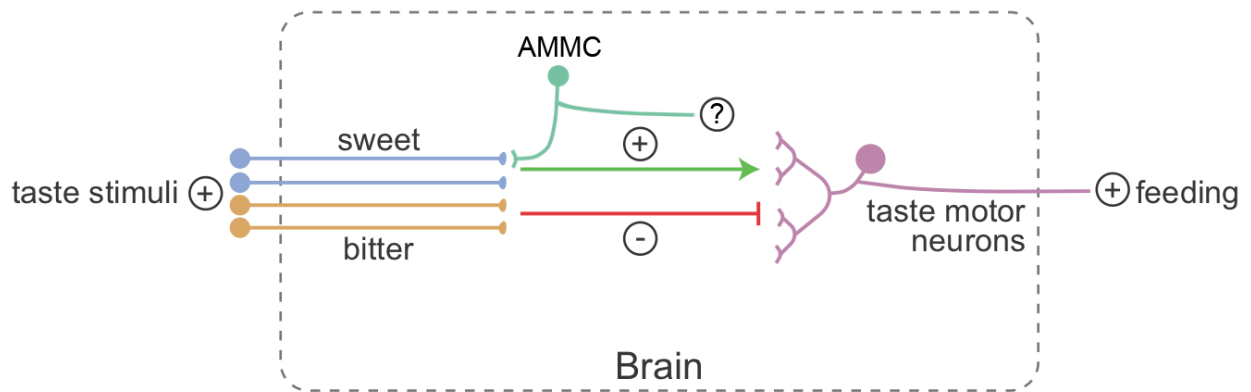
Figure 4. Gustatory receptor neurons detect sweet (blue) and bitter (orange) compounds at the periphery. When sweet sensory neurons are activated they evoke motor programs that initiate feeding behaviour (green). When bitter sensory neurons are activated they will prevent feeding behaviour through presynaptic GABAergic inhibition on sweet sensory neurons (red). The only known second order gustatory neuron is the sweet gustatory projection neuron (sGPN) in the AMMC (teal).

information to other unidentified interneurons in the sweet circuit, or to neurons controlling motor output of the proboscis.

1.5 FEEDING BEHAVIOUR IN DROSOPHILA

In the fruit fly, feeding is a modular process that consists of sub-programs, each of which in itself is a distinct behaviour (Pool and Scott, 2014). These sub-programs are hierarchical, and the decision to engage in any further sub-program comes from signals that pertain not only the palpability of the food in question but also depend on, for example, the fly's current nutritional state, or reproductive needs, among other factors. The sub-programs of fly feeding include: foraging, detection of food sources on the legs, proboscis, and pharynx, and ingestion (Pool and Scott, 2014).

Adult flies, when nutritionally deprived, will forage for food in the environment, using olfaction as their guiding chemical cue. It has been shown that olfactory sensitivity increases during starvation, through a short neuropeptide F (sNPF)-dependent mechanism that improves the foraging capabilities of the fly (Root et al., 2011). During starvation, low levels of circulating insulin relieve suppression on the short neuropeptide F receptor (sNPFR). More sNPFR is thus transcribed and integrated into the membrane of olfactory receptor neurons (ORNs). This increase in sNPFR in ORNs mediates the starvation-induced increase in odour sensitivity by directly facilitating their response, and that of their downstream projection neurons (PNs).

As flies are guided through their environment by smell, the legs are used to actively sample the taste of potential food sources. Detection of a favourable food source on the legs causes a cessation of locomotion (Mann, Gordon and Scott, 2013). The activation of appetitive GRNs on the legs increases the probability that a fly will extend its proboscis and initiate feeding (Dethier, 1976). Contacting food with the proboscis allows for further evaluation of the food source by activating GRNs on the labellum of the proboscis (Pool and Scott, 2014). If the food source is judged to be favourable for ingestion, feeding is initiated. The fly's pharyngeal sense organs contain the last GRNs to be activated by a food source, and can monitor quality of the incoming food and sustain ingestion. Instructions to terminate ingestion come from post-ingestive signals, such as physical stretching of the gut (Pool et al., 2014) and detection of circulating levels of fructose in the hemolymph by nutrient sensors in the brain (Miyamoto et al., 2012).

1.6 INTEGRATION OF HUNGER AND SATIETY SIGNALS IN DROSOPHILA

Hunger signals and taste perception must act together to guide feeding and ensure homeostasis. In *Drosophila*, the influence of internal physiological states on feeding behaviour has become an increasingly studied topic. Differential responses to similar compounds (i.e, acceptance or rejection) can occur depending on the fly's current level of nutritional deficit (Inagaki, Panse and Anderson, 2014). This behavioural shift comes from changes in sensory neuron sensitivity and from modulation of sensory neurons by other neurons. A multitude of studies have been published that describe sets of neurons that change their activity based on the

satiety state of the fly (Marella, Mann and Scott, 2012; Iganaki et al., 2012; Inagaki, Panse and Anderson, 2014; Kain and Dahanukar, 2015; Yapici et al., 2016). These neurons are therefore poised to modulate sensory neuron sensitivity as the nutritional requirements of the fly change.

Starvation increases the fly's sensitivity to sugar, while simultaneously decreasing its sensitivity to bitters. This system serves to increase the fly's receptivity to sugars while decreasing its aversion to bitter substances that it may normally reject in the fed state. Enhancement in sugar sensitivity is caused by a dopaminergic interneuron residing in the SEZ called the tyrosine hydroxylase positive ventral unpaired medial (TH-VUM) neuron (Marella, Mann and Scott, 2012). TH-VUM activity increases in starved flies, which increases the amount of dopamine released. It is thought that TH-VUM acts through the dopamine/ecdysteroid receptor, DopEcR, which is present in Gr5a axon terminals, to enhance the activity of Gr5a neurons under conditions of starvation (Inagaki et al., 2012). Behaviourally, this potentiation of Gr5a activity by starvation causes increased PER to lower concentrations of sugar compared to fed flies (Inagaki et al., 2012).

The one second-order sweet neuron to be connected to labellar Gr5a sensory neurons has also been shown to increase its activity in response to dopamine (Kain and Dahanukar, 2015). Both starvation and feeding of L-Dopa increases the calcium output of sPGNs, mimicking the increase seen in the Gr5a sensory neurons themselves, perhaps by TH-VUM acting to release dopamine on both Gr5a and sPGNs. neuropeptide F (dNPF) positive neurons can also increase the sensitivity of

sweet sensory neurons, and act upstream of dopaminergic neurons (Inagaki, Panse and Anderson, 2014). Starvation-induced sugar sensitivity caused by dNPF is abolished in DopEcR mutants. One other set of neurons, the ingestion neurons (IN1) has been empirically shown to be part of the sweet taste pathway but these neurons are postsynaptic to sweet sensory neurons originating in the pharynx (Yapici et al., 2016). These neurons also have altered activity depending on the satiety state of the animal, with increased activity in the starved state. It is unknown whether the activity of these neurons can be increased by dopamine, or whether their increased response is a product of the increased sweet sensory neuron activity.

Much less is known about the composition of the bitter taste circuit in *Drosophila* and how it is regulated in response to hunger. Peptidergic modulators responsible for the desensitization of primary bitter sensory neurons have been identified (Iganaki, Panse and Anderson, 2015). Flies lacking sNPF do not show reduced bitter sensitivity when starved, but retain normal sugar sensitivity. Adipokinetic hormone (AKH) is released upon starvation and silencing AKH neurons or deletion of the AKH receptor has been shown to suppress starvation induced behaviours, such as increased foraging (Lee and Park, 2004). Abolishing AKH cells eliminates the starvation induced decrease in bitter sensitivity. AKH was shown to act upstream of sNPF to achieve this reduction, as activating AKH cells in sNPF mutant flies resulted in no change in bitter sensitivity. However, sNPF does not act directly on the Gr66a bitter neurons themselves as knockdown of sNPFR in these sensory neurons has no effect on bitter sensitivity. Therefore, sNPF positive

neurons must act on another cell population upstream of the Gr66a sensory neurons, but this population was not identified (Inagaki, Panse and Anderson, 2015).

Other neurons have also been shown to influence hunger-dependent behaviours, but it is unclear how these neurons fit into the taste circuit. A group of serotonergic neurons appear to elicit hunger responses in fed flies. When these neurons are artificially activated fed flies show PER to lower concentrations of sucrose than fed controls and correspondingly increase their consumption (Albin et al., 2015). Another independent group of peptidergic neurons, those expressing myoinhibitory peptide (MIP), regulate body weight by conveying a sated signal and suppressing food intake (Min et al., 2016). Both of these subsets of neurons appear to modulate sucrose sensitivity depending on the satiety state of the fly, but is still unknown where and how these neurons interact with sweet sensory neurons.

1.7 FLY NEUROGENETICS

The fruit fly, *Drosophila melanogaster,* has become a prominent model organism in neuroscience research over the last decade. *Drosophila* provides us with the ability to genetically manipulate distinct cell types and circuits, which will ultimately aid in the dissection of neural mechanisms governing perception and behaviour (Venken, Simpson and Bellen, 2011). In particular, this genetically tractable model system is advantageous because it can be used in combination with *in vivo* imaging techniques (calcium imaging) that allow for the observation of activity in neuron populations over time.

1.7.1 The Gal4/UAS Binary Expression System

In flies, the Gal4/UAS binary expression system is a powerful means of manipulating gene expression. This technique uses genomic promoters or enhancers to spatially control the expression of transgenes in subpopulations of neurons (Duffy, 2002). Gal4 is a yeast transcription activator protein that binds to an upstream activating sequence (UAS) to initiate gene transcription of downstream elements (Brand and Perrimon, 1993). Transgenes containing genes of interest can be inserted downstream of UAS sequences, and tissue-specific expression of this transgene is determined by which enhancers and/or promoters regulate the expression of a separate transgene containing Gal4. The spatial and temporal activity of Gal4 can be further restricted using Gal80, a protein that interacts with Gal4 by binding to its transcriptional activation domain, preventing the binding of Gal4 to UAS regions.

Pfeiffer et al. (2008) created a large collection of Gal4 promoter lines that express in small subsets of neurons in the Drosophila brain. To create this collection, 3-kb DNA fragments flanking genes expressed in the brain were cloned upstream of Gal4 and inserted into the genome using site-specific recombination (Groth et al., 2004). This process has now been used extensively to create large collections of Gal4 promoter lines, including Janelia and Vienna Tile (VT) collections. Crossing these promoter lines to flies that contain a UAS-GFP transgene allows for visualization of the subset of cells where Gal4 is active. Researchers can then

visually screen large collections of Gal4 promoter lines and select those that contain neuronal Gal4 expression within circuits of interest.

1.7.2 Using Gal4/UAS to Manipulate Neuronal Activity

The Gal4/UAS system can be used to activate or silence different populations of cells to determine their effect on behaviour. The inwardly rectifying potassium channel 2.1 (Kir2.1) maintains an open confirmation at resting potential. As a result, potassium exits neurons expressing this channel, hyperpolarizing the membrane and preventing depolarization by presynaptic neurons (Hodge, 2009). Similarly, expressing *Drosophila* TRPA1 (dTRPA1) in Gal4 lines allows for inducible activation of neurons at temperatures around 30°C. dTRPA1 is a member of the highly conserved transient receptor potential (TRP) family of cation channels. The channel conductance of dTRPA1 varies with alterations in temperature, and thus these channels can be expressed exogenously in populations of neurons to cause acute activation in response to warming (Hamada et al., 2008). Warming of flies expressing dTRPA1 causes a more robust depolarization of neurons than the alternative optogenetic channel, Channelrhodopsin-2 (ChR2; Bernstein, Garrity and Boyden, 2012). In addition, the voltage-gated bacterial sodium channel NaChBac can be expressed in neurons to cause increased membrane excitability (Nitabach et al., 2006). NaChBac may be a good alternative to dTRPA1 in situations where heating the fly while performing an assay is not feasible.

1.7.3 Using Gal4/UAS to Measure Neuronal Activity Through Calcium Imaging

The Gal4/UAS system can also be used to express the calcium sensor GCaMP in specific populations of neurons, allowing measurement of neural activity via a change in fluorescence. GCaMP is a fluorescent calcium indicator derived from circularly permuted GFP (cpGFP), the calcium binding protein calmodulin (CaM), and the CaM binding peptide M13 (Crivici and Ikura, 1995; Baird, Zacharias and Tsien, 1999; Nagai et al., 2001). As calcium enters the cell during neuronal depolarization, CaM binds calcium through an E-F hand motif (Mank and Griesbeck, 2008; McCombs and Palmer, 2008; Whitaker, 2010). Binding of calcium by CaM causes a conformational change as a result of its interaction with M13, which subsequently closes a pore in the GFP barrel. This change causes a fluorescence increase compared to the baseline conformational state, thus acting as a proxy for neuronal activity and allowing us to measure the activity in many cells simultaneously.

In flies, GCaMP offers advantages in measuring neural activity when compared to electrophysiology. It provides the ability to measure calcium transients in specific neural populations in response to a behaviour or stimulus, without damaging cells. The small size of fly neurons also makes cell recordings particularly challenging. GCaMP3 was the first indicator capable of detecting single action potentials in populations of mammalian neurons, and fluorescence of GCaMP3 caused by calcium influx increased linearly with an increase in the number of action potentials (Tian et al., 2009). In *Drosophila* antennal lobe neurons, GCaMP3

provided a 4 – 6x increase in stimulus-evoked fluorescent responses when compared to earlier version of GCaMP (Tian et al., 2009). Yet GCaMP3 still did not surpass the ability of synthetic calcium indicator, OGB-1, in terms of photostability and the calcium binding rate. It also had lower sensitivity to calcium, a smaller dynamic range, and slower decay kinetics. However, synthetic OGB-1 must be injected into the brain making it difficult to target specific neurons. So researchers were forced to trade-off between specificity in labelling neurons of interest and using a superior indicator of neural activity. Further versions of GCaMP have since been designed, improving on the structure of the original GCaMP by using protein structure guided-mutagenesis (Tian et al., 2009; Akerboom et al., 2012). With the advent of GCaMP6, the abilities of genetically encoded calcium indicators came to rival synthetic indicators. GCaMP6 exceeded the ability of OGB-1 in terms of sensitivity to calcium and had an improved signal to noise ratio. GCaMP6 was also able to detect single action potentials *in vivo* (Chen et al., 2013).

1.8 OBJECTIVE OF THE CURRENT EXPERIMENTS

One of the main objectives in neuroscience research is to understand the neural circuits that govern behaviour. This is of fundamental importance in order decipher the relationship between neural activity and behaviour in both healthy and diseased states. *Drosophila* is an excellent model system in which to address this challenge. We can make use of the powerful genetic tools available in *Drosophila* to precisely manipulate neuronal circuits and combine these tools with live imaging to measure corresponding neural activity. These techniques provide a promising

avenue towards comprehension of the causal relationships between the formation of percepts and the generation of appropriate behavioural outputs.

1.8.1 Chapter 2

In chapter 2, we sought to characterize the role of pharyngeal neurons in taste perception and feeding behaviour using multiple techniques. We were interested in whether pharyngeal GRNs could drive feeding decisions and whether they had a function that was independent from that of taste receptors on the labellum. We identified subsets of sweet Grs that reside in the internal taste organs of the pharynx using double labeling. By using calcium imaging, we determined that these Grs are functional and respond to sugars. By abolishing all peripheral taste using a *pox-neuro* (*poxn*) mutant, we determined that these pharyngeal GRNs alone can be used to discriminate sweet compounds. Pharyngeal GRNs are also required for sustained ingestion of sweet compounds, suggesting that they have a unique role in feeding separate from that of GRNs on the labellum.

1.8.2 Chapter 3

In chapter 3, we identified a small set of neurons, the OA-VL neurons, that modulate primary sensory bitter neuron output during starvation. Using GRASP, we identified that these octopaminergic/tyraminergic neurons are in close physical proximity to bitter sensory neuron axon terminals. During starvation OA-VL neurons decrease their firing rate, suggesting that they act to potentiate bitter neuron output in the fed state. We genetically silenced OA-VL neurons and

performed both PER assays and calcium imaging of bitter axon terminals. When OA-VL neurons are silenced, bitter neuron output is reduced to levels observed in starved flies. Consistent with this reduction, flies show less inhibition to sucrose mixed with increasing concentrations of bitter in a PER assay. Knockdown of the Oct-TyrR, a receptor that binds both octopamine and tyramine, in bitter sensory neurons attenuated starvation-induced bitter sensitivity. We also applied octopamine or tyramine or both to the brain while measuring the bitter axon terminals and found that each had the ability to potentiate bitter neuron output in starved flies. Combined, this data suggests that OA-VLs act directly on bitter sensory neurons to potentiate their activity in the fed state, and during starvation depotentiation caused by reduced OA-VL activity allows flies to consume potentially contaminated foods to survive.

CHAPTER 2   THE ROLE OF PHARYNGEAL SENSE ORGANS IN FEEDING DECISIONS IN *DROSOPHILA MELANOGASTER*

2.1 INTRODUCTION

Sweet taste plays a key role in promoting ingestion of nutritionally rich sources of carbohydrates. Adult *Drosophila* express sweet taste receptors in GRNs located in the legs, labellum and a set of three pharyngeal sense organs collectively referred to as the internal mouthparts (Stocker, 1994). Sweet GRNs in the legs and labellum are broadly tuned to sugar stimuli, and their activation initiates feeding behaviours including the proboscis extension reflex (Wang et al., 2004; Dahanukar et al., 2007; Freeman, Wisotsky and Dahanukar, 2014; Marella et al., 2006; Deithier, 1976). However, neither the physiology nor the behavioural roles of pharyngeal GRNs have been described.

The pharyngeal sense organs consist of the DCSOs and VCSOs and the more distal LSO (Stocker, 1994; Nayak et al., 1983). The DCSO has two gustatory sensilla on each side of the midline, each housing three GRNs. Each side of the VCSO and LSO has three gustatory sensilla housing a total of eight and ten GRNs, respectively (Nayak et al., 1983; Gendre, 2004). Axons from pharyngeal GRNs project via the pharyngeal nerve to the SEZ of the brain, where they target an area that is distinct from the projections of the leg and labellar GRNs (Stocker, 1994; Wang et al., 2004). Mapping of body parts to different areas of the SEZ raises the possibility that taste detection by the legs, labellum and pharyngeal sense organs may each have distinct ethological functions.

The *Drosophila* genome encodes 68 members of the Gr family, with nine classified as sweet receptors (Wang et al., 2004; Dahanukar et al., 2007; Freeman, Wisotsky and Dahanukar, 2014; Marella et al., 2006; Deithier, 1976; Jiao et al., 2007; Slone et al., 2007; Scott et al., 2001; Miyamoto et al., 2012). Eight of these, Gr5a, Gr61a and Gr64a–64f, are closely related in sequence and are the defining members of a clade of insect sweet taste receptors (Kent and Robertson, 2009). Both expression and functional analyses suggest that sweet GRNs co-express multiple sweet receptors (Dahanukar, et al., 2007; Jiao, et al., 2007; Slone et al., 2007; Miyamoto et al., 2012; Jiao et al., 2008). Furthermore, mapping of Gr promoter-GAL4 expression patterns to identified sensilla in the labellum and tarsi suggests that individual sweet Grs may be expressed in overlapping but distinct subsets of sweet GRNs (Nayak et al., 1983; Gendre, 2004; Weiss et al., 2011; Ling et al., 2014). In addition to the sweet clade, a highly conserved receptor, Gr43a, also functions as a sugar receptor (Freeman, Wisotsky and Dahanukar, 2014; Miyamoto et al., 2012; Sato, Tanaka and Touhara, 2011). Interestingly, Gr43a, which is expressed in a few neurons in the protocerebrum, appears to be restricted to some tarsal and pharyngeal GRNs in the gustatory system (Miyamoto et al., 2012; Weiss et al., 2011; Ling et al., 2014).

In addition to sweet taste, mounting evidence suggests that the caloric content of sugars can drive feeding preferences in both insects and mammals (Sclafani, 2006; de Araujo et al., 2011; Dus et al., 2011; Dus et al., 2013; Burke and Waddell, 2011; Burke et al., 2012; Fujita and Tanimura, 2011; Stafford et al., 2012). To distinguish between the nutritional and gustatory effects of various sugars, it would

be beneficial to examine animals that completely lack taste sensory input (de Araujo et al., 2011; Dus et al., 2011; Dus et al., 2013). One proposed means of achieving this effect in flies has been to use *poxn* mutants, in which external taste bristles are transformed into mechanosensory bristles (Dus et al., 2011; Dus et al., 2013; Nottebohm et al., 1994; Awasaki and Kimura, 1997). However, there is evidence that the pharyngeal sense organs of *poxn* mutants retain expression of at least some gustatory genes (Galindo and Smith, 2001). If *poxn* mutants have functional pharyngeal taste sensilla, this could account for their observed preference for caloric sugars (Dus et al., 2011; Dus et al., 2013).

2.2 MATERIALS AND METHODS

2.2.1 Fly Stocks

Flies were raised on standard cornmeal fly food at 25 °C and 70% relative humidity. The following fly lines were used: Gr5a-GAL4, Gr43a-GAL4, Gr64a-GAL4, Gr61a-GAL4, Gr64c-GAL4, Gr64d-GAL4 and Gr64e-GAL4 (Weiss et al., 2011); Gr64f^{LexA} and Gr43a^{GAL4} (Miyamoto et al., 2012); UAS-GCaMP3 (Tian et al., 2009); UAS-KIR2.1 (Baines et al., 2001); UAS-TdTomato and UAS-GFP (Bloomington stock centre); *poxn*$^{\Delta M22-B5}$ (Boll, 2002); and *poxn*^{70} (Awasaki and Kimura, 1997).

2.2.2 Immunohistochemistry

Immunohistochemistry was carried out as previously described (Gordon and Scott, 2009). The primary antibodies used were rabbit anti-GFP (1:1000, Invitrogen) and mouse nc82 (1:50, Developmental Studies Hybridoma Bank). The secondary

antibodies used were goat anti-rabbit Alexa-488 and goat anti-mouse Alexa-568 (Invitrogen). Images are maximum intensity projections of confocal z-stacks acquired using a Leica SP5 II confocal microscope with 25 × water immersion objective or 63 × oil immersion objective. Images were taken sequentially with a scanning speed of 200 lines per second, a line average of 2, and a resolution of 1024 × 1024 pixels.

2.2.3 Gr Expression Mapping

Expression of sweet Gr promoter-GAL4 lines was mapped in the three pharyngeal sense organs by visualizing UAS-mCD8::GFP fluorescence in live tissue. Proboscis tissue was dissected and mounted in 80% glycerol in 1 × PBS before imaging with a Leica SP5 laser scanning confocal microscope. For double-driver analysis, the UAS-mCD8::GFP transgene was under the control of two different Gr promoter-Gal4 transgenes and the number of GFP-labelled neurons were compared with those in flies containing a single-Gal4 driver alone.

2.2.4 GCaMP Imaging

Female flies aged 2–12 days were used for calcium imaging. To prepare flies for imaging, they were briefly anaesthetized and all the legs were removed to allow unobstructed access to the proboscis. Using a custom chamber, each fly was mounted by inserting the cervix into individual collars. To further immobilize the head, nail polish was applied in a thin layer to seal the head to the chamber. Melted wax was applied using a modified dental waxer to adhere the fully extended

proboscis to the chamber rim. The antennae and associated cuticle covering the SEZ were removed and adult hemolymph-like (AHL) buffer with ribose (Liu et al., 2012) was immediately injected into preparation to cover the exposed brain. A coverslip was inserted into the chamber to keep the proboscis dry and separated from the bath solution.

GCaMP3 fluorescence was viewed with a Leica SP5 II laser scanning confocal microscope equipped with a tandem scanner and HyD detector. The relevant area of the SEZ was visualized using the 25 × water objective with an electronic zoom of eight. Images were acquired at a speed of 8000 lines per second with a line average of four, resulting in collection time of 60 ms per frame at a resolution of 512 × 200 pixels. The pinhole was opened to 2.68 AU. Stimuli were applied to the proboscis using a pulled glass pipette, and flies were allowed to ingest solutions during imaging. The maximum change in fluorescence ($\Delta F/F$) was calculated as the peak intensity change divided by the average intensity over 10 frames before stimulation.

2.2.5 Behavioural Assays

2.2.5.1 Proboscis Extension Reflex Assay

For PER, adult female flies were aged 3–10 days and starved on 1% agar at room temperature (~22 °C) for 24 h before testing. For tarsal PER, flies were mounted on glass slides using nail polish. For labellar PER, flies were placed inside a pipette tip cut to size so that only the head was exposed. Flies were then sealed into the tube with tape, and then adhered to a glass slide with doublesided tape. Flies

were allowed 1–2 h to recover before testing began. Flies were stimulated with water on their front tarsi or labella for tarsal and labellar PER, respectively, and allowed to drink until satiated. Each fly was then stimulated with a tastant on either the tarsi or labella, and responses to each of three trials were recorded. Flies were provided with water between each tastant. All stimuli were delivered with a 1 mL syringe attached to a 20 mL pipette tip. For statistical purposes, each trial was treated as an independent unit of analysis.

2.2.5.2 Binary Choice Assay

Binary choice preference tests were performed similarly to previous descriptions (Weiss et al., 2011; Dus et al., 2011; Riberio and Dickson, 2010). Female flies aged 3–8 days were sorted into groups of 10 at least 2 days before the experiment, and starved on 1% agar at room temperature (~22 °C) for 24 h before testing. For the assay, flies were transferred into standard vials containing six 10 μL dots of agar that alternated in color. The agar substrates were 1% agar with or without the test stimulus at a concentration of 100 mM. Each choice contained either 0.125 mg mL^{-1} blue (Erioglaucine, FD&C Blue#1) or 0.5 mg ml^{-1} red (Amaranth, FD&C Red#2) dye, and half the replicates for each stimulus were done with the dyes swapped to control for any dye preference. Flies were allowed to feed for 2 h in the dark at 25 °C and then frozen and scored for abdomen color. PI for sugar was calculated as ((number of flies labelled with the stimulus colour)−(number of flies labelled with the plain agar colour))/(total number of flies that fed).

2.2.5.3 Temporal Consumption Assay

The temporal consumption assay was performed on flies deprived of food for 24 h (Pool et al., 2014). As described above for PER, flies were mounted on glass slides using nail polish and allowed to recover for 1–2 h in a humidified chamber. Water-satiated flies were then offered 50 mM arabinose on their labella. Once they initiated feeding, the time between starting and stopping their first feeding bout was recorded (first bout length). The fly was then offered arabinose again and if they failed to reinitiate feeding for three consecutive stimulations, the assay was terminated for that fly. If flies began a new bout of feeding on stimulation, the time of the subsequent bouts was added to the first bout to determine total consumption time and the number of bouts was recorded.

2.3 RESULTS

2.3.1 Pharyngeal GRNs Express Sweet Receptors

Pharyngeal GRNs express sweet receptors. Previous studies have suggested that sweet receptors are expressed in internal pharyngeal GRNs (Dahanukar et al., 2007; Miyamoto et al., 2012; Wisotsky et al., 2011). To assign selected sweet receptors to identified pharyngeal GRNs, we used seven previously reported *Gr promoter-GAL4* transgenes (Weiss et al., 2011; Ling et al., 2014) and a *LexA* knock-in allele of *Gr64f* (Miyamoto et al., 2012). Mapping was based on the examination of GFP expression in the LSO, VCSO and DCSO (Figure 5) of flies carrying each *Gr-GAL4* or *Gr^LexA* driver, as well as analysis of GFP expression in flies carrying two different

drivers. Six of the seven sweet *Gr-GAL4* drivers, as well as *Gr64f^LexA*, showed

expression in the pharynx and suggest that the LSO and VCSO are innervated by

sweet GRNs (Figure 5). We did not detect any *Gr5a-GAL4* expression in the pharynx,

and none of the sweet *Gr-GAL4* lines tested showed expression in the DCSO (not

shown). The map identifies two candidate sweet GRNs per side of the LSO, which co-

express Gr43a and Gr64e with other members of the sweet clade. The VCSO also

contains two candidate sweet GRNs per side, both of which express *Gr43a-GAL4* and

Gr64e-GAL4. Thus, *Gr43a-GAL4* and *Gr64e-GAL4* offer two tools to explore the

physiological and behavioural roles of the pharyngeal sense organs. Both are

expressed in all identified candidate pharyngeal sweet neurons, as well as GRNs in

the legs; however, *Gr64e-GAL4* is also expressed in the taste hairs and taste pegs of

the labellum, while *Gr43a-GAL4* lacks labellar expression, but is expressed in sugar-

sensing neurons in the protocerebrum (Miyamoto et al., 2012; Weiss et al., 2011;

Ling et al., 2014; Wisotsky et al., 2011).

2.3.2 Pharyngeal GRNs Detect a Variety of Sugars

To examine the role of sweet taste detected by the pharyngeal sense organs,

we began by measuring the response properties of pharyngeal neurons expressing

Gr43a. We expressed GCaMP3 under the control of *Gr43a-GAL4* and used an in vivo

imaging preparation to measure the calcium responses of GRN axon terminals in the

SEZ during consumption of sweet stimuli (Figure 6). Pharyngeal Gr43a$^+$ GRNs
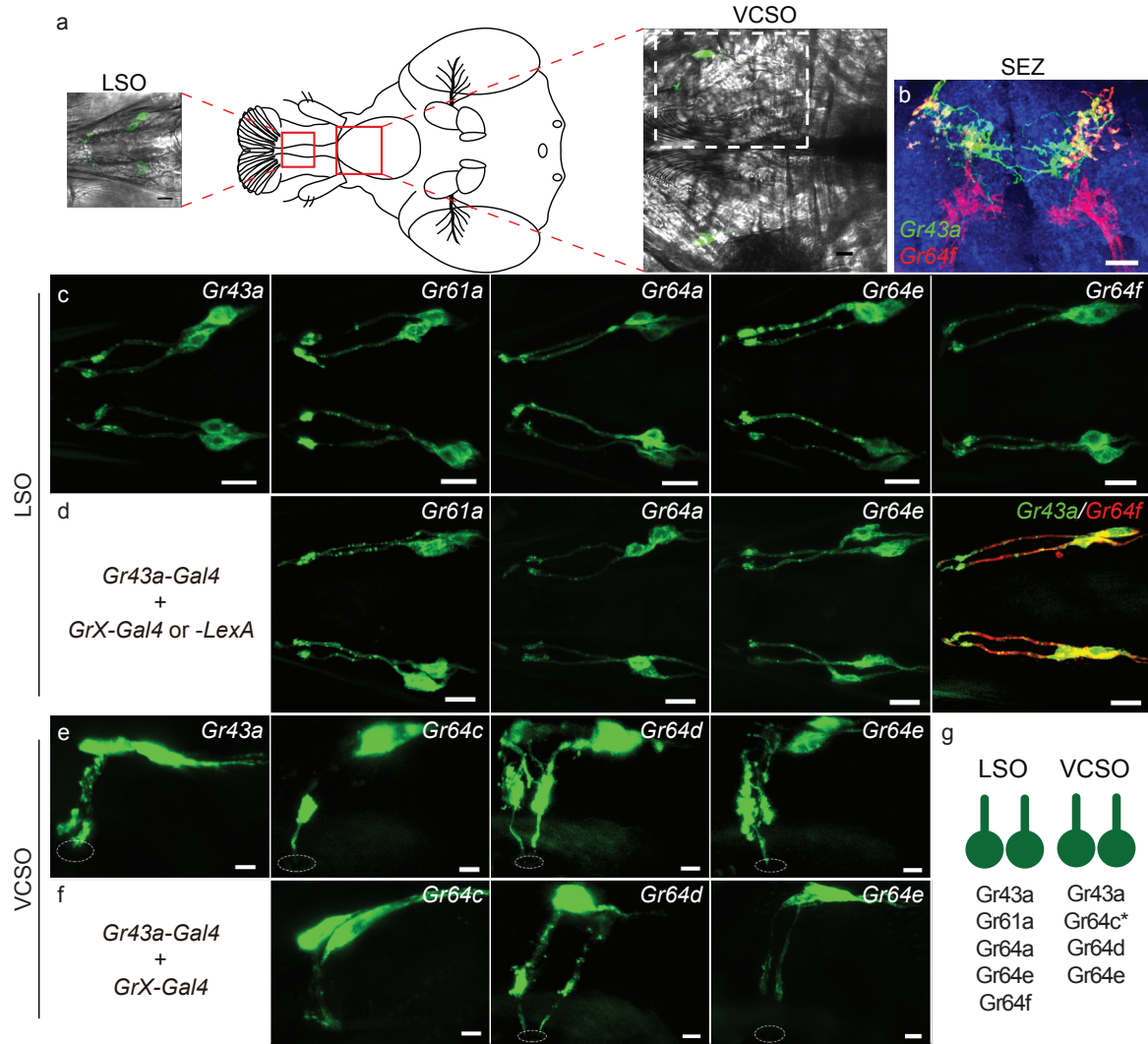
Figure 5. Pharyngeal GRNs express sweet Grs (a) Cartoon showing the positions of
the LSO and VCSO, with associated images of each structure from a fly expressing
GFP under control of *Gr43a-GAL4*. Dotted white box indicates area shown in e-f. (b)
Axonal projections of *Gr43a-GAL4* (green) and *Gr64f^LexA* (red) to the SEZ.
Overlapping regions are from LSO projections. (c) *Gr-GAL4*-driven GFP expression in
LSO. Scale bars are 10 μm in c-d. (d) LSO GFP expression from flies carrying *Gr43a-
GAL4* and indicated second *Gr-GAL4* or *Gr64f^LexA*. (e) *Gr-GAL4*-driven GFP expression
in VCSO. (f) VCSO GFP expression from flies carrying Gr43a-GAL4 and indicated

second Gr-GAL4. Scale bars are 5 µm in e-f. Dotted circles indicate the cuticular pore of sensilla. (g) Schematic of observed sweet Gr expression in LSO and VCSO GRNs. Asterisk indicates that *Gr64c- GAL4* expression is seen in only one neuron per side of the VCSO.

Gr43a-GAL4 ; UAS-GCaMP3
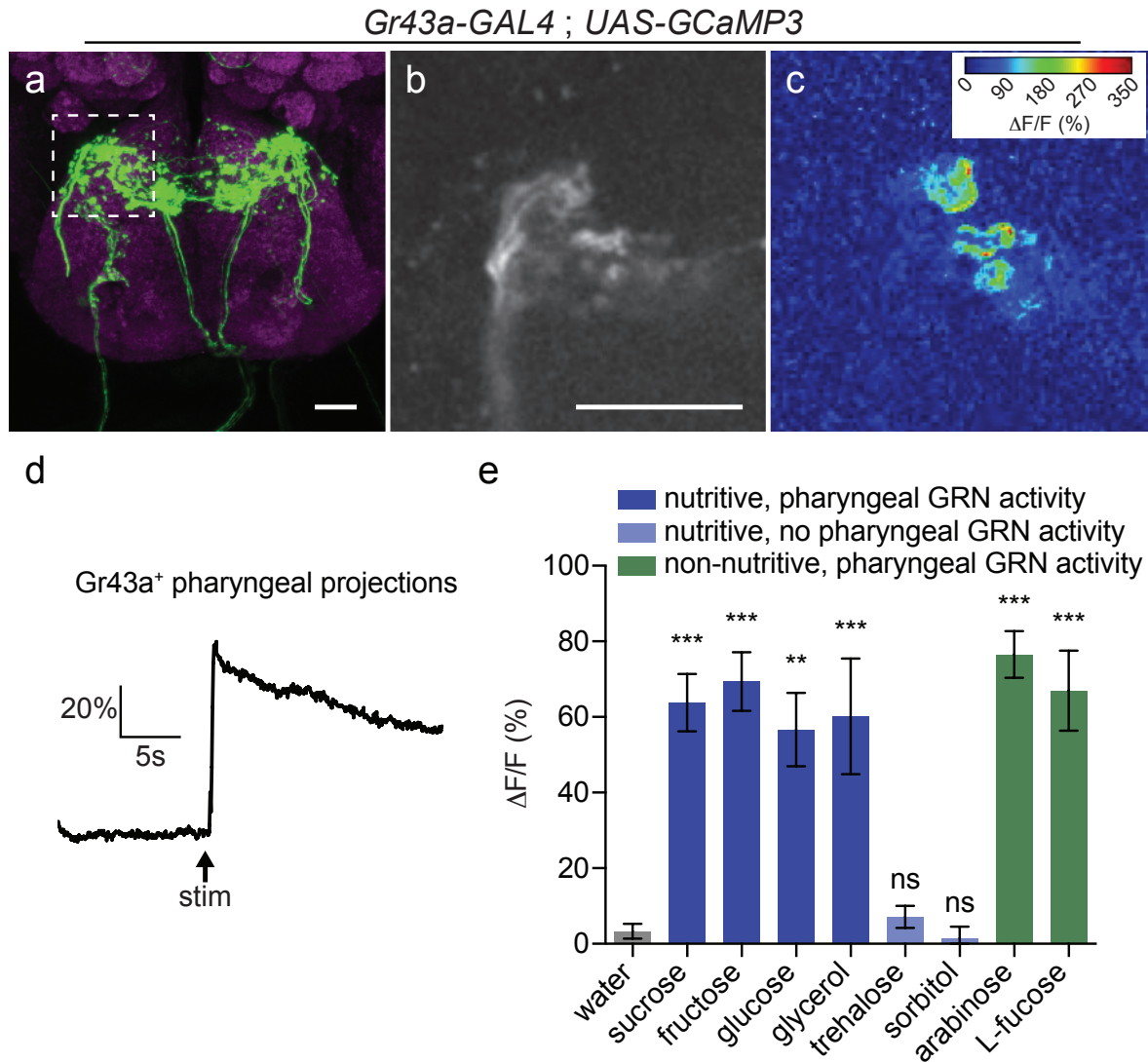
Figure 6. Pharyngeal GRNs respond to sweet compounds (a) Immunofluorescence of anti-GFP (green) and nc82 (magenta) in the SEZ of flies expressing GCaMP3 under control of *Gr43a-GAL4*. Dotted line shows area imaged in panel (b). (b) Single optical section of baseline GCaMP3 fluorescence in pharyngeal GRN axon terminals. Scale bars are 20 μm in a-b. (c) Heat map showing change in GCaMP3 fluorescence during ingestion of 1 M fructose. (d) Representative trace of fluorescence change of GCaMP3 in Gr43a axon terminals during ingestion of 1 M fructose. Arrow indicates

time at which stimulus is applied to the proboscis to initiate feeding. (e) Peak

fluorescence changes of GCaMP3 in Gr43a axon terminals during ingestion of 1 M

solutions of the indicated compounds. Values represent mean +/− SEM. for n = 5

flies per stimulus (n = 4 for sorbitol), with data collected over at least 2 days.

Asterisks indicate significant difference from water by one-way ANOVA with

Bonferroni correction for multiple comparisons: **$p < 0.01$, ***$p < 0.001$, ns = not

significant.

exhibited broad tuning to sweet compounds, with responses to both nutritive (sucrose, fructose and glucose) and non-nutritive (arabinose and L-fucose) sugars, as well as the sweet sugar alcohol glycerol (Figure 6e). Consistent with the reported lack of *Gr5a* expression in the pharyngeal sense organs, we did not observe responses to the Gr5a ligand trehalose (Wang et al., 2004; Dahanukar et al., 2007; Freeman, Wisotsky and Dahanukar, 2014). We also saw no response to the nutritive sugar alcohol sorbitol, which is generally considered tasteless to the fly. Taken together, these data confirm that, as predicted by their Gr expression, a subset of pharyngeal GRNs is activated by the ingestion of sweet compounds. In addition, it is notable that calcium responses in pharyngeal GRNs were sustained much longer than those previously observed from stimulation of labellar GRNs (Marella et al., 2006; Chu et al., 2014).

2.3.3 *poxn* Mutants Retain Functional Pharyngeal Sense Organs

To investigate the behavioural role of internal pharyngeal GRNs, we began by examining null mutants for the transcription factor *poxn*. *poxn* mutants lack external taste bristles, which are instead transformed into mechanosensory bristles (Awasaki and Kimura, 1997). This has resulted in the use of *poxn* mutants as taste-blind flies (Dus et al., 2011; Dus et al., 2013). However, the pharyngeal sense organs of *poxn* mutants have been reported to express a reporter for the apparently gustatory-specific odorant- binding protein, *OBP56b*, raising the possibility that taste sensilla may be intact in these tissues (Galindo and Smith, 2001). Moreover, many of the neurons in the pharyngeal organs of the adult originate in the embryo

and persist through the larval stages and metamorphosis, which contrasts with the general principle that adult sensory structures are born during metamorphosis and suggests that the pharyngeal organs may depend on an entirely distinct developmental program (Gendre et al., 2004). We, therefore, began by asking whether *poxn* mutants have functional pharyngeal taste sensilla.

Transheterozygotes for two *poxn* null alleles (poxn[70] and poxn[ΔM22-B5]) showed normal expression of *Gr43a-GAL4* in GRNs of the LSO and VCSO (Figure 7a,b). In addition, the brains from *poxn* null mutants had morphologically normal projections from pharyngeal GRNs, while they lacked the leg projections seen in otherwise wild-type flies (Figure 7e,f). Examining *Gr64e-GAL4* expression in the *poxn* background confirmed these results and additionally demonstrated that labellar taste peg GRNs are also present in *poxn* mutants (Figure 7c,d,g,h). To ask whether the pharyngeal GRNs of *poxn* mutants are functional, we expressed GCaMP3 under the control of *Gr43a-GAL4* in the *poxn* null mutant background, and measured calcium responses during ingestion of sweet compounds. We observed robust activation of Gr43a^{+} pharyngeal GRNs on ingestion of fructose and glycerol but not sorbitol (Figure 7i). Due to the technical difficulties in stimulating flies lacking external taste sensation to ingest sweet tastants during calcium imaging, we did not expand our analysis to a larger panel of compounds. However, it is very likely that *poxn* Gr43a^{+} pharyngeal neurons retain the same receptive fields seen in a wild-type background (Figure 6e). By contrast, Gr64e^{+} taste peg GRNs did not respond to any of the sweet compounds tested but were activated by carbonated water (Figure 7j), as previously reported for taste pegs in a wild-type background
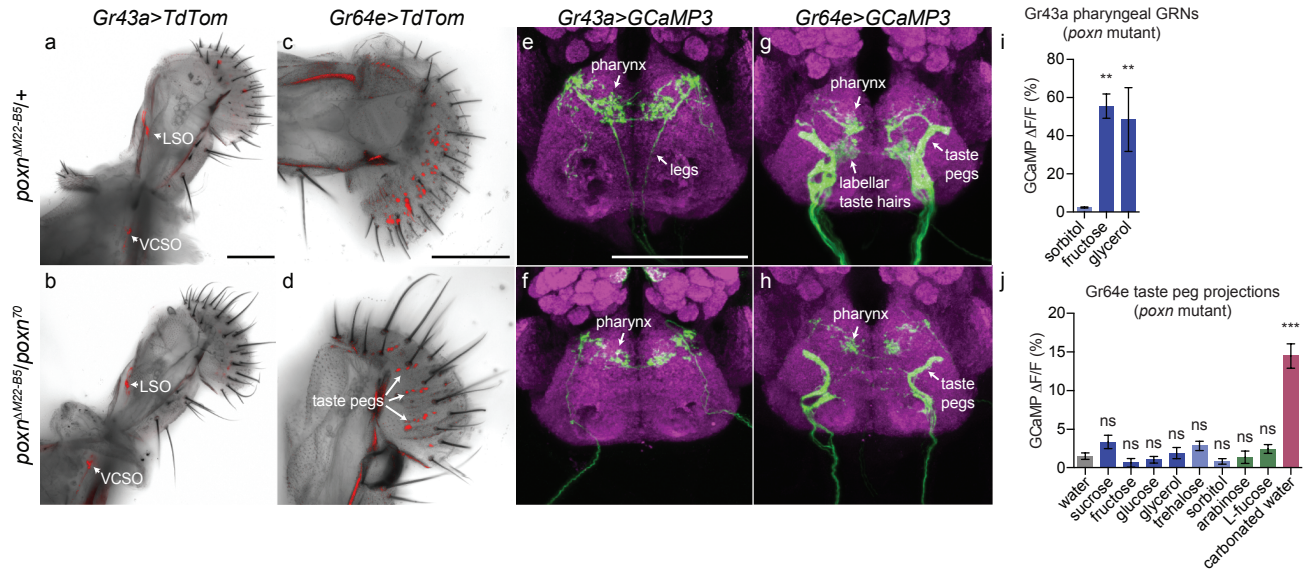
Figure 7. *poxn* null mutants retain functional pharyngeal sense organs (a,b) Pharyngeal GRNs labeled by *Gr43a-GAL4* driving *UAS-TdTomato* in $poxn^{\Delta M22-B5}$/+ heterozygotes (a) and $poxn^{\Delta M22-B5}$/$poxn^{70}$ null mutants (b). Arrows point to GRNs in the LSO and VCSO. (c,d) Labellar GRNs labeled by *Gr64e-GAL4* driving *UAS-TdTomato* in $poxn^{\Delta M22-B5}$/+ heterozygotes (a) and $poxn^{\Delta M22-B5}$/$poxn^{70}$ null mutants (b). Arrows point to taste peg GRNs in d. (e-h) Immunofluorescence of anti-GFP (green) and nc82 (magenta) in the brains of $poxn^{\Delta M22-B5}$/+ heterozygotes (e,g) and $poxn^{\Delta M22-B5}$/$poxn^{70}$ null mutants (f,h) expressing GCaMP3 under control of *Gr43a-GAL4* (e,f) or *Gr64e-GAL4* (g,h). Arrows point to GRN projections originating from the various body locations. (i-j) Peak fluorescence changes of GCaMP3 in *Gr43a-GAL4* pharyngeal (i) or *Gr64e-GAL4* taste peg (j) axon terminals in $poxn^{\Delta M22-B5}$/$poxn^{70}$ null mutants during ingestion of the indicated compounds. Values represent mean +/− SEM. for n = 5 flies, with data collected over at least 2 days. Asterisks indicate significant difference from sorbitol (i) or water (j) by one way ANOVA with

Bonferroni correction for multiple comparisons: **p < 0.01, ***p < 0.001, ns = not significant. Scale bars are 100 μm.

(Fischler et al., 2007). Together, these data demonstrate unequivocally that *poxn* mutants retain functional pharyngeal taste sensilla that are capable of responding to sweet compounds. Moreover, while functional taste peg GRNs also exist in these mutants, they do not respond to sweet compounds, and thus are unlikely to affect our subsequent behavioural analyses of sweet taste preferences driven by the pharyngeal sense organs.

2.3.3 *poxn* Mutants Prefer Sweet Compounds

Given that *poxn* mutants have functional pharyngeal sweet taste, but apparently lack all peripheral sweet taste sensation, we asked whether pharyngeal taste is sufficient to direct consumption of a variety of sweet compounds. First, we verified that *poxn* null mutants lack peripheral sweet taste responses by performing PER (Dethier, 1976; Gordon and Scott, 2009; Rajashekhar and Singh, 1994). We used ribose as a negative control stimulus, as it evokes no significant response from L-type taste sensilla on the labellum (Dahanukar et al., 2007). However, our observation that ribose elicits a mean PER response of ~20% in control flies suggests that it may stimulate some appetitive taste neurons at a low level (Figure 8). Alternatively, these responses could be the result of osmotic differences between the ribose solution and water used to water satiate the flies before testing (Cameron et al., 2010; Chen, Wang and Wang, 2010). Nevertheless, control flies (w^{1118}) showed robust PER to all sweet compounds tested, responding at frequencies significantly higher than those elicited by ribose (Figure 8a,c). By contrast, PER was entirely abolished in *poxn* mutants following stimulation of either the labellum or the tarsal
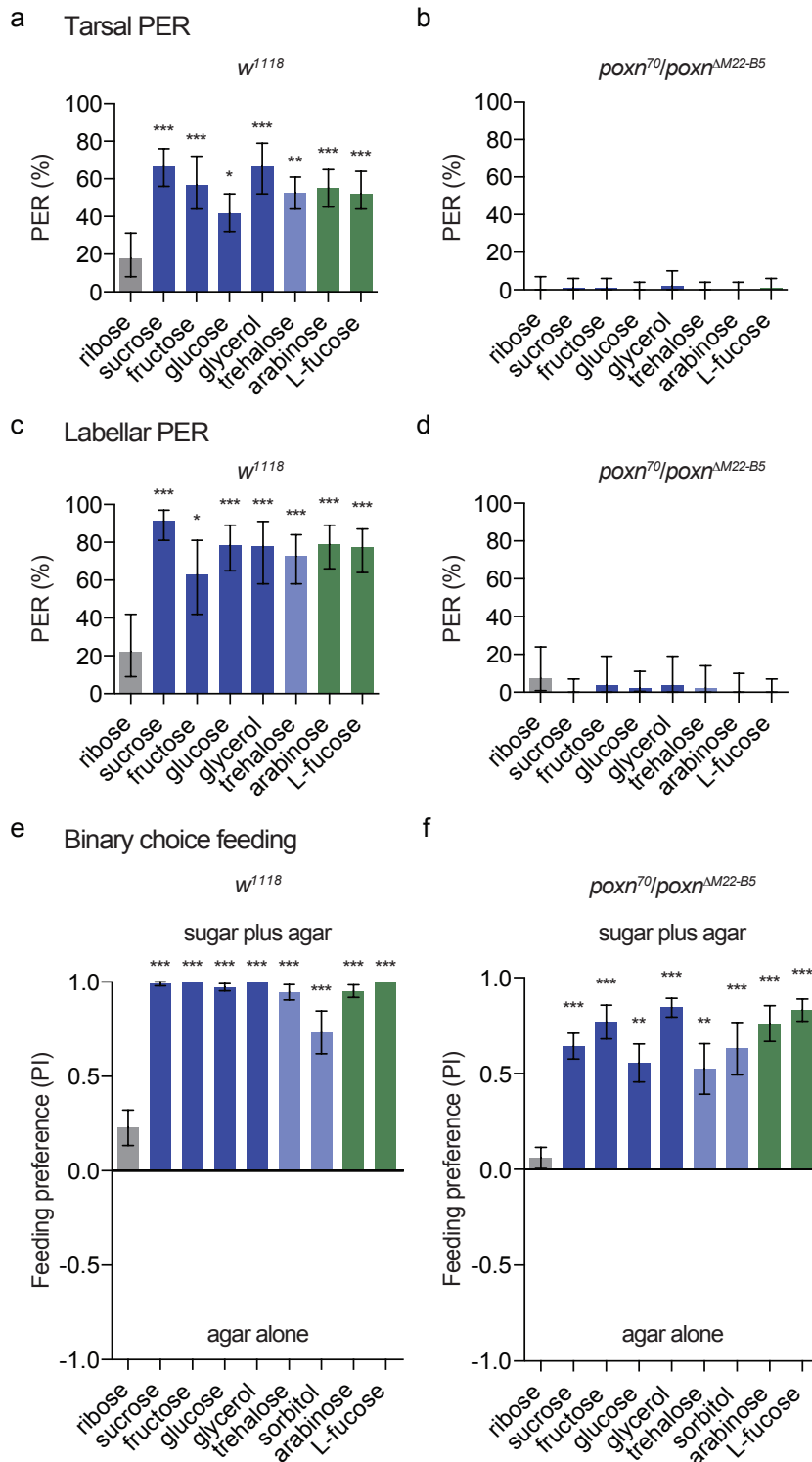
Figure 8. *poxn* null mutants lack peripheral taste responses but prefer sweet

compounds (a-d) PER responses of w^{1118} (a,c) and $poxn^{\Delta M22-B5}/poxn^{70}$ null mutant

(b,d) flies following stimulation of the tarsi (a,b) or labellum (c,d) with the indicated compounds. Values represent percentage of stimulations resulting in a positive response; error bars show 95% binomial confidence interval, and asterisks indicate significant difference from ribose stimulation: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by Fisher's exact test. n = 17- 50 flies for w^{1118} tarsal PER, n = 17-33 flies for *poxn* tarsal PER, n = 9-19 flies for w^{1118} labellar PER, and n = 9-17 flies for *poxn* labellar PER. (e,f) Preference of w^{1118} (e) and *poxn*$^{\Delta M22\text{-}B5}$/*poxn*^{70} null mutant flies (f) for 100 mM solutions of the indicated compounds in 1% agar versus agar alone. Values represent mean +/− SEM. for n = 10 groups of 10 flies each, with independent replicates performed over at least 2 days. Asterisks indicate significant difference from ribose preference by one-way ANOVA with Bonferroni correction for multiple comparisons: **$p < 0.01$, ***$p < 0.001$.

segment of the legs (Figure 8b,d).

Next, we subjected *poxn* mutants and controls to a variation of the binary feeding choice paradigm in which groups of food-deprived flies were allowed to feed for 2 h on drops of 1% agar with or without a given test compound. Again, we used ribose as a negative control because it has little to no taste response or nutritional value (Dahanukar et al., 2007; Stafford et al., 2012). Control flies showed a small preference for ribose (PI = 0.23±0.09) over plain agar, which may be due to weak taste responses or the osmolarity differences discussed above (Figure 8e). This effect was reduced in *poxn* mutants (PI = 0.06±0.06).

As expected, *poxn* mutants and controls displayed robust preference for sucrose, fructose, glucose and glycerol (Figure 8f). All of these compounds are nutritional (Burke and Waddell, 2011; Fujita and Tanimura, 2011; Stafford et al., 2011) and activate Gr43a+ pharyngeal GRNs (Figure 6e). The potential role of nutritional content in guiding feeding decisions is supported by the observation that poxn mutants preferred two compounds, trehalose and sorbitol, that are caloric but did not stimulate pharyngeal GRNs in our experiments (Figure 8f). Importantly, *poxn* mutants also strongly preferred arabinose and L-fucose, both of which stimulate sugar taste but offer no caloric value (Dahanukar et al., 2007; Burke and Waddell, 2011; Fujita and Tanimura, 2011; Stafford et al., 2012). These data strongly suggest that activation of pharyngeal taste neurons is sufficient to drive consumption behaviour, and that this activation accounts for at least part of the previously reported preference of *poxn* mutants for caloric sugars (Dus et al., 2011;

Dus et al., 2013).

To further examine the role of pharyngeal taste in driving the preference of poxn mutants for sweet compounds, we silenced Gr64e^{+} pharyngeal GRNs in the poxn mutant background through expression of the inward rectifying potassium channel KIR2.1 (Baines et al., 2001). Importantly, the insertion of *Gr64e-GAL4* used (*Gr64e-GAL4[II]*) lacks expression in the taste pegs (Figure 9a), meaning that silencing specifically affects pharyngeal GRNs. Silencing of Gr64e^{+} GRN function in *poxn* mutants resulted in a complete loss of preference for arabinose and L-fucose over agar alone, indicating that Gr64e^{+} pharyngeal GRNs are necessary for the preference for non-caloric sweet sugars in poxn mutants (Figure 9b). This suggests that *Gr64e-GAL4* likely labels the complete set of pharyngeal sweet GRNs, and that *poxn* mutants lacking Gr64e^{+} pharyngeal GRN function may be sweet-blind.

Since *poxn*, Gr64e-silenced flies lack both peripheral and identified pharyngeal sweet taste, we used them to re-evaluate the role of post-ingestive nutrient sensing in driving preference for a number of sugars. Silencing of pharyngeal taste caused only a mild, nonsignificant decrease in the preference of *poxn* mutant flies for sorbitol (Figure 9b), consistent with previously reported post-ingestive mechanisms promoting consumption of this compound, and its reported tastelessness (Miyamoto et al., 2012). However, it is worth noting that the preference for sorbitol in these experiments was weak, so it is difficult to confidently ascribe a taste-independent effect. In contrast to sorbitol, the preference for trehalose was significantly reduced following silencing of Gr64e^{+} pharyngeal
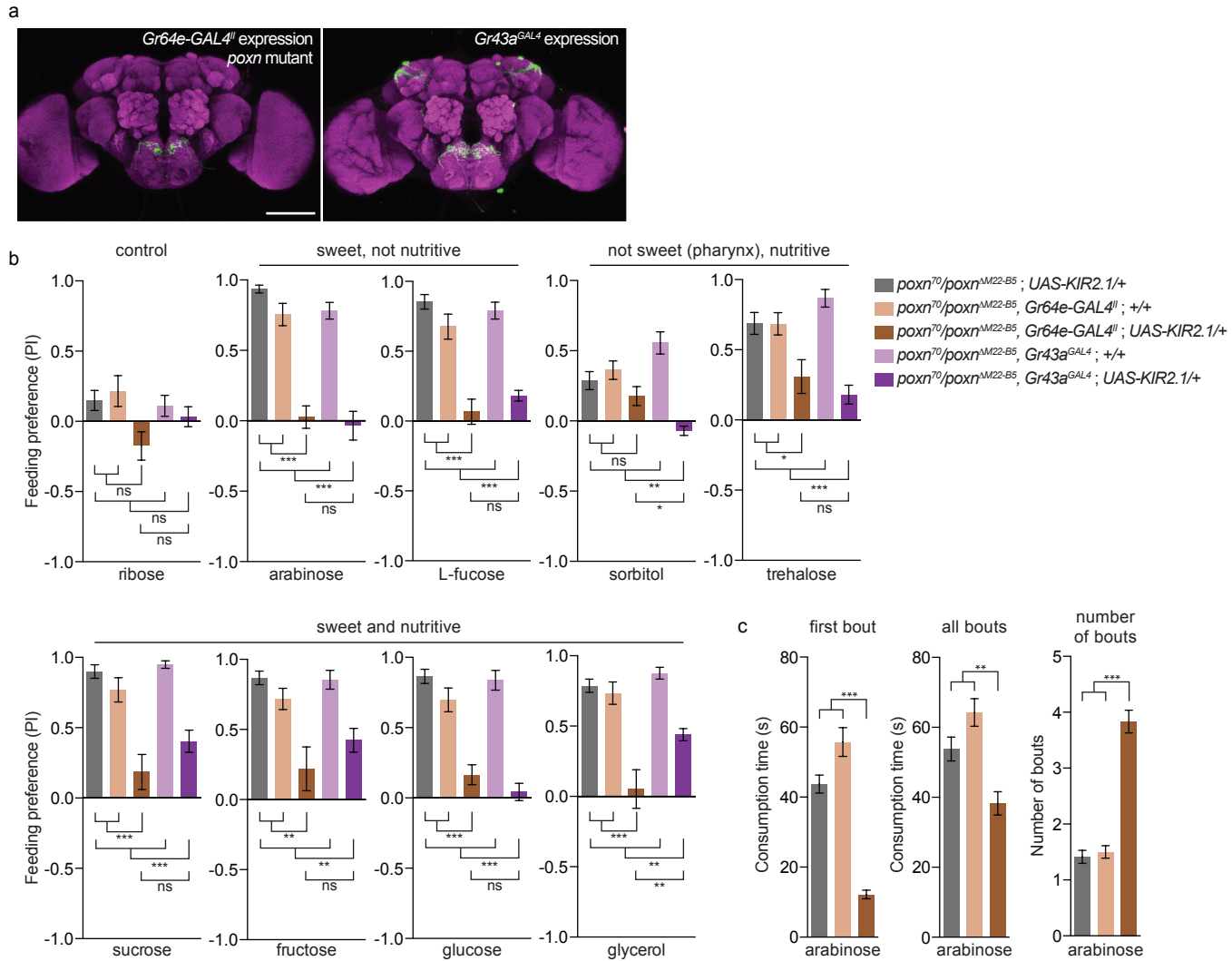
Figure 9. Pharyngeal GRNs are necessary for the preference of *poxn* mutants for

sweet compounds (a) Immunofluorescence of anti-GFP (green) and nc82 (magenta),

showing expression of the *Gr64e-GAL4[II]* and *Gr43a[GAL4]* drivers used in the

behavioural experiments shown. *Gr64e-GAL4* is shown in a *poxn* null mutant

background, while *Gr43a[GAL4]* is in a *poxn/+* heterozygous background. Scale bars are

100 μm. (b) Preference of indicated genotypes for 100 mM solutions of the specified

compounds in 1% agar (positive) versus agar alone (negative). (c) Temporal

consumption characteristics of the indicated genotypes in response to stimulation

with 50 mM arabinose. Values represent mean +/− SEM. for n = 10 groups of 10 flies each in b and n = 29-60 flies in c, with independent replicates performed over at least 2 days. Asterisks indicate significant difference by one-way ANOVA with Bonferroni correction for multiple comparisons: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, ns = not significant.

GRNs, suggesting that this sugar may stimulate pharyngeal GRNs at a level below the sensitivity of our calcium imaging but enough to affect behaviour.

Strikingly, silencing Gr64e^{+} pharyngeal GRNs markedly reduced the preference of *poxn* flies for all four sweet and nutritive compounds tested (Figure 9b). These data suggest that taste is the dominant driver of feeding preference in our short-term binary choice assay, and, in contrast to previous reports (Dus et al., 2011; Dus et al., 2013), taste-independent post-ingestive sugar sensing has little, if any, effect on consumption behaviour in this context.

To further probe the factors influencing sugar preference, we repeated the silencing experiment using a *GAL4* knock-in allele of *Gr43a* that is expressed in the same complement of pharyngeal GRNs as *Gr64e-GAL4^{II}* but also shows additional expression in identified sugar-sensing neurons in the protocerebrum and a population of neurons in the proventricular ganglion (Miyamoto et al., 2012). Consistent with a role for Gr43a^{+} brain neurons in promoting the ingestion of sorbitol, we observed a complete lack of sorbitol preference in Gr43a-silenced *poxn* mutants (Figure 9b). Notably, this was a significant reduction compared with both genetic controls and Gr64e-silenced flies. Interestingly, while the behavioural assay used may lack the resolution to tease out small differences between Gr64e and Gr43a silencing, we observed a trend towards increased preference for sweet and nutritive sugars in Gr43a-silenced mutants compared with Gr64e-silenced flies. This trend, which was nonsignificant for sucrose and fructose but significant for glycerol, could reflect weaker silencing with the *Gr43a^{GAL4}* driver, a difference in genetic

background, or the previously reported role for Gr43a⁺ brain neurons in promoting feeding termination of nutritive sweet sugars in some contexts (Miyamoto et al., 2012). Nevertheless, overall, our silencing results strongly support the conclusion that short-term sugar preferences are primarily taste-mediated, even in *poxn* mutants lacking external gustatory sensilla.

2.3.3 Pharyngeal Sweet GRNs Sustain Ingestion

On the basis of the anatomical position of pharyngeal GRNs, we wondered whether they might affect food preference by preferentially sustaining the ingestion of sweet compounds. To test this, we subjected *poxn*, Gr64e-silenced flies to a temporal consumption assay (Pool et al., 2014) and compared their behaviour with that of the *poxn* controls with intact pharyngeal GRN function (Figure 9c). We found that *poxn* flies with silenced pharyngeal GRNs displayed a dramatic reduction in the duration of consumption during the first bout of feeding on a solution of sweet, non-nutritive arabinose compared with non-silenced controls, as well as a reduction in total feeding time over multiple bouts and an elevated number of feeding bouts before 'satiety' (defined here as the refusal to initiate further feeding). These data support the notion that pharyngeal GRNs indeed function to sustain ingestion of sweet, appetitive food sources.

2.4 DISCUSSION

Much is known about sugar sensing through peripheral sweet taste neurons in the fly's legs and labellum, and there is growing interest in mechanisms that sense

dietary sugars following ingestion (Itskov and Ribeiro, 2013; Miyamoto, Wright and Amrein, 2013). Sugar sensing by the pharyngeal sense organs, however, has remained virtually unexplored due to their inaccessibility to electrophysiology and the lack of genetic tools to specifically manipulate their function. This represents an important gap in our understanding of how sugar feeding is regulated, since the pharyngeal sense organs must operate following the initiation of feeding, but before ingestion. They are, therefore, poised to provide feedback during feeding, contributing to the ongoing decision of whether to maintain or terminate ingestion.

Our calcium imaging data demonstrated that the receptive fields of pharyngeal sweet GRNs are in line with predictions based on the receptor expression and specificities (Dahanukar et al., 2007; Freeman, Wisotsky and Dahanukar, 2014; Jiao, Moon and Montell, 2007; Slone, Daniels and Amrein, 2007; Miyamoto et al., 2012; Jiao et al., 2008; Miyamoto et al., 2013). All putative pharyngeal sweet GRNs co-express Gr43a and Gr64e, which are receptors tuned to fructose and glycerol, respectively, and we observed robust calcium responses to those two compounds. Expression of Gr61a in the LSO likely accounts for the response to glucose, since Gr61a-dependent glucose responses are seen in tarsal GRNs lacking Gr5a (Miyamoto et al., 2013). Likewise, sucrose and L-fucose responses can be accounted for by the expression of Gr64a in the LSO (Dahanukar et al., 2007; Freeman, Wisotsky and Dahanukar, 2014; Jiao, Moon and Montell, 2007). Notably, we did not observe responses to trehalose, consistent with the lack of Gr5a expression, which is necessary for trehalose responses in the labellum (Dahanukar et al., 2007). However, our behavioural data suggested that Gr64e^{+} pharyngeal GRNs

mediate some attraction to trehalose. This apparent discrepancy could be explained if trehalose excites sweet pharyngeal GRNs at a level too low to observe significant calcium responses in our preparation, a possibility supported by previous reports that overexpression of Gr43a or Gr64e confers trehalose sensitivity to tarsal GRNs or ab1C olfactory receptor neurons, respectively (Freeman, Wisotsky and Dahanukar, 2014; Miyamoto et al., 2012).

Using *poxn* mutants lacking peripheral taste, we demonstrated that pharyngeal sweet GRNs are sufficient to drive preference for sweet compounds in binary choice feeding assays. These results indicate that appetitive taste is not absolutely necessary for feeding initiation, and that flies must 'sample' potential food sources in the absence of sweet taste input from the legs and labellum. Moreover, we provide evidence that activation of pharyngeal sweet GRNs prolongs feeding by providing a positive feedback signal to sustain ingestion. This proposed role for pharyngeal GRN function is also consistent with their physiology, which exhibits prolonged activation during consumption of sweet compounds compared with previously reported responses in the labellum (Marella et al., 2006; Chu et al., 2014). It is likely that this prolonged activation functions to maintain ingestion during feeding bouts lasting up to several seconds. In the future, it will be interesting to examine whether pharyngeal sweet taste plays any specific roles outside of feeding. For example, pharyngeal bitter GRNs appear to be important in selection of egg laying substrates (Joseph and Heberlein, 2012).

A key component of our work is the demonstration that *poxn* mutants have a

functional pharyngeal taste system that is critical in guiding their preference for sweet compounds, which therefore precludes their experimental use as 'taste-blind' flies. The behavioural preference we observed of *poxn* mutant flies for the non-nutritional sweetners L-fucose and arabinose contrasts with the conclusion reached by Dus et al. 2011 who performed a similar experiment with the artificial sweetner sucralose. One possible explanation for this difference is that arabinose and L-fucose may activate pharyngeal sweet GRNs more potently than sucralose. Another possible source of the discrepancy is that Dus et al. 2011 analysed their feeding data by plotting the total proportion of flies to eat each option in the binary choice assay, while we analysed relative preference between the options. Nevertheless, the strong dependence of *poxn* mutants on Gr64e^{+} GRNs for preferred consumption of both nutritive and non-nutritive sweeteners demonstrates that the majority of the preference of these mutants for sweet compounds is mediated by pharyngeal taste sensitivity.

By silencing Gr64e^{+} GRNs in a *poxn* mutant background, we created for the first time a fly that may completely lack sweet taste, allowing one to re-evaluate the taste-independent role for nutrient-sensing mechanisms in behaviour. While we did observe some evidence for taste-independent selection of nutritive carbohydrates, the observed effect was much weaker than previously suggested (Dus et al., 2011; Dus et al., 2013). Although ample additional evidence supports the existence of taste-independent carbohydrate sensing (Miyamoto et al., 2012; Burke and Wadell, 2011; Burke et al., 2012; Fujita and Tanimura, 2011; Stafford et al., 2012), its specific role in regulating feeding remains unclear. Why do we observe such weak

preference of putatively sweet-blind flies for nutritive sugars? One possibility is the particular behavioural assay used, which operates over only 2 h. We have previously reported increasing effects of caloric content on feeding preferences over the course of a 16-h assay (Stafford et al., 2012). It will be instructive to re-evaluate the feeding behaviour of *poxn*, sweet GRN-silenced flies over longer time periods using different behavioural paradigms.

Our newly established putatively sweet-blind flies also afford the opportunity to examine potential nutrient-sensing mechanisms in a taste-independent context. For example, by comparing Gr43a-silenced with Gr64e-silenced flies in a *poxn* mutant background, we were able to observe effects for non-taste Gr43a$^+$ populations that are largely consistent with those previously reported (Miyamoto et al., 2012). Further evaluation of these and other putative nutrient-sensing cell populations in a sweet taste-blind background will continue to shed light on the apparently complex interactions between the taste-dependent and taste-independent mechanisms that ultimately guide critical feeding decisions.

CHAPTER 3   STARVATION-INDUCED DEPOTENTIATION OF BITTER TASTE IN
                     DROSOPHILA

3.1 INTRODUCTION

Animals depend on the appropriate interpretation of sensory information to make favorable behavioural decisions. To achieve this, neural circuits underlying innate behaviours, such as feeding and mating, must possess some intrinsic flexibility to reflect changing internal states. *Drosophila* offers an extensive genetic toolbox to parse the mechanisms of how internal motivational states modify neural circuit function to achieve behavioural plasticity.

Starvation induces a powerful internal state that drives extensive behavioural modifications. Notably, starvation promotes food seeking and consumption at the expense of behaviours that satisfy other essential needs like sleep (Farhadian et al., 2012; Keene et al., 2010; Root et al., 2011). This behavioural shift is driven in part by changes in chemosensory processing. For example, starvation potentiates or suppresses sensory neuron output in specific glomeruli of the fly olfactory system through the activity of two distinct neuropeptides – sNPF and tachykinin (DTK), respectively (Root et al., 2011; Lebreton et al., 2015; Ko et al., 2015). Moreover, dNPF acts as a gate for the starvation-dependent expression of appetitive olfactory memories (Krashes et al., 2009). Thus, starvation affects food-seeking behaviours through modulation of both peripheral and central olfactory neurons.

While olfaction drives long-range food search behaviours, taste serves as a final checkpoint for evaluating the suitability of foods for consumption. In the fly

taste system, segregated classes of GRNs respond to particular classes of tastants (Meunier et al., 2003; Marella et al., 2006). The two most well characterized GRN types are sweet and bitter, each of which expresses distinct members of the Gr family (Wang et al., 2004; Thorne et al., 2004; Weiss et al., 2011). Sweet GRNs express combinations of nine identified sweet Grs, including Gr5a and Gr64f, and activation of these neurons promotes feeding behaviours such as PER (Wang et al., 2004; Gordon and Scott, 2009; Fujii et al., 2015; Slone, Daniels and Amrein, 2007; Jiao et al., 2008). Bitter GRNs express Gr66a along with combinations of approximately 32 additional bitter Grs (Wang et al., 2004; Thorne et al., 2004; Weiss et al., 2011). Activation of bitter GRNs drives avoidance behaviour and terminates PER, preventing flies from ingesting toxic food (Meunier et al., 2003; Marella et al., 2006; Keene and Masek, 2012). Both sweet and bitter GRNs project their axons into distinct areas of the SEZ, suggesting that taste information is segregated by modality at the first synapse in the fly brain (Wang et al., 2004; Thorne et al., 2004; Kwon et al., 2014).

Like olfaction, taste sensory input is modulated by satiety state. Starvation potentiates sweet GRN synaptic output, while independently suppressing the synaptic output of bitter GRNs. This reciprocal modulation of sweet and bitter GRNs depends on the activity of distinct neuropeptides: dNPF for sweet, and sNPF and AKH for bitter (Inagaki, Panse and Anderson, 2014). Downstream of dNPF, sweet GRN potentiation is mediated by dopamine signaling through the dopamine/ecdysteroid receptor, DopEcR (Srivastava et al., 2005; Inagaki et al., 2012). Starvation also enhances the firing of a single modulatory dopaminergic

neuron in the SEZ, TH-VUM, the activation of which is sufficient to induce PER (Marella et al., 2012). Although it has never been formally demonstrated, it is appealing to infer that TH-VUM is the source of the direct dopaminergic signal to sweet GRNs, and possibly the target of dNPF regulation.

The direct source of bitter GRN modulation is unknown. It has been suggested that AKH acts upstream of sNPF, which signals through a GABAergic intermediate to suppress bitter sensitivity (Inagaki, Panse and Anderson, 2014). However, neither the signals that act on bitter GRNs nor any starvation-regulated neurons in the bitter circuit have been identified.

Here we describe a small cluster of interneurons that produce octopamine (OA) and tyramine (TA), named OA-VLs (ventrolateral cluster of octopaminergic neurons (Busch et al., 2009)), which act to modulate bitter GRN output during starvation. OA-VL projections are in close proximity to bitter GRN axon terminals in the SEZ and have presynaptic terminals in the same region. Patch clamp recordings show that OA-VL firing decreases significantly as flies become starved, suggesting that their activity may provide a positive signal to potentiate bitter GRN output in the fed state. Consistent with this model, genetic silencing of OA-VL activity in fed flies mimics the behavioural and physiological effects of starvation on the bitter circuit – bitter GRN calcium responses are suppressed, and flies become less sensitive to the inhibitory effects of bitter on PER. Moreover, knockdown of Octopamine-Tyramine receptor (Oct-TyrR) in bitter GRNs also suppresses bitter sensitivity, suggesting direct modulation of bitter sensory neuron output by OA-VLs. Finally, pharmacological addition of OA or TA to the brain is sufficient to potentiate

bitter GRN output in starved, but not fed, flies. Thus, OA-VL neurons may represent the missing link between peptidergic hunger signals and bitter modulation, allowing starved flies to consume suboptimal foods.

3.2 MATERIALS AND METHODS

3.2.1 Fly Stocks

Fly stocks were raised on standard cornmeal fly food at 25°C and 70% humidity. The following lines were used: *VT026002-Gal4, VT049128-Gal4, VT045791-Gal4*, *w1118* control (from the Vienna Tile collection); *GMR38A06-Gal4* (from the Janelia Rubin Gal4 Collection); *UAS-Oct-TyrR*RNAi (Stock Number 26877 VDRC); *Gr5a-LexA::VP16*, *UAS-CD4::spGFP1-10*, *LexAop-CD4::spGFP11* (Gordon and Scott, 2009); *UAS-mCD8::GFP* (Lee and Luo, 1999); *UAS-synaptotagmin-GFP* (Zhang, Rodesch and Broadie, 2002); *hs-FLP, MKRS* (Bloomington stock center); *UAS-GCaMP6f* and *LexAop-GCaMP6f* (Chen et al., 2013); *UAS-ORK* (Nitabach et al., 2004); *Gr66a-Gal4* (Wang et al., 2004); *Gr66a-LexA::VP16* (Thistle et al., 2012); *UAS-KIR2.1, tub-Gal80*ts (Baines et al., 2001); and *PBDPGal4U*, an enhancerless Gal4 line from the Janelia collection used as a control with no Gal4 expression (Pfeiffer et al., 2008).

3.2.2 Immunohistochemistry

Immunohistochemistry was carried out as described previously (Gordon and Scott, 2009). For GRASP, the primary antibodies used were mouse anti-GFP (1:100, Sigma, cat# G6539) and rat anti-CD8 (1:500, Cedarlane, cat# CL168AP). Other primary antibodies used were rabbit anti-GFP (1:1000, Invitrogen, cat# A11122),

mouse anti-nc82 (1:50, Developmental Studies Hybridoma Bank), rabbit anti-DsRed (1:2000, Clontech, cat# 632496) and rabbit anti-tdc2 (1:2000, Covalab, cat# pab0822-P). The secondary antibodies used were goat anti-mouse Alexa-488 (Invitrogen, cat# A11029), goat anti-rabbit Alexa-488 (Invitrogen, cat# A11008), goat anti-mouse Alexa-568 (Invitrogen, cat# A11036), and goat anti-rat Alexa-568 (Invitrogen, cat# A11077). Images are maximum intensity projections of confocal z-stacks acquired using a Leica SP5 II confocal microscope with the 25x water immersion objective or 63x oil immersion objective.

3.2.3 Physiology

Both calcium imaging and cell-attached recordings were performed on female flies aged 2 – 12 days were used. Flies were anaesthetized using CO_2 and the legs were removed using scissors. Flies were mounted by the cervix into individual collars of a custom chamber. Nail polish was applied behind the head in a thin layer to hold the fly in place. A dental waxer was used to apply melted wax to the sides of the extended proboscis, adhering it to the rim of the chamber.

3.2.3.1 GCaMP Imaging

For calcium imaging, the antennae and cuticle covering access to the SEZ were removed using forceps. AHL buffer with ribose (Liu et al., 2012) was used to cover the open brain. A coverslip was inserted into a slot of the chamber, keeping the proboscis free from the buffer solution to allow stimulation of the labellum with tastants. GCaMP6f fluorescence was monitored using a Leica SP5 II scanning confocal with a tandem scanner and HyD detector. The SEZ was viewed with the 25x

water objective and an electronic zoom of eight. Images were acquired at a speed of 8,000 lines per second with a line average of four, resulting in collection time of 60 ms per frame at a resolution of 512 x 200 pixels. The pinhole was opened to 2.68 - 4 AU. Using a manual micromanipulator, a glass pipette was used to briefly apply tastant stimuli to the proboscis after acquiring 5 – 10 seconds of baseline fluorescence. Octopamine hydrochloride and tyramine hydrochloride (Sigma Aldrich) were dissolved as 0.1 mM stocks in AHL buffer with ribose. Both the octopamine and tyramine solutions were applied directly to the brain preparation and were used at a final concentration of 1 μM. The stimulus was delivered one minute after drug addition. For all Gr66a GRN imaging, flies were stimulated with 0.07mM lobeline. The change in fluorescence ($\Delta F/F_0$) was calculated by the 5-frame average peak intensity minus a ten-frame average pre-stimulus intensity (F_0), divided by F_0. The heat map in Figure 10 was created using Image J. To generate this image, 10 frames prior to stimulation and 8 frames following stimulation were each averaged, and then a threshold was applied (values < 10 = 0) to reduce background noise. A Gaussian blur with a pixel radius of 2 was applied, and then the two images were subtracted to give the change in fluorescence. The resulting image was divided by the prestimulus image to give $\Delta F/F_0$.

3.2.3.2 Electrophysiology

For cell-attached recordings, we made use of two different dissection methods depending on whether the fly was being stimulated with a tastant. In both cases the antennae and cuticle covering the SEZ were removed. For preparations

where taste stimulations were carried out, the top head cuticle was also cut away, and the perineural sheath was removed lateral to the base of the antennal lobe using sharp forceps. For baseline recordings where the proboscis was not required, both the proboscis and surrounding cuticle were removed. The perineural sheath was then removed at the bottom of the brain in the area where the optic lobe meets the SEZ.

All electrophysiological recordings were performed using glass electrodes (~1.5-3 MOhm) containing AHL, with patch resistances from 50-500 MOhm. OA-VL1 or OA-VL2 neurons were identified by driving *UAS-mCD8::GFP* with *VT026002-Gal4* or *VT049128-Gal4*, respectively. Spiking was recorded in voltage-clamp mode with a multiclamp 700B recorder at 20 kHz. Recordings were first passed through a low-pass filter at 5kHz and then band-pass filtered between 100 and 3000 Hz with a Butterworth filter. A custom Python script was used for spike detection. Taste stimuli used were: 1 mM denatonium, 20 mM lobeline, or a cocktail of 5 mM berberine mixed with 100 mM caffeine (bitter); 1 M sucrose or 1 M glucose (sweet). All tastants were delivered to the proboscis of the fly using a glass pipette controlled by a manual micromanipulator. At contact, a stimulus artifact could be seen in the recording. Prestimulation and taste-evoked firing were calculated from the average firing rate during the five seconds preceding and two seconds following this artifact, respectively. Tonic firing for food-deprived flies (0, 12, 24, 40 hours) was calculated by averaging 30 – 140 seconds of steady activity.

3.2.4 Behavioural Assays

For PER, female flies were aged 3 – 10 days. Flies were shifted to 29°C for 48 hours to induce production of KIR2.1 in cells of interest. Flies were starved on 1% agar at 29°C for the last 18 hours of this induction period and then mounted inside pipette tips that were cut to size so that only the head was exposed. The tubes were sealed with tape, positioned on a glass slide with double-sided tape, and allowed to recover for 1 – 2 hours in a humidified chamber. Before testing began, flies were stimulated with water and allowed to drink until satiated. Flies were then stimulated on the labellum with increasing concentrations of the bitter tastant L-canavanine (25, 50, 75, 100 mM) in 200 mM sucrose, using a 20µL pipette attached to a 1mL syringe. Flies were presented with each stimulus three times and PER responses were recorded. For analysis, each fly's responses were treated as an independent replicate, and 5-10 flies of each genotype were tested on at least three different days. The order of genotypes tested on each day was random.

3.2.5 Statistical Analyses

We used the lme4 1.1-7 package in R 3.2.1 (Bates et al., 2015) to model the binary PER at each concentration using generalized linear mixed models with a binomial error distribution and a random effect of fly ID to account for repeated measures of individual flies. Twenty-five flies of each genotype were measured 3 times at each concentration. We obtained p-values from these models to compare each genotype with the two controls (40 tests total), and classified these comparisons as statistically significant only if $p < 0.05$ even after applying the

positive false discovery rate procedure to correct for multiple tests (Benjamini and

Hochberg, 1995) using the q-value 2.2.2 package in R. This procedure controls the

expected rate of false positives (as a proportion of all positive results) to be 0.05,

and is thus a more appropriate way to maintain statistical power while performing a

large number of comparisons than the Bonferroni procedure (Benjamini and

Hochberg, 1995; Nakagawa, 2004). All other statistical tests were performed using

GraphPad Prism 6 and we used the Bonferroni correction to adjust p-values for

other experiments with a small number of comparisons.

3.3 RESULTS

3.3.1 OA-VL Neurons are in Close Proximity to Bitter GRNs

To identify novel neurons in the *Drosophila* taste circuit, we conducted an

anatomical screen using GRASP (Gordon and Scott, 2009; Feinberg et al., 2008). We

selected 20 lines from the Vienna Tile (VT) or Janelia Rubin Gal4 collections that

showed sparse SEZ labeling without detectable GRN expression, and tested each for

GRASP with sweet and bitter GRNs. This led us to identify two lines of interest,

VT026002-Gal4 and *VT049128-Gal4*, which both showed strong GRASP with bitter

GRNs, and little to no GRASP with sweet GRNs (Figure 10A-D). Each line labeled a

similar prominent bilateral pair of neurons in the SEZ; however, double labeling of

both *VT026002-Gal4* and *VT049128-Gal4* demonstrated that the neurons labeled by

each line are distinct.

Using antibody markers for candidate neurotransmitter pathways, we

determined that the prominent SEZ neurons in each line were positive for tyrosine
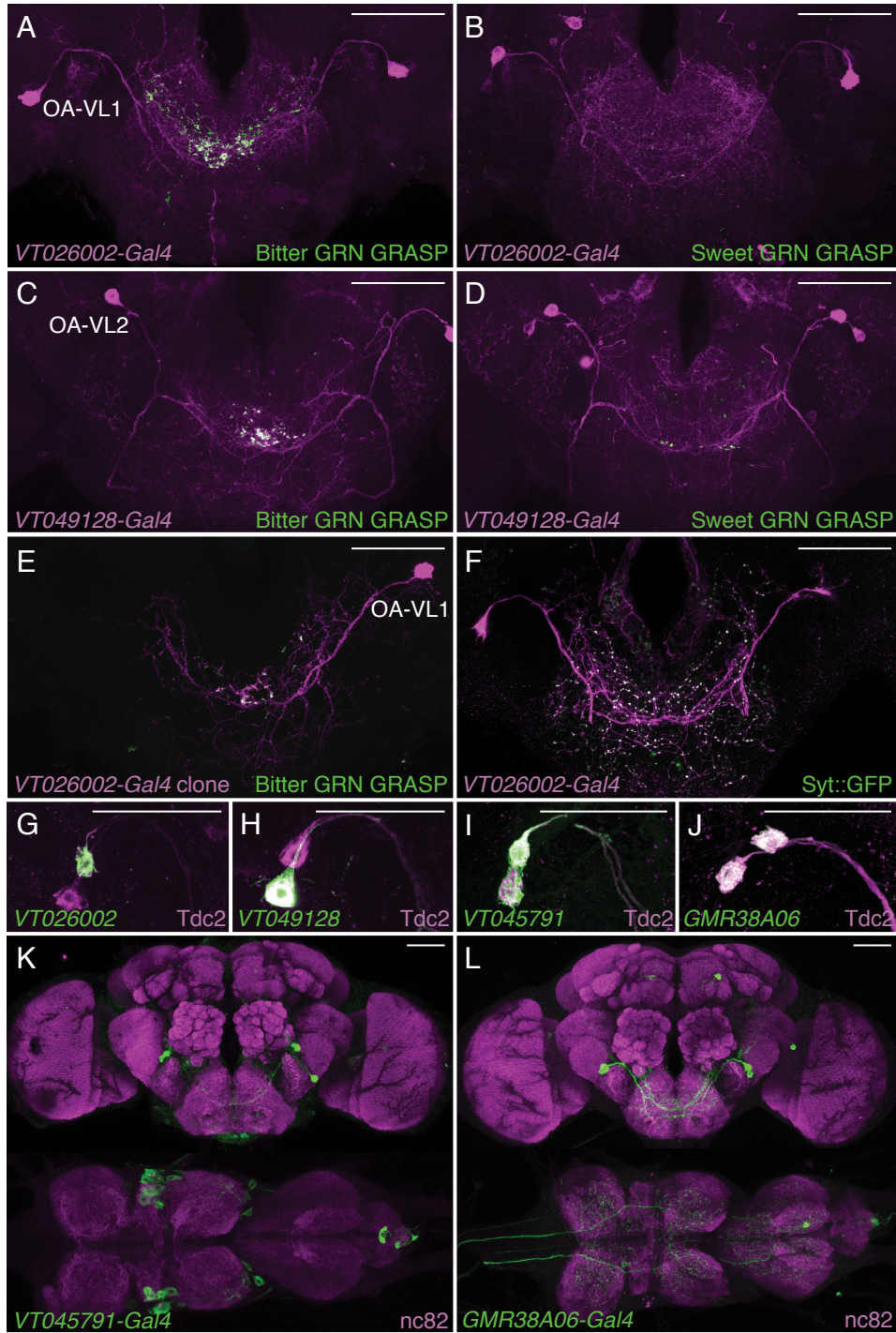
Figure 10. OA-VL neurons show specific proximity to bitter sensory inputs. (A-D) *VT026002-Gal4* and *VT049128-Gal4* each drive expression in a single OA-VL neuron per side of the SEZ (magenta) that shows extensive GRASP (green) with bitter GRNs

(A,C), but little to no GRASP with sweet GRNs (B,D). (E) A single OA-VL1 neuron

from *VT026002-Gal4* (magenta), labeled using FLP-out mosaics, displays prominent

GRASP (green) with bitter GRNs. (F) OA-VL1 presynaptic zones labeled with

synaptotagmin-GFP (green). OA-VL processes labeled with anti-dsRed (magenta).

(G-J) Anti-Tdc2 immunofluorescence (magenta) detects two OA-VL neurons per side

of the brain. The four Gal4 lines used to label OA-VLs in this study label either OA-

VL1 (G), OA-VL2 (H), or both OA-VLs (I,J). (K-L) Full brain and ventral nerve cord

expression of *VT045791-Gal4* (K) and *GMR38A06-Gal4* (L) driving CD8::GFP.

Neuropil is labeled with nc82 (magenta). All scale bars are 50 μm.

Full genotypes: (A) *Gr66a-LexA::VP16/+; lexAop-CD4::GFP11/+; VT026002-Gal4/UAS-CD4::GFP1-10, UAS-CD8::dsRed*. (B) *Gr5a-LexA::VP16/+; lexAop-CD4::GFP11/+; VT026002-Gal4/UAS-CD4::GFP1-10, UAS-CD8::dsRed*. (C) *Gr66a-LexA::VP16/+; lexAop-CD4::GFP11/+; VT049128-Gal4/UAS-CD4::GFP1-10, UAS-CD8::dsRed*. (D) *Gr5a-LexA::VP16/+; lexAop-CD4::GFP11/+; VT049128-Gal4/UAS-CD4::GFP1-10, UAS-CD8::dsRed*. (E) *tub>Gal80>/+; Gr66a-LexA::VP16/LexAop-CD4::GFP11, UAS-CD8::dsRed; VT026002-Gal4, UAS-CD4::GFP1-10/MKRS, hs-FLP*. (F) *VT026002-Gal4/+, UAS-CD8::dsRed/UAS-syt::GFP*. (G) *UAS-CD8::GFP/+, VT026002-Gal4/+*. (H) *UAS-CD8::GFP/+, VT049128-Gal/+*. (I,K) *UAS-CD8::GFP/+, VT045791-Gal4/+*. (J,L) *UAS-CD8::GFP/+, GMR38A06-Gal4/+*

decarboxylase 2 (Tdc2), a key enzyme in the synthesis of TA and its product OA (Figure 10G,H; Cole et al., 2005). Previous anatomical studies have defined these two neurons in each hemisphere as the OA-VL cluster, and shown they are immunoreactive for both OA and TA (Busch et al., 2009). By comparing the morphology of each neuron to the published images, we determined that *VT026002-Gal4* labels OA-VL1 and *VT049128-Gal4* labels OA-VL2 (Busch et al., 2009). Importantly, mosaic analysis of *VT026002-Gal4* confirmed that OA-VL1 exhibits a strong GRASP signal with bitter GRNs (Figure 10E). Although we cannot formally rule out contributions from non-OA-VL neurons to the full *VT026002-Gal4* GRASP signal, we did not find evidence for other GRASP-positive neurons in our mosaics. To examine the structure of OA-VLs in more detail, we expressed Synaptotagmin-GFP (Syt-GFP) under the control of *VT026002-Gal4*. This revealed extensive presynaptic labeling in the SEZ (Figure 10F), suggesting that OA-VLs may release OA and/or TA locally to modulate circuit function.

In addition to labeling one of the OA-VL pairs, *VT026002-Gal4* and *VT049128-Gal4* each labels a number of other neurons in the brain (Figure 11A,B). Therefore, we sought additional lines for functional analyses. By visually screening the VT and Janelia Gal4 collections, we identified *VT045791-Gal4* and *GMR38A06-Gal4*, each of which has sparse expression patterns that include both OA-VL1 and OA-VL2 (Figure 10I,J). In addition to the OA-VLs, *VT045791-Gal4* has weak activity in a group of neurons in the ventral SEZ and stronger activity in a small cluster of local or motor neurons in the ventral nerve cord (vnc) (Figure 10K), while *GMR38A06-Gal4* drives expression in 1-2 neurons in the protocerebrum and a few ascending neurons in the
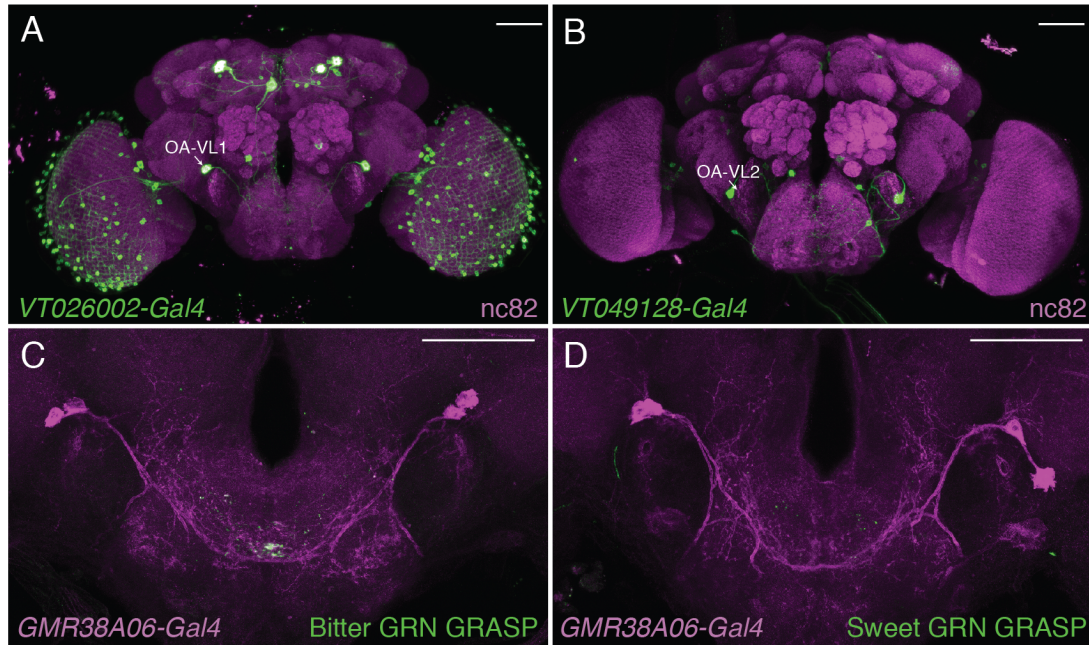
Figure 11. (A-B) Full brain expression of *VT026002-Gal4* (A) and *VT049128-Gal4* (L) driving CD8::GFP. Neuropil is labeled with nc82 (magenta). (C-D) GRASP between *GMR38A06-Gal4* neurons and bitter (C) or sweet (D) GRNs.

Full genotypes: (A) *UAS-CD8::GFP/+, VT026002-Gal4/+.* (B) *UAS-CD8::GFP/+, VT049128-Gal/+.* (C) *Gr66a-LexA::VP16/+; lexAop-CD4::GFP11/+; GMR38A06-Gal4/UAS-CD4::GFP1-10, UAS-CD8::dsRed.* (D) *Gr5a-LexA::VP16/+; lexAop-CD4::GFP11/+; GMR38A06-Gal4/UAS-CD4::GFP1-10, UAS-CD8::dsRed.*

vnc (Figure 10L). To further confirm the robustness of our GRASP results, we tested GRASP between the OA-VLs and GRNs using *GMR38A06-Gal4*. As expected, we observed a strong GRASP signal between the OA-VLs and bitter, but not sweet, GRNs (Figure 11C,D).

3.3.2 OA-VL Neurons are Regulated by Satiety State

To investigate possible functions of OA-VLs in bitter processing, we began by testing whether OA-VLs receive input from GRNs. Using cell-attached electrophysiological recordings, we measured OA-VL1 spike rates before and after stimulation of the fly labellum with either sugar or a cocktail of bitter compounds. Neither sweet nor bitter stimulation evoked a significant change in OA-VL firing, suggesting that these neurons do not receive excitatory or inhibitory input from sweet or bitter GRNs (Figure 12).

Our observation of OA-VL presynaptic terminals in the region of bitter GRN axon terminals (Figure 10F), and their lack of bitter-evoked activity, suggested an alternative mechanism for OA-VL function in bitter processing: OA and TA, released by OA-VLs, could directly modulate bitter GRNs. Given that satiety state is known to affect bitter sensitivity, we tested whether starvation may affect OA-VL activity. Indeed, we observed a progressive decrement in OA-VL firing rate following starvation for up to 40 hours (Figure 13A-C). These results, along with the previous observation that starvation lowers bitter sensitivity (Inagaki, Panse and Anderson, 2014), led us to examine the hypothesis that OA-VL activity potentiates bitter GRN output, and that this potentiation is decreased upon starvation.
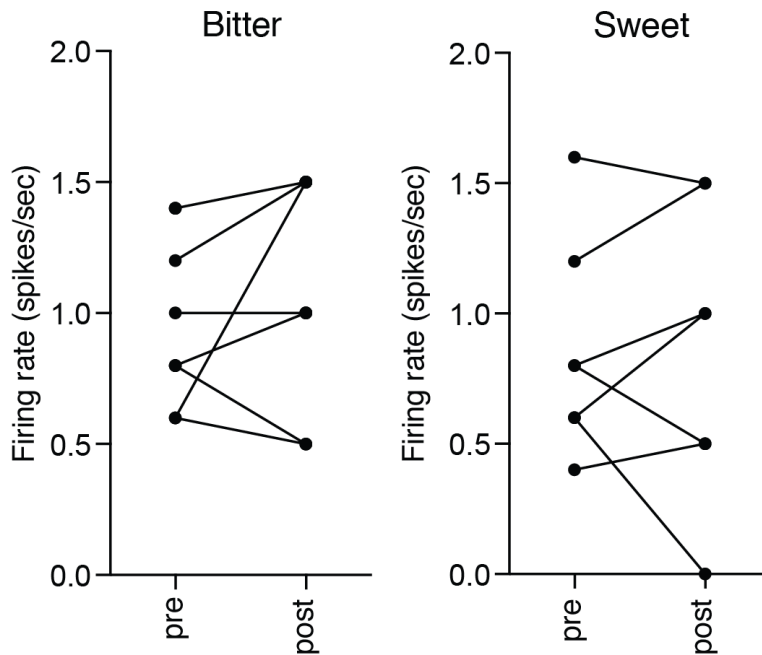
Figure 12. OA-VL1 activity prior to and following stimulation with bitter (left) or

sweet (right) tastants. Each line represents a single OA-VL1 neuron, and joins the

pre-stimulus and post-stimulus values. Bitter stimuli used were: 1 M denatonium,

20 mM lobeline, or a cocktail of 5 mM berberine mixed with 100 mM caffeine. Sweet

stimuli were 1 M sucrose or 1 M glucose.
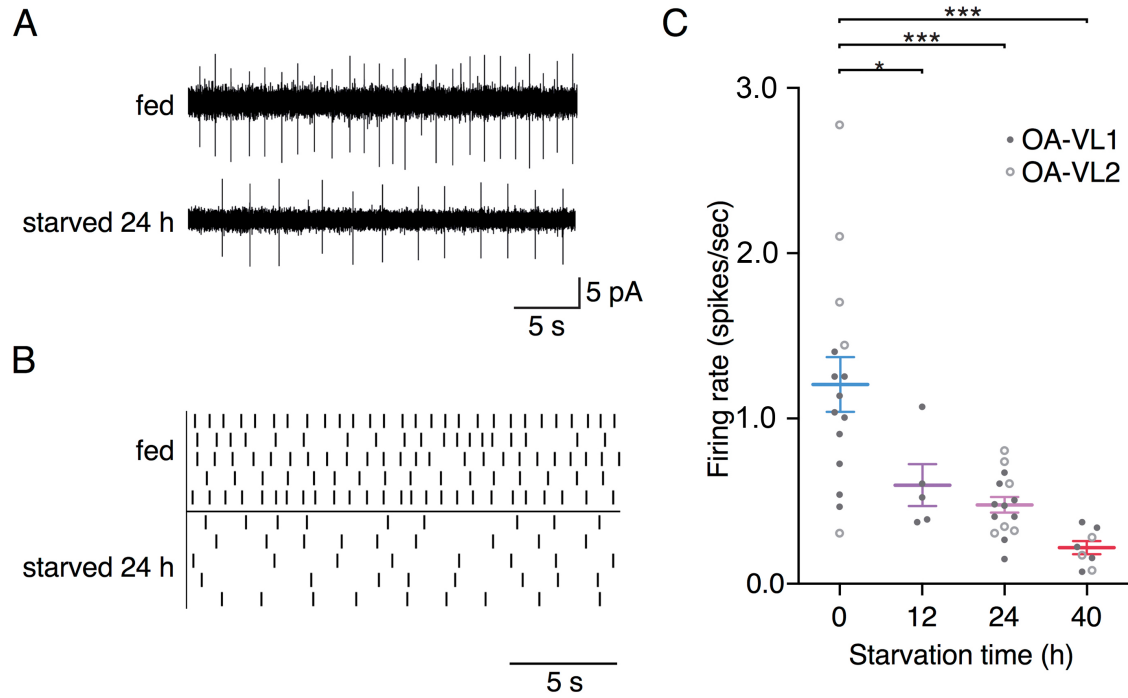
Figure 13. OA-VL neuron activity is regulated by satiety state. (A) Representative cell-attached recordings of OA-VL1 in flies that were fully fed (top) or starved for 24 h (bottom). (B) Raster plots of OA-VL1 activity in five flies that were fully fed (top) or starved for 24 h (bottom). (C) Summary plot of OA-VL1 and OA-VL2 activity as the duration of starvation increases. Lines and error bars represent mean +/- SEM for OA-VL1 and OA-VL2 recordings combined. Asterisks indicate statistical significance in a one-way ANOVA with Bonferroni correction, * p < 0.05, *** p < 0.001.

3.3.3 OA-VLs Modulate Bitter GRN Output

To directly test whether OA-VL activity potentiates bitter GRN output, we performed calcium imaging of bitter GRN axon terminals in the SEZ using GCaMP6f (Figure 14A). As reported by Inagaki and colleagues (2014), we found that starvation reduces calcium responses in bitter GRNs evoked by stimulation with a low concentration of lobeline (Figure 14A,B). We then examined bitter-evoked calcium responses in flies with OA-VLs silenced through expression of the *Drosophila* open rectifier potassium channel (ORK) under the control of *VT045791-Gal4* (Nitabach et al., 2004). Strikingly, artificial reduction of OA-VL firing in fed flies led to bitter GRN calcium responses indistinguishable to those seen following starvation (Figure 14B). Moreover, no further reduction in the bitter response was observed following starvation of OA-VL-silenced flies. These results indicate that OA-VLs are necessary for the potentiation of taste-evoked bitter GRN calcium signals in fed flies. Surprisingly, we observed similar effects from silencing just the OA-VL1s, suggesting that lowering the activity of just this single pair of neurons is sufficient to confer a starvation-like phenotype to bitter GRNs (Figure 15).

OA-VL neurons were previously identified as octopaminergic based on immunoreactivity for OA (Busch et al., 2009). However, they are also strongly immunoreactive for the OA precursor TA (Busch et al., 2009), raising the possibility that OA-VLs co-release these two neurotransmitters. To assess the roles of OA and TA in bitter modulation, we measured the impact of each on the bitter GRN calcium response. Pharmacological addition of OA or TA to the brain of starved flies caused a significant elevation in the taste-evoked calcium transients of bitter GRNs, and
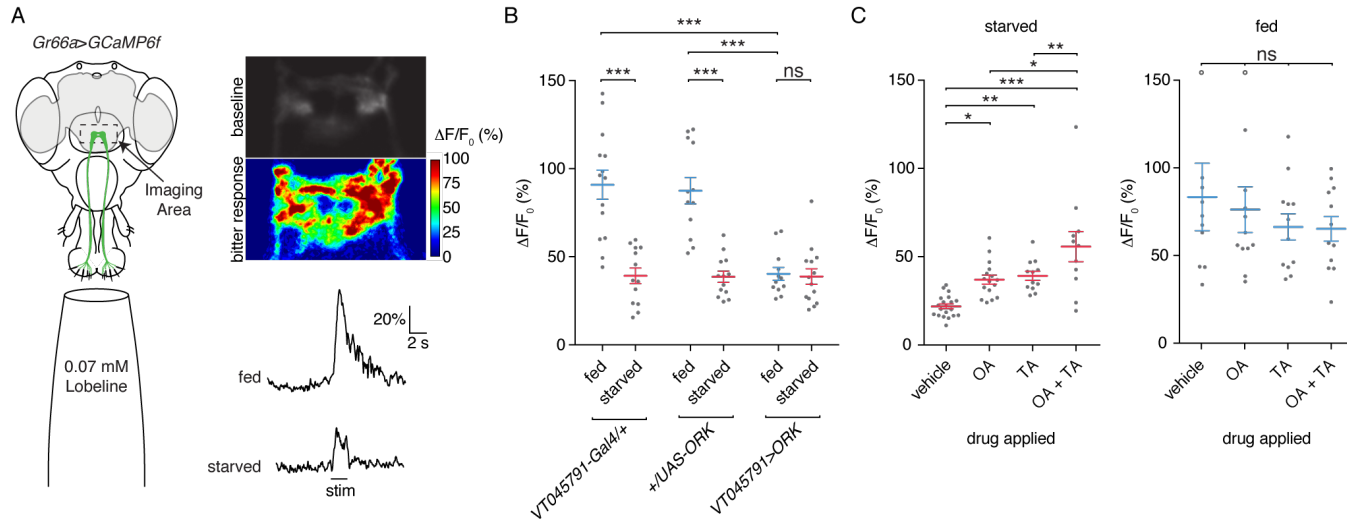
Figure 14. OA-VL activity modulates bitter GRN calcium responses. (A) Schematic of bitter GRN calcium imaging paradigm, along with sample heat map of taste-evoked activity in bitter GRNs and traces showing the change in fluorescence over time. (B) Average peak GCaMP6f fluorescence change in Gr66a axon terminals following stimulation with 0.07 mM lobeline, for indicated genotypes under fed and 40h starved conditions. Lines represent mean +/- SEM, with blue lines representing fed flies and red lines representing starved flies. Grey dots indicate values for individual flies. Asterisks indicate statistical significance in a two-way ANOVA with Bonferroni correction, *** p < 0.001, ns = not significant. We also found a significant interaction between the fed/starved condition and genotype for both controls compared to OA-VL-silenced flies. *VT045791>ORK* is short form for *VT045791-Gal4/UAS-ORK*, and all indicated genotypes also had *Gr66a-LexA::VP16* and *LexAop-GCaMP6f* in the background. (C) Average peak bitter GRN responses in *Gr66a-Gal4, UAS-GCaMP6f* flies following addition of the indicated compound to the brain at a final

concentration of 1 µM. Lines represent mean +/- SEM, grey dots represent values for

individual flies, and open grey circles are values that are higher than the y-axis

maximum. Asterisks indicate a statistically significant difference from the vehicle

condition by one-way ANOVA with Bonferroni correction, $*p < 0.05$, $**p<0.01$, $***$ p

< 0.001, ns = not significant.
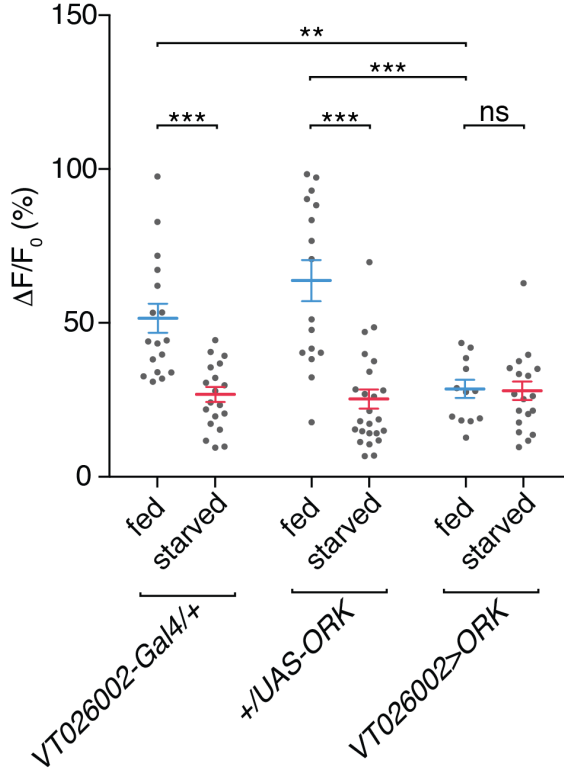
Figure 15. Average peak GCaMP6f fluorescence change in Gr66a axon terminals following stimulation with 0.07 mM lobeline, for indicated genotypes under fed and 40h starved conditions. Lines represent mean +/- SEM, with blue lines representing fed flies and red lines representing starved flies. Grey dots indicate values for individual flies. Asterisks indicate significance by two-way ANOVA with Bonferroni correction, *** p < 0.001, ns = not significant. We also found significant interactions between the fed/starved condition and genotype for both controls compared to OA-VL-silenced flies. *VT026002>ORK* is short form for *VT026002-Gal4/UAS-ORK*, and all indicated genotypes also had *Gr66a-LexA::VP16* and *LexAop-GCaMP6f* in the background.

addition of both together produced a roughly additive effect (Figure 14C). Importantly, application of OA or TA, or both, had no effect on the bitter GRN responses measured in fed flies, supporting the conclusion that reduction of OA and/or TA signaling underlies the starvation-dependent depotentiation of bitter GRN responses.

To confirm the behavioural relevance of bitter modulation by OA-VLs, we measured their role in PER inhibition by bitter compounds. We conditionally silenced OA-VLs in adult flies with KIR2.1 expression under the temporal control of Gal80[ts], and then measured PER to sucrose plus increasing concentrations of the bitter compound L-canavanine (Figure 17). L-canavanine was chosen because, unlike lobeline and most other bitter compounds, it does not directly suppress the firing of sweet GRNs, and therefore should inhibit PER exclusively through the activation of bitter GRNs (Jeong et al., 2013; Chu et al., 2014). We performed PER following 18 h of starvation, which increases sugar sensitivity and the robustness of PER, but is not sufficient to decrease bitter GRN sensitivity (Inagaki, Panse and Anderson, 2014). Silencing of both OA-VLs with either *VT045791-Gal4* or *GMR38A06-Gal4* led to a significant reduction in PER inhibition by L-canavanine, demonstrating that OA-VLs are necessary for the inhibition of PER by bitter compounds (Figure 17). We infer that this reduction in bitter sensitivity upon OA-VL silencing is due to the reduced bitter GRN output we observed with calcium imaging. Also consistent with the results of our calcium imaging, silencing of either the *VT026002-Gal4* or *VT049128-Gal4* population produced a similar phenotype, suggesting that both OA-VL1 and OA-VL2 are necessary for the potentiation of bitter
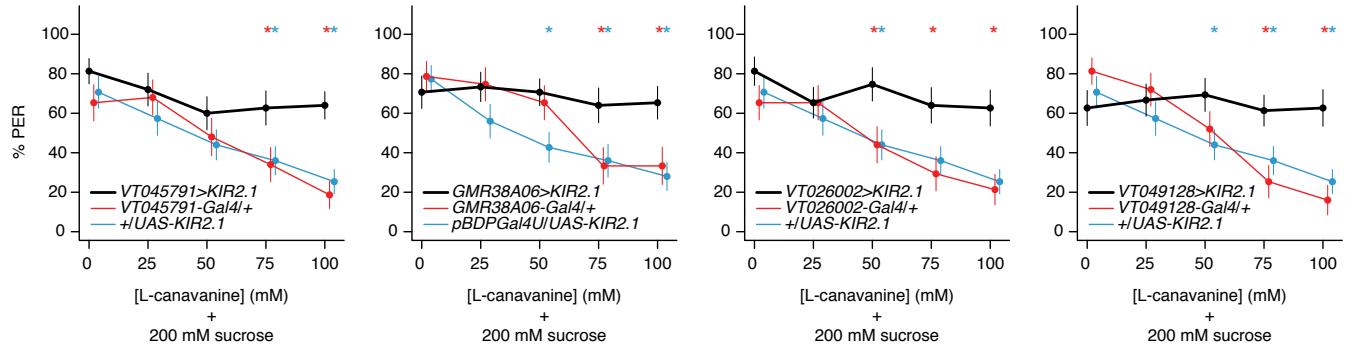
Figure 16. OA-VL neurons regulate behavioural bitter taste sensitivity. The PER of flies with silenced OA-VLs is significantly less inhibited by high concentrations of the bitter compound L-canavanine. Each panel shows the percent PER (mean +/- SEM) of flies starved for 18 h. All genotypes with *UAS-KIR2.1* also have *tub-Gal80*[ts] for temporal control of KIR2.1 expression. "+" indicates the w^{1118} strain from VDRC used to make the Vienna Tile (VT) collection; *PBDPGal4U* is an enhancerless Gal4 control for the Janelia lines. A colored asterisk indicates that the genotype shown in black was significantly more likely to exhibit PER than its red or blue control line, after a false discovery rate correction. n = 25 flies per genotype at each L-canavanine concentration.

sensitivity (Figure 17). Although none of our Gal4 lines exclusively label OA-VLs, our expression analyses suggest it is highly unlikely that any non-OA-VL expression is shared by all four lines. Thus, we are confident that the observed phenotypes are produced by manipulation of the OA-VLs.

3.3.1 OA and TA Act Directly on Bitter Neurons

Given the anatomical and functional characteristics of OA-VLs, the most parsimonious model is that OA and TA released by OA-VLs directly modulate bitter GRN sensitivity. To provide additional support for this model, we tested the function of OA and TA receptors in bitter GRNs.

There are seven OA and TA receptors in *Drosophila* (El-Kholy et al., 2015). To identify likely candidate receptors functioning in bitter GRNs, we made use of a published microarray dataset comparing gene expression in the labella of control flies to that of *poxn* mutants with reduced numbers of GRNs (Cameron et al., 2010). Two OA and/or TA receptor genes showed evidence of possible enrichment in taste tissue: *Octβ1R* (CG6919) and *Oct-TyrR* (CG7485). We performed PER following knockdown of each receptor in bitter GRNs, and found that *Oct-TyrR* knockdown significantly reduced bitter sensitivity in flies that had been starved 18 hours (Figure 17A). This result suggests that OA and TA, released by OA-VLs, acts directly on bitter GRNs to potentiate their output through the activity of Oct-TyrR (Figure 17B). Moreover, the previous observation that Oct-TyrR expressed in heterologous cells mediates similar calcium responses following addition of either OA or TA (Robb et al., 1994) may explain why either ligand is sufficient to potentiate bitter
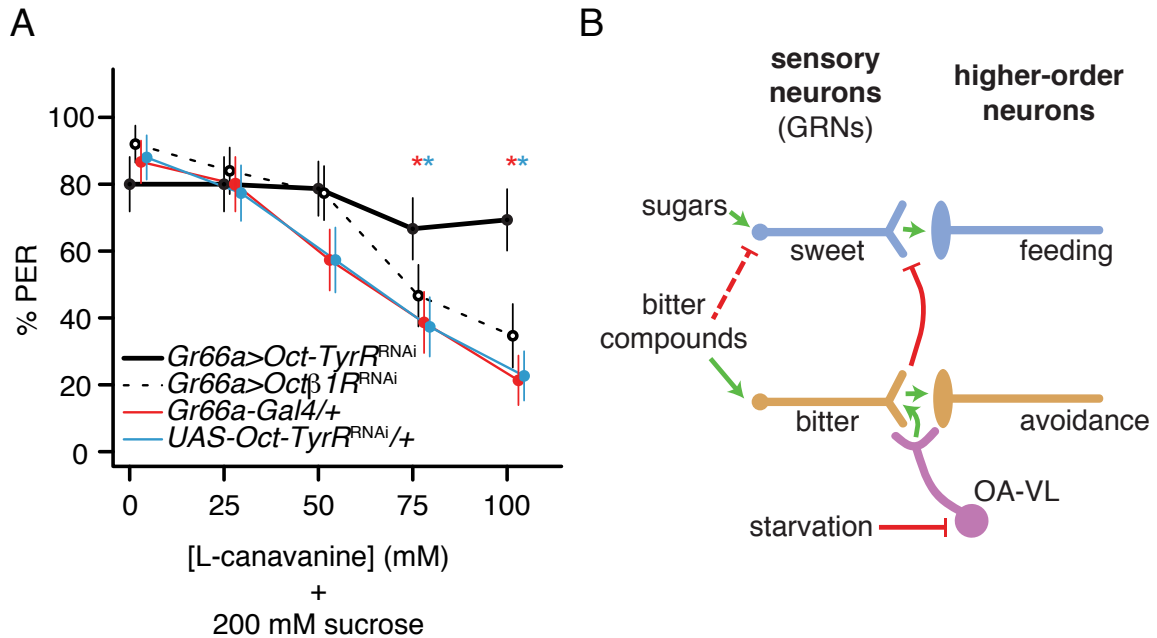
Figure 17. Oct-TyrR mediates the potentiation of bitter sensitivity. (A) The PER of flies with *Oct-TyrR* knocked down in bitter sensory neurons is significantly less inhibited by high concentrations of the bitter compound L-canavanine. Plot shows the percent PER (mean +/- SEM) of flies starved for 18 h. The colored asterisks indicate that the *Gr66a>Oct-TyrR[RNAi]* flies were significantly more likely to exhibit PER than the red or blue control lines, after a false discovery rate correction. (B) Model for the effect of OA-VLs on bitter sensitivity and feeding behaviour. Green arrows indicate excitation, red lines indicate inhibition. The dashed line indicates that most, but not all, bitter compounds directly inhibit sweet neurons. Synaptic inhibition of sweet GRNs by bitter GRN activity is based on previous work (Chu et al., 2014).

GRNs (Figure 14C).

3.4 Discussion

Fly chemosensation has emerged as a leading model for understanding the neuromodulatory effects of starvation on sensory processing (Su and Wang, 2014). In the gustatory system, sweet and bitter taste show orthogonal responses to starvation – sweet taste is potentiated, while bitter taste is suppressed (Inagaki, Panse and Anderson, 2014). While sweet GRNs are known to be directly modulated by dopamine, the signal that suppresses bitter sensitivity was unknown. Here, we identify a novel class of starvation-regulated octopaminergic/tyraminergic neurons that directly modulate bitter GRN output, demonstrating a key link in the mechanism of bitter taste regulation (Figure 17).

3.4.1 Independent and Reciprocal Regulation of Sweet and Bitter Taste

Inagaki and colleagues (2014) demonstrated that sweet and bitter taste sensitivities were independently modulated by starvation through the activity of dNPF and sNPF, respectively. This raised the question of how these two related peptide systems, both of which are thought to increase following starvation, could affect opposite physiological responses in two distinct taste neuron classes. Our results explain this phenomenon: sweet and bitter GRNs are both potentiated by their respective neuromodulators (DA for sweet, OA/TA for bitter), but the neurons releasing these signals are regulated in opposite directions. While the starvation-induced increase in TH-VUM activity potentiates sweet taste (Marella, Mann and Scott, 2012), the concomitant decrease in OA-VL activity depotentiates bitter taste.

We expect that the inhibition of OA-VL activity may be from the activity of a GABAergic intermediate suggested previously (Inagaki, Panse and Anderson, 2014).

One intriguing aspect to our results is that silencing of a single OA-VL pair (OA-VL1) was sufficient to confer starved-like bitter sensitivity. We believe the most likely explanation for this observation is that silencing one OA-VL reduces OA/TA levels below a specific threshold that signals starvation. It is worth noting that 24 hours of starvation, which has been shown to produce near minimal levels of bitter sensitivity (Inagaki, Panse and Anderson, 2014), resulted in only an approximate 50% reduction in OA-VL firing. Thus, it is conceivable that genetic silencing of one OA-VL neuron pair, which is likely a more potent inhibitor of firing than starvation, reduces the total OA-VL output enough to confer the starved state. Another possibility is that the OA-VLs could be synaptically coupled to provide coordinated regulation, and expression of KIR2.1 or ORK in one of them leads to decreased activity in both.

3.4.2 OA/TA Regulation of Gustation and Starvation-Dependent Behaviours

Functional roles for TA have been rather obscure, with the exception of its recent implication in courtship drive (Huang et al., 2016) and evidence for differential roles of OA and TA in larval locomotion (Saraswati et al., 2004). However, OA is known to modulate numerous fly behaviors, including sleep, visual guidance, and social behaviors such as aggression and courtship (Wasserman, Salomon and Frye, 2013; van Breugel, Suver and Dickinson, 2014; Nall and Sehgal, 2014; Zhou, Rao and Rao, 2008; Certel et al., 2010; Andrews et al., 2014). Of

particular relevance to our work is a report that bitter GRNs – which detect some male pheromones in addition to canonical bitter compounds – provide input to a cluster of OA neurons in the SEZ that regulates male aggression and courtship behaviours (Andrews et al., 2014). *TBh* mutant males, unable to synthesize octopamine, showed reduced aggression towards other males and elevated male-male courtship, and this was phenocopied by ablation of bitter GRNs. Additionally, GRASP and calcium imaging suggested functional excitatory connections between bitter GRNs and SEZ OA neurons (Andrews et al., 2014). While the specific OA neurons receiving this input were not identified, the cluster examined with calcium imaging did not include OA-VLs. Thus, it appears that OA may modulate some gustatory behaviours in multiple ways, including presynaptic control of GRN output and modulation of downstream circuitry. It would be interesting to examine whether OA-VLs are regulated by any starvation-independent motivational states, including those that may have more salience in social behaviours.

A role for OA in starvation-induced hyperactivity has also been recently demonstrated (Yang et al., 2015). In this case, however, mutants lacking OA displayed activity levels comparable with the fed state, suggesting that OA promotes hyperactivity in starved flies. Thus, there may exist a subset of OA neurons that is positively regulated by starvation, in contrast to the starvation-induced reduction OA-VL activity shown here. Further characterization of OA/TA populations and their relationship to identified nutrient sensors and other starvation-regulated neural circuits will greatly enhance our understanding of how internal states modify perception and behavior.

CHAPTER 4   DISCUSSION

4.1 DISCUSSION

4.1.1 Implications for the Field of Drosophila Taste

How animals are able to sense the nutritional value of food independently of taste is becoming an increasingly popular area of research interest (de Araujo, 2011). Specifically, there appears to be a mechanism by which animals have the ability to detect the caloric value of food without sweetness. This mechanism is also separate from other satiety signals that reinforce the cessation of ingestion, such as gut stretching and release of insulin. The seminal papers on this topic in the field of *Drosophila* gustation made use of what were thought to be taste-blind flies to tease apart taste from nutrient sensing (Dus et al., 2011; Dus et al., 2013). These flies were either mutants for the developmental gene *poxn*, which had all external taste sensilla transformed into mechanosensory neurons, or flies that were double-mutants for the sugar receptors Gr5a and Gr64a and were reported to not detect sweet tastes. These studies used the putatively taste-blind flies to determine whether flies could sense the caloric value of food when it was uncoupled from its appetitive taste. Researchers were interested in whether flies could use this caloric information alone to make appropriate feeding choices, and if so, what the underlying mechanisms were.

However, these early studies that purported to lay the foundation of our understanding of this phenomenon were confounded. First, the double mutants used in these experiments are not sugar blind, only limited in their perception of

certain sugars (Miyamoto et al., 2012). Loss of Gr5a results in the inability to detect trehalose, whereas loss of Gr64a compromises detection of sucrose, maltose and glucose. Secondly, although at the time no one had formally shown that the pharyngeal neurons played a role in detecting appetitive tastes, it was well established that the pharynx did indeed contain taste sensilla that were anatomically similar to those on other parts of the body (Nayak and Singh, 1983; Stocker, 1994). The study presented as chapter 2 of this thesis examines the role of these sorely overlooked taste receptors in evaluating food sources and controlling feeding behaviour. The initial nutrient sensing studies do not acknowledge that the internal pharyngeal sense organs may play a role in feeding, in the absence of peripheral taste. Therefore, the work in chapter 2 serves to address a fundamental gap in the literature by concretely showing that even without functional peripheral taste receptors, the pharyngeal taste receptors alone are enough to drive consumption of sweet compounds over tasteless ones. Moreover, my work shows that much, if not all, the preference for sweet compounds that was originally attributed to post-ingestive nutrient sensing, is likely to be a function of pharyngeal sweet taste. Thus, this study provides a new framework in which to interpret past results. In addition, with the identification of the receptors in the taste sensilla of pharyngeal sense organs, we provide a method to create a more realistic sweet-blind fly, which could aide in future studies wishing to implicate mechanisms in taste-independent nutrient sensing.

Animal survival depends on the neural circuitry underlying taste perception and feeding. *Drosophila* feeding operates as series of hierarchical subprograms, with

taste receptors on different body organs playing a role at discrete stages. Detecting

sugar is the first step in feeding and the sweet receptors contained in taste hairs of

the legs provide the first signal that a potential food has nutritional value. The

detection of sugar on the legs plays a key role in commencing feeding: it terminates

locomotion thereby allowing the fly to extend its proboscis. The legs contain two

distinct groups of taste neurons. One set projects to the thoracic ganglion, called

segmental tarsal GRNs (stGRNs) and another set projects into the SEZ, called the

ascending tarsal GRNs (atGRNs; Thoma et al., 2016). Interestingly, each group

serves a distinct behavioural function. The stGRNs are required for the cessation of

locomotion when the fly encounters sugar, and silencing these neurons prolongs

locomotion of starved flies on foods with high concentration of sucrose (Thoma et

al., 2016). The atGRNs detect sweet compounds that initiate feeding via extension of

the proboscis. Once the proboscis contacts a sweet compound, the labellar GRNs can

further examine the quality of the food source and may play a role in opening of the

labial palps to allow food to enter the mouth parts (Thoma et al., 2016). Once food

enters the pharynx it is further monitored by the pharyngeal GRNs. This is the last

point of evaluation for a food source before entering the gut. Our study empirically

shows that pharyngeal GRNs are required for sustaining consumption bouts of

appetitive compounds in hungry flies, which confers them a unique function in

feeding.

Chapter 3 details a number of experiments that led to the identification of the

first higher-order components of the bitter circuit in *Drosophila*. As discussed in the

introduction, a number of independent studies have been published that describe

higher-order taste neurons. In particular, there are several reports of sweet-responsive neurons, or putative sweet circuit neurons, in the fly brain (Flood et al., 2013; Miyazaki et al., 2015; Kain and Dahanukar, 2015, Yapici et al., 2016), and neuron populations that affect starvation-dependent behaviors (Marella, Mann and Scott, 2006; Inagaki et al., 2012; Inagaki et al., 2015; Min et al., 2016). These studies have become numerous in our field, and although they provide valuable information on which future researchers will build, without identifying how each fits with each other or with known inputs and outputs of taste circuits, we remain far from understanding how taste information is processed in the fly brain. Therefore, we must work towards integrating the current studies in our field to place these neurons in their appropriate hierarchy in taste circuits, and further build on our understanding of these neurons to determine how they function in a network.

The study described in chapter 3 describes a bilateral pair of neurons that modulate the primary sensory bitter neuron at their first synapse in the taste center of the fly brain. The discovery of these neurons answered a long-standing question in the field of how the sensitivity of bitter neurons was altered in a state-dependent manner. With the identification of these neurons, we now know how sweet and bitter neurons become reciprocally sensitized and desensitized, respectively, when the fly is starved. This adds a valuable piece of information to the overall body of literature on the much less well studied and understood bitter circuit, and to our comprehensive understanding of mechanisms by which taste perception is altered in response to changes in energy deficits that threaten homeostasis.

4.1.2 Caveats and Future Directions in our Current Studies

4.1.2.1 Chapter 2

In chapter 2, we showed that in the absence of both peripheral and pharyngeal sweet taste, flies showed little preference for either caloric or non-caloric sweet foods. It is possible that we were unable to discern the effects of post-ingestive nutrient sensing due to the short time frame of our binary feeding assay. It would be of interest to evaluate the protein, named Cupcake, that Dus et al. (2013) identified to be responsible for taste-independent nutrient sensing in our sweet-blind flies. Because post-ingestive mechanisms are likely to operate over a longer time scale than can be detected by the original assay we used in chapter 2, it would have been ideal for us to use a long-term quantitative feeding assay. The most appropriate assay for this purpose would be the capillary feeding (CAFE) assay, which is a quantitative assay that can measure feeding over multiple days (Ja et al., 2007). Unfortunately, it was not possible to use this assay in conjunction with *poxn* mutant flies. The CAFE assay requires that flies feed in an inverted position at the tip of a capillary. This assay is incompatible with *poxn* mutants because they show a motor defect that hinders their ability to climb onto the capillary and maintain the inverted position necessary to feed from these tubes. Although this motor defect associated with the *poxn* mutant gene is not well described, it could stem from a documented defect in the segmentation of the tarsi (Awasaki and Kimura, 2001). This may explain their difficulties in walking on the glass capillaries and maintaining the position necessary to feed in the CAFE assay. Even with the arrival of new

apparatuses designed to better measure feeding dynamics, such as Expresso or the FlyPad, none have yet been designed that would provide both easy access to food for flies that have a *poxn* mutant background and could measure feeding choices over multiple days (Yapici et al., 2016; Itskov et al., 2014).

Another possible alternative is that post-ingestive effects may require sweet taste to guide feeding, and this is why we see no preference for caloric sweet foods in our experiment. It may be that sweet taste acts a permissive signal for taste-independent function, perhaps by allowing flies to form memories associating sweet taste with the caloric value of a food compound, and without this initial signal, i.e., the sweet taste, taste-independent functions like post-ingestive sensing have no impact. This may be because sweet taste is the guiding indicator of caloric value in a food, and nutrient value contributes to the reinforcement of appetitive memories, while non-caloric sugars do not produce strong appetitive memories. Sorbitol, a tasteless but nutritive sugar alcohol, does not impart an appetitive memory unless mixed with a sweet tastant (Burke and Waddell, 2011). In addition, flies do not form long-term appetitive memories to sweet but not nutritive sugars like arabinose and xylose (Burke and Waddell, 2011). In the case of sorbitol, the lack of appetitive memory is thought to be due to insufficient ingestion of the taste-less sugar alcohol, while no long-term appetitive memory is formed with arabinose due to its lack of calories. However, it may be the case that both the sweet taste and caloric value are needed. The sweet taste might be necessary as a reinforcing component that underlies post-ingestive effects. It could be that in the absence of sweet taste in our

poxn mutant flies with silenced pharyngeal neurons, there is no signal for nutrient sensing to reinforce, thus the preference for these sugars is lost.

4.1.2.2 Chapter 3

One of the issues we attempted to address in this study, but could not fully resolve was the use of a GAL4 driver line that was completely selective for OA-VL neurons. It would be ideal to have GAL4 lines that label the both of OA-VL pairs and each individual pair of neurons, OA-VL1 and OA-VL2. This would allow us to determine conclusively that the OA-VL neurons are responsible for depotentation of bitter neurons in the starved state, and would be beneficial moving forward with further experiments to address how OA-VL neurons themselves are being regulated, whether it be through a peptidergic mechanism as suggested by Iganaki et al. (2014) or GABA inhibition, or some combination of both. One possible way to address this would be to use a mosaic analysis (Gordon and Scott, 2009), but this was not feasible in our case. Most importantly mosaic analysis creates a random expression pattern that varies from fly to fly and thus requires a large number of replicates. With our behavioural experiments, this may have been feasible but the phenotype of each fly would still vary depending on the random expression pattern created. There would undoubtedly be a large overlap between populations of neurons labelled and the corresponding behavioural phenotype, making it difficult to examine only the effect caused by OA-VLs.

Iganaki et al. (2014) showed compelling evidence that implicates sNPF acts through GABA to decrease bitter sensitivity during starvation, independently of the

increase in sugar sensitivity. Both 24 and 48 hour starved sNPF homozygous

mutant flies showed an increased sensitivity to bitter compounds compared to wild-

type controls. Because there are roughly 4000 sNPF positive neurons in the fly brain

Iganaki et al (2014) tested 11 individual sNPF-GAL4 lines that labeled smaller

subsets of the overall sNPF population. They drove expression of KIR2.1 in these

lines in an attempt to identify a smaller subset of sNPF positive neurons that were

responsible for regulating bitter sensitivity. Lines in which a defect in bitter

sensitivity was seen after starvation had one subset of sNPF neurons in common: a

set of 11 – 12 neurons called the lateral neurosecretory cells (LNCs). The cell bodies

of the LNCs reside in the central brain, but these neurons send their axonal

projections the SEZ and are thus poised to modulate bitter sensory neurons.

Iganaki et al (2014) also examined whether sNPF acted directly on bitter

GRNs through the one sNPF receptor in *Drosophila*. To do this they knocked down

sNPFR in bitter neurons using Gr66a-GAL4 and then performed calcium imaging in

bitter GRNs. If sNPF were acting directly on bitter GRNs through the sNPFR it would

reduce bitter sensitivity in the fed state. However, this experiment resulted in no

defect in the modulation of the sensitivity of bitter neurons during starvation,

showing that sNPF does not directly act on bitter GRNs through sNPFR. Iganaki et al

(2014) then postulated that sNPF could be acting through GABAergic interneurons

to decrease the sensitivity of bitter neurons during starvation. To test this

hypothesis, they knocked down sNPFR in GABAergic neurons using the dvGAT-

GAL4 driver. When sNPF was unable to act through GABAergic neurons, starvation-

dependent modulation of bitter sensitivity was abolished. The evidence provided by

these experiments show that sNPF acts through GABAergic interneurons to induce a change in bitter sensitivity when flies are starved.

Therefore, we believe that OA-VL neurons may be receiving GABAergic inhibition from local interneurons in the SEZ that are in turn being acted upon by the sNPF positive LNCs. Our own experiments show that double labeling of GFP under the control of GABA$_B$R2-GAL4 and anti-tdc2 reveals overlap with both OA-VL neurons and GABA$_B$R2 expression. However, the expression pattern of GABA$_B$R2-GAL4 is extremely broad and therefore does not provide sufficient evidence that this double labeling reflects the endogenous expression of GABA$_B$R2 in OA-VL neurons. It would be of interest to test the role GABA may have in setting the inhibitory tone in OA-VL neurons during starvation in a number of ways. First, GABA$_B$R2 RNAi could be expressed in OA-VL neurons using the sparse GAL4 lines we identified that label each OA-VL neuron pair separately, with little expression elsewhere. With this fly, in which GABA$_B$R2 has been knocked down specifically in OA-VL neurons, we could do both behavioural and physiological experiments to test whether this receptor is acting in OA-VL neurons and controlling their reduced response in the starved state. For example, if GABA$_B$R2 were knocked down in OA-VL neurons, we would expect the frequency of firing in OA-VL neurons to resemble that seen in the fed state, which allows for high bitter sensory neuron output. In this case, we would expect starved flies to continue to show aversion to bitter compounds, as assessed by PER, similar to that seen in the fed state. We could also measure the tonic firing rate of OA-VL neurons in OA-VL > GABA$_B$R2 knockdown flies. We would expect that OA-VL neurons in starved flies without GABA inhibition would show a comparable tonic

firing rate to that measured in fed flies. The decrement seen when measuring the firing over a starvation time course would be lost. We could also perform an experiment where we add sNPF directly to the brain while recording from OA-VL neurons. If sNPF acts on GABAergic interneurons, which in turn act on OA-VL neurons, the addition of sNPF to the brain could cause a decrease in their firing rate, and depotentiate bitter sensory neurons mimicking the starved state, and this reduction would be blocked with pharmacological inhibitors of GABA receptors.

4.1.3 Implications for Human Health

Drosophila provides an excellent model for understanding the elaborate neural circuits underlying taste perception and feeding. Compared to mammals, the fruit fly has a complex, yet manageably sized, nervous system and the genetic tools available to dissect circuits offer an advantage over mammalian models. Elucidation of feeding circuits in *Drosophila* could provide valuable information that helps uncover how similar mechanisms regulate food intake in mammalian models, and eventually humans. Human obesity has become an increasingly impactful health issue over the last few decades, and obesity is linked to many serious diseases, including type 2 diabetes, heart disease and even cancer.

The rampant increase in human obesity has also lead to widespread consumption of artificial sweeteners. Artificial sweeteners impart sweet taste without providing any nutrition and were originally directed for use in people who were required to limit their sugar intake, such as diabetics. Now these sweeteners have become increasingly common within foods marketed to the general population.

However, we know little about how they affect the human body and there is intense debate within the literature as to their safety as an effective way to reduce sugar intake and eliminate weight gain (Tandel, 2011). Studies have shown a connection between these artificial sweeteners and human metabolic dysregulation, but the mechanisms underlying these changes are not well understood (Dhringa et al., 2007). A recent study by Wang et al. (2016) revealed the mechanism through which sucralose affects the metabolism of the fruit fly. Flies that were fed a sucralose and yeast diet showed a number of effects that are paralleled in human studies, the main similarities being an increased overall consumption and an increased tolerance to glucose. This study shows the value in using the simple fruit fly model to identify mechanisms underlying general phenomena observed across species. The findings of these studies can then allow researchers to fine tune their experimental approach in mammalian models, thus increasing the efficiency of these studies.

REFERENCES

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

Akerboom J, Chen TW, Wardill TJ, Tian L, Marvin JS, Mutlu S, Calderon NC, Esposti F, Borghuis BG, Sun XR, Gordus A, Orger MB, Portugues R, Engert F, Macklin JJ, Filosa A, Aggarwal A, Kerr RA, Takagi R, Kracun S, Shigetomi E, Khakh BS, Baier H, Lagnado L, Wang SS, Bargmann CI, Kimmel BE, Jayaraman V, Svoboda K, Kim DS, Schreiter ER, Looger LL (2012) Optimization of a GCaMP calcium indicator for neural activity imaging. Journal of Neuroscience 32: 13819 – 13840.

Albin SD, Kaun KR, Knapp JM, Chung P, Heberlein U, Simpson JH (2015) A subset of serotonergic neurons evokes hunger in adult Drosophila. Current Biology 25: 2435 – 2440.

Andrews JC, Fernández MP, Yu Q, Leary GP, Leung AKW, Kavanaugh MP, Kravitz EA, Certel SJ (2014) Octopamine neuromodulation regulates Gr32a-linked aggression and courtship pathways in Drosophila males. PLoS Genetics 10 e1004356.

Awasaki T, Kimura K (1997) pox-neuro is required for development of chemosensory bristles in Drosophila. Journal of Neurobiology 32: 707 – 721.

Baines RA, Uhler JP, Thompson A, Sweeney ST, Bate M (2001) Altered electrical properties in Drosophila neurons developing without synaptic transmission. Journal of Neuroscience 21: 1523 – 1531.

Baird GS, Zacharias DA, Tsien RY (1999) Circular permutation and receptor insertion within green fluorescent proteins. Proceedings of the National Academy of Sciences 96: 11241 – 11246.

Bates D, Maechler M, Bolker BM, Walker SC (2015) Fitting Linear Mixed-Effects Models Using lme4. Journal of Statistical Software 67: 1 – 48.

Benton R, Dahanukar A (2011) Electrophysiological recording from Drosophila taste sensilla. Cold Spring Harbor Protocols 2011 (7): 839 – 850.

Benton R, Vannice KS, Gomez-Diaz C, Vosshall LB (2009) Variant ionotropic glutamate receptors chemosensory receptors in *Drosophila*. Cell 136: 149 – 162.

Bernstein JG, Garrity PA, Boyden ES (2012) Optogenetics and thermogenetics: technologies for controlling the activity of targeted cells within intact neural circuits. Current Opinions in Neurobiology 22: 61 – 71.

Bradbury J (2004) Taste perception: cracking the code. PLoS Biology 2(3): e64. doi:10.1371/journal.pbio.0020064.

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

van Breugel F, Suver MP, Dickinson MH (2014) Octopaminergic modulation of the visual flight speed regulator of Drosophila. Journal of Experimental Biology 217: 1737 – 1744.

de Brito Sanchez G, Giurfa M (2011) A comparative analysis of neural taste processing in animals. Philosophical Transactions of the Royal Society 366: 2171 – 2180.

Burke CJ, Waddell S (2011) Remembering nutrient quality of sugar in Drosophila. Current Biology 21: 746 – 750.

Burke CJ, Huetteroth W, Owald D, Perisse E, Krashes MJ, Das G, Gohl D, Silies M, Certel S, Waddell S (2012) Layered reward signaling through octopamine and dopamine in Drosophila. Nature 492: 433 – 437.

Busch S, Selcho M, Ito K, Tanimoto H (2009) A map of octopaminergic neurons in the Drosophila brain. Journal of Comparative Neurology 513: 643 – 667.

Cameron P, Hiroi M, Ngai J, Scott K (2010) The molecular basis for water taste in *Drosophila*. Nature 465: 91 – 95.

Certel SJ, Leung A, Lin C-Y, Perez P, Chiang A-S, Kravitz EA (2010) Octopamine Neuromodulatory Effects on a Social Behaviour Decision-Making Network in Drosophila Males. PLoS ONE 5 e13248.

Chandrashekar J, Kuhn C, Oka Y, Yarmolinsky DA, Hummler E, Ryba NJP, Zuker CS (2010) The cells and peripheral representation of sodium taste in mice. Nature: 297 – 301.

Chen T, Wardill TJ, Sun Y, Pulver SR, Renninger SL, Baohan A, Schreiter ER, Kerr RA, Orger MB, Jayaraman V, Looger LL, Svoboda K, Kim DS (2013) Ultra-sensitive fluorescent proteins for imaging neuronal activity. Nature 499: 295 – 300.

Chen X, Gabito M, Peng Y, Ryba NJP, Zuker CS (2011) A gustotopic map of taste qualities in the mammalian brain. Science 333(6047): 1262 – 1266.

Chu B, Chui V, Mann K, Gordon MD (2014) Presynaptic gain control drives sweet and bitter taste integration in Drosophila. Current Biology 24: 1978 – 1984.

Chu, B (2014) The role of GABA in modulating taste neuron output in *Drosophila*. (Master's Thesis) Retrieved from UBC Electronic Theses and Dissertation Database cIRcle doi: 10.14288/1.0165955.

Clyne PJ, Warr CG, Carlson JR (2000) Candidate taste receptors in *Drosophila*. Science, 287:1830 – 1834.

Cole S, Carney G, McClung C, Willard S, Taylor B, Hirsh J (2005) Two functional but noncomplementing Drosophila tyrosine decarboxylase genes: distinct roles for neural tyramine and octopamine in female fertility. Journal of Biological Chemistry 280: 14948 – 14955.

Crivici A, Ikura M (1995) Molecular and structural basis of target recognition by calmodulin. Annual Review of Biophysics and Biomolecular Structure 24: 85 – 116.

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: 1182 – 1186.

Dahanukar A, Lei Y, Kwon J, Carlson J (2007) Two Gr genes underlie sugar reception in Drosophila. Neuron 56: 503 – 516.

Damak S, Rong M, Yasumatsu K, Kokrashvili Z, Varadarajan V, Zou S, Jiang P, Ninomiya Y, Margolskee RF (2003) Taste in the absence of taste receptor T1r3. Science 301: 850 – 853.

Dethier VG (1976) The hungry fly. (Cambridge: Harvard University Press).

Duffy JB (2002) GAL4 system in Drosophila: a fly geneticist's Swiss army knife. Genesis 34: 1 – 15.

Dunipace L, Meister S, McNealy C, Amrein H (2001) Spatially restricted expression of candidate taste receptors in *Drosophila* gustatory system. Current Biology 11: 822 – 835.

Dus M, Min S, Keene AC, Lee GY, Suh GSB (2011) Taste-independent detection of caloric content of sugar in Drosophila. Proceedings of the National Academy of Sciences 108: 11644 – 11649.

Dus M, Ai M, Suh GSB (2013) Taste-independent nutrient selection is mediated by a brain-specific Na^+/solute cotransporter in Drosophila. Nature Neuroscience 16: 526 – 528.

El-Kholy S, Stephano F, Li Y, Bhandari A, Fink C, Roeder T (2015) Expression analysis of octopamine and tyramine receptors in Drosophila. Cell Tissue Research 361: 669 – 684.

Falk R, Bleiser-Avivi N, Atidia J (1976) Labellar taste organs of Drosophila melanogaster. Journal of Morphology 150: 327 – 342.

Farhadian SF, Suárez-Fariñas M, Cho CE, Pellegrino M, Vosshall, LB (2012) Post-fasting olfactory, transcriptional, and feeding responses in *Drosophila*. Physiology & Behaviour 105: 544 – 553.

Feinberg E, Vanhoven M, Bendesky A, Wang G, Fetter R, Shen K, Bargmann C (2008) GFP Reconstitution Across Synaptic Partners (GRASP) defines cell contacts and synapses in living nervous systems. Neuron 57: 353 – 363.

Fischler W, Kong P, Marella S, Scott K (2007) The detection of carbonation by the *Drosophila* gustatory system. Nature 448: 1054 – 1057.

Flood TF, Iguchi S, Gorczyca M, White B, Ito K, Yoshihara M (2013) A single pair of interneurons commands the *Drosophila* feeding motor program. Nature 499: 83 – 87.

Freeman EG, Dahanukar A (2015) Molecular neurobiology of Drosophila taste. Current Opinion in Neurobiology 34: 140 – 148.

Freeman EG, Wisotsky Z, Dahanukar A (2014) Detection of sweet tastants by a conserved group of insect gustatory receptors. Proceedings of the National Academy of Sciences 111: 1598 – 1603.

Fujii S, Yavuz A, Slone J, Jagge C, Song X, Amrein H (2015) Drosophila Sugar Receptors in Sweet Taste Perception, Olfaction, and Internal Nutrient Sensing. Current Biology 25: 1 – 8.

Fujita M, Tanimura T (2011) Drosophila evaluates and learns the nutritional value of sugars. Current Biology 21: 751 – 755.

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

Gao N, Lu M, Echeverri F, Laita B, Kalabat D, Williams ME, Hevezi P, Zlotnik A, Moyer BD (2009) Voltage-gated sodium channels in taste bud cells. BMC Neuroscience 10: 20.

Gendre N, Lüer K, Friche S, Grillenzoni N, Ramaekers A, Technau GM, Stocker RF (2004) Integration of complex larval chemosensory organs into the adult nervous system of Drosophila. Development 131: 83 – 92.

Gordon MD, Scott K (2009) Motor control in a *Drosophila* taste circuit. Neuron 61: 373 – 384.

Groth AC, Fish M, Nusse R, Calos MP (2004) Construction of transgenic Drosophila by using the site-specific integrase from phage phiC31. Genetics 166: 1775 – 1782.

Hamada FN, Rosenzweig M, Kang K, Pulver SR, Ghezzi A, Jegla TJ, Garrity PA (2008) An internal thermal sensor controlling temperature preference in *Drosophila*. Nature 454: 217 – 220.

Hiroi M, Marion-Poll F, Tanimura T (2002) Differential response to sugars among labellar chemosensilla in Drosophila. Zoological Science 19: 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.

Hodge JJ (2009) Ion channels to inactivate neurons in *Drosophila*. Frontiers in Molecular Neuroscience 2: 1 – 10.

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

Huang J, Liu W, Qi Y-X, Luo J, Montell, C. (2016) Neuromodulation of Courtship Drive through Tyramine-Responsive Neurons in the Drosophila Brain. Current Biology

Hussain A, Zhang M, Ucpunar HK, Svensson T, Quillery E, Gompel N, Ignell R, Grunwald Kadow IC (2016) Ionotropic chemosensory receptors mediate the taste and smell of polyamines. PLoS Biology 14(5): e1002454. doi:10.1371/journal.pbio.1002454

Iguchi N, Ohkuri T, Slack JP, Zhong P, Huang L (2011) Sarco/endoplasmic reticulum Ca2+ATPases (SERCA) contribute to GPCR-mediated taste perception. PLoS One 6(8): e23165. doi:10.1371/journal.pone.0023165.

Inagaki HK, Ben-Tabou de-Leon S, Wong AM, Jagadish S, Ishimoto H, Barnea G, Kitamoto T, Axel R, Anderson DJ (2012) Visualizing neuromodulation in vivo: TANGO-mapping of dopamine signaling reveals appetite control of sugar sensing. Cell 148: 583 – 595.

Inagaki HK, Panse KM, Anderson DJ (2014) Independent, reciprocal neuromodulatory control of sweet and bitter taste sensitivity during starvation in *Drosophila*. Neuron 84: 806 – 820.

Itskov PM, Ribeiro C (2013) The dilemmas of the gourmet fly: the molecular and neuronal mechanisms of feeding and nutrient decision making in Drosophila. Frontiers in Neuroscience 7: 12.

Jeong YT, Shim J, Oh SR, Yoon HI, Kim CH, Moon SJ, Montell C (2013) An odorant-binding protein required for suppression of sweet taste by bitter chemicals. Neuron 79: 725 – 737.

Jiao Y, Moon SJ, Montell C (2007) A Drosophila gustatory receptor required for the responses to sucrose, glucose, and maltose identified by mRNA tagging. Proceedings of the National Academy of Sciences 104: 14110 – 14115.

Jiao Y, Moon SJ, Wang X, Ren Q, Montell C (2008) Gr64f Is Required in Combination with Other Gustatory Receptors for Sugar Detection in Drosophila. Current Biology 18: 1797 – 1801.

Joseph RM, Heberlein U (2012) Tissue-specific activation of a single gustatory receptor produces opposing behavioral responses in Drosophila. Genetics 192: 521 – 532.

Kain P, Dahanukar A (2015) Secondary taste neurons that convey sweet taste and starvation in the *Drosophila* brain. Neuron 85: 819 – 832.

Keene AC, Duboué ER, McDonald DM, Dus M, Suh GSB, Waddell S, Blau J (2010) Clock and cycle limit starvation-induced sleep loss in Drosophila. Current Biology 20: 1209 – 1215.

Keene AC, Masek P (2012) Optogenetic induction of aversive taste memory. Neuroscience 222: 173 – 180.

Ko KI, Root CM, Lindsay SA, Zaninovich OA, Shepherd AK, Wasserman SA, Kim SM, Wang JW (2015) Starvation promotes concerted modulation of appetitive olfactory behavior via parallel neuromodulatory circuits. eLife 4, 221.

Krashes MJ, Dasgupta S, Vreede A, White B, Armstrong JD, Waddell S (2009) A neural circuit mechanism integrating motivational state with memory expression in Drosophila. Cell 139: 416 – 427.

Kwon JY, Dahanukar A, Weiss LA, Carlson JR (2014) A map of taste neuron projections in the Drosophila CNS. Journal of Biosciences 39: 565 – 574.

Lebreton S, Trona F, Borrero-Echeverry F, Bilz F, Grabe V, Becher PG, Carlsson MA, Nässel DR, Hansson BS, Sachse S, et al (2015) Feeding regulates sex pheromone attraction and courtship in Drosophila females. Science Reports 5: 13132

Lee G, Park JH (2004) Hemolymph sugar homeostasis and starvation-induced hyperactivity affected by genetic manipulations of the adipokinetic hormone-encoding gene in *Drosophila melanogaster*. Genetics 167: 311 – 323.

Lee T, Luo L (1999) Mosaic analysis with a repressible cell marker for studies of gene function in neuronal morphogenesis. Neuron 22: 451 – 461.

Lienhard MC, Stocker RF (1987) Sensory projection patterns of supernumerary legs and aristae in D. melanogaster. Journal of Experimental Zoology 244: 187 – 201.

Ling F, Dahanukar A, Weiss LA, Kwon JY, Carlson JR (2014) The molecular and cellular basis of taste coding in the legs of *Drosophila*. The Journal of Neuroscience 34(21): 7148 – 7164.

Liu C, Placais PY, Yamagata N, Pfeiffer BD, Aso Y, Friedrich AB, Siwanowicz I, Rubin GM, Preat T, Tanimoto H (2012) A subset of dopamine neurons signals reward for odour memory in Drosophila 488: 512 – 516.

Mank M, Griesbeck O (2008) Genetically encoded calcium indicators. Chemical Reviews 108: 1550 – 1564.

Mann K, Gordon MD, Scott K (2013) A pair of interneurons influences the choice between feeding and locomotion in *Drosophila*. Neuron 79: 754 – 765.

Manzo A, Silies M, Gohl DM, Scott K (2012) Motor neurons controlling fluid ingestion in Drosophila. Proceedings of the National Academy of Sciences 109: 6307 – 6312.

Marella S, Fischler W, Kong P, Asgarian S, Rueckert E, Scott K (2006) Imaging taste responses in the fly brain reveals a function map of taste category and behaviour. Neuron 49: 285 – 295.

Marella S, Mann K, Scott K (2012) Dopaminergic modulation of sucrose acceptance behaviour in *Drosophila*. Neuron 73: 941 – 950.

McCombs JE, Palmer AE (2008) Measuring calcium dynamics in living cells with genetically encodable calcium indicators. Methods 46: 152 – 159.

Meunier N, Marion-Poll F, Rospars JP, Tanimura T (2003) Peripheral coding of bitter taste in Drosophila. Journal of Neurobiology 56: 139 – 152.

Min S, Chae HS, Jang YH, Choi S, Lee S, Jeong YT, Jones WD, Moon SJ, Kim YJ, Chung J (2016) Identification of a peptidergic pathway critical to satiety responses in *Drosophila*. Current Biology 26: 1 – 7.

Mitchell BK, Itagaki H, Rivet MP (1999) Peripheral and central structures involved in insect gustation. Microscopy Research and Technique 47: 401 – 415.

Miyamoto T, Slone J, Song X, Amrein H (2012) A fructose receptor functions as a nutrient sensor in *Drosophila* brain. Cell 151(5): 1113 – 1125.

Miyamoto T, Wright G, Amrein H (2013) Nutrient sensors. Current Biology 23: R369 – R373.

Montell C (2009) A taste of the Drosophila gustatory receptors. Current Opinions in Neurobiology 19: 345 – 353.

Mueller KL, Hoon MA, Erlenbach I, Chandrashekar J, Zuker CS, Ryba NJP (2005) The receptors and coding logic for bitter taste. Nature 434: 225 – 229.

Nagai T, Sawano A, Park ES, Miyawaki A (2001) Circularly permuted green fluorescent proteins engineered to sense Ca^{2+}. Proceedings of the National Academy of Sciences 98: 3197 – 3202.

Nakagawa S (2004) A farewell to Bonferroni: the problems of low statistical power and publication bias. Behavioural Ecology 15: 1044 – 1045.

Nall A, Sehgal A (2014) Monoamines and sleep in Drosophila. Behavioural Neuroscience 128: 264 – 272.

Nayak SV, Singh RN (1983) Sensilla on the tarsal segments and the mouthparts of adult Drosophila melanogaster. International Journal of Insect Morphology and Embryology 12: 273 – 291.

Nelson G, Hoon MA, Chandrashekar J, Zhang Y, Ryba NJP, Zuker CS (2001) Mammalian sweet taste receptors. Cell 106: 381 – 390.

Nitabach MN, Wu Y Sheeba V, Lemon WC, Strumbos J, Zelensky PK, White BH, Holmes TC (2006) Electrical hyperexcitation of lateral ventral pacemaker neurons desynchronizes downstream circadian oscillators in the fly circadian circuit and induces multiple behavioural periods. Journal of Neuroscience 26: 479 – 489.

Oka Y, Butnaru M, von Buchholtz L, Ryba NJP, Zuker CS (2013) High salt recruits aversive taste pathways. Nature 494: 472 – 476.

Pfeiffer BD, Jenett A, Hammonds AS, Ngo TB, Misra S, Murphy C, Scully A, Carlson JW, Wan KH, Laverty TR, Mungall C, Svirskas R, Kadonaga JT, Doe CQ, Eisen MB, Celniker S, Rubin GM (2008) Tools for neuroanatomy and neurogenetics in *Drosophila*. Proceedings of the National Academy of Sciences 28: 9715 – 9720.

Pool AH, Kvello P, Mann K, Cheung SK, Gordon MD, Wang L, Scott K (2014) Four GABAergic interneurons impose feeding restraint in *Drosophila*. Neuron 83: 164 – 177.

Pool AH, Scott K (2014) Feeding regulation in *Drosophila*. Current Opinion in Neurobiology 0: 57 – 63.

Rajashekhar KP, Singh RN (1994) Neuroarchitecture of the tritocerebrum of *Drosophila melanogaster*. Journal of Comparative Neurology 349: 633 – 645.

Ribeiro C, Dickson BJ (2010) Sex peptide receptor and neuronal TOR/S6K signaling modulate nutrient balancing in Drosophila. Current Biology 20: 1000 – 1005.

Robb S, Cheek TR, Hannan FL, Hall LM, Midgley JM, Evans PD (1994) Agonist-specific coupling of a cloned Drosophila octopamine/tyramine receptor to multiple second messenger systems. EMBO Journal 13: 1325 – 1330.

Roberston HM, Warr CG, Carlson JR (2003) Molecular evolution of the insect chemoreceptor gene superfamily in *Drosophila melanogaster*. Proceedings of the National Academy of Sciences 100: 14537 – 14542.

Root CM, Ko KI, Jafari A, Wang JW (2011) Presynaptic facilitation by neuropeptide signaling mediates odor-driven food search. Cell 145(1): 133 – 144.

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: 661 – 673.

Shiraiwa T, Carlson JR (2009) Proboscis extension response (PER) assay in Drosophila. Journal of Visualized Experiments 3: 193.

Slone J, Daniels J, Amrein H (2007) Sugar receptors in Drosophila. Current Biology 17: 1809 – 1816.

Spector AC, Travers SP (2005) The representation of taste quality in the mammalian nervous system. Behavioural and Cognitive Neuroscience Reviews 4: 143 – 191.

Srivastava DP, Yu EJ, Kennedy K, Chatwin H, Reale V, Hamon M, Smith T, Evans PD (2005) Rapid, nongenomic responses to ecdysteroids and catecholamines mediated by a novel Drosophila G-protein-coupled receptor. Journal of Neuroscience 25: 6145 – 6155.

Stafford JW, Lynd KM, Jung AY, Gordon MD (2012) Integration of taste and calorie sensing in Drosophila. The Journal of Neuroscience 32: 14767 – 14774.

Steiner JE, Glaser D, Hawilo ME, Berridge KC (2001) Comparative expression of hedonic impact: affective reactions to taste by human infants and other primates. Neuroscience & Biobehavioural Reviews 25: 53 – 74.

Stocker RF (1994) The organization of the chemosensory system in Drosophila melanogaster: a review. Cell and Tissue Research 275: 3 – 26.

Storey JD (2002) A direct approach to false discovery rates. Journal of the Royal Statistical Society: Series B (Statistical Methodology) 64: 479 – 498.

Su C-Y, Wang JW (2014) Modulation of neural circuits: how stimulus context shapes innate behaviour in Drosophila. Current Opinion in Neurobiology 29: 9 – 16.

Thistle R, Cameron P, Ghorayshi A, Dennison L, Scott K (2012) Male-male repulsion and male-female attraction during *Drosophila* Courtship. Cell 149: 1140 – 1151.

Thorne N, Chromey C, Bray S, Amrein H (2004) Taste perception and coding in Drosophila. Current Biology 14: 1065 – 1079.

Tian L, Hires SA, Mao T, Huber D, Chiappe ME, Chalasani SH, Petreanu L, Akerboom J, McKinney SA, Schreiter ER, Bargmann CI, Jayaraman V, Svoboda K, Looger LL (2009) Imaging neural activity in worms, flies and mice with improved GCaMP calcium indicators. Nature Methods 6(12): 875 – 881.

Venken KJT, Simpson JH, Bellen HJ (2011) Genetic manipulation of genes and cells in the nervous system of the fruit fly. Neuron 72: 202 – 230.

Vosshall LB, Stocker RF (2007) Molecular architecture of smell and taste in Drosophila. Annual Review of Neuroscience 30: 505 – 533.

Wang Z, Singhvi A, Kong P, Scott K (2004) Taste representations in the Drosophila brain. Cell 117: 981 – 991.

Wasserman S, Salomon A, Frye MA (2013) Drosophila Tracks Carbon Dioxide in Flight. Current Biology 23: 301 – 306.

Weiss LA, Dahanukar A, Kwon JY, Banerjee D, Carlson JR (2011) The Molecular and Cellular Basis of Bitter Taste in Drosophila. Neuron 69: 258 – 272.

Whitaker M (2010) Genetically encoded probes for measurement of intracellular calcium. Methods in Cell Biology 99: 153 – 182.

Wisotsky Z, Medina A, Freeman E, Dahanukar A (2011) Evolutionary differences in food preference rely on Gr64e, a receptor for glycerol. Nature Neuroscience 1534 – 1541.

Yang Z, Yu Y, Zhang V, Tian Y, Qi W, Wang L (2015) Octopamine mediates starvation-induced hyperactivity in adult Drosophila. Proceedings of the National Academy of Sciences USA 112: 5219 – 5224.

Yapici N, Cohn R, Schusterreiter, Ruta V, Vosshall LB (2016) A taste circuit that regulates ingestion by integrating food and hunger signals. Cell 165: 1 – 15.

Yarmolinsky DA, Zuker CS, Ryba NJP (2009) Common sense about taste: from mammals to insects. Cell 139: 234 – 244.

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: 293 – 301.

Zhang YV, Ni J, Montell C (2013) The molecular basis for attractive salt taste coding in Drosophila. Science 340: 1334 – 1338.

Zhang YQ, Rodesch CK, Broadie K (2002) Living synaptic vesicle marker: Synaptotagmin-GFP. Genesis 34: 142 – 145.

Zhao GQ, Zhang Y, Hoon MA, Chandrashekar J, Erlenbach I, Ryba NJP, Zuker CS (2003) The receptors for mammalian sweet and umami taste. Cell 115: 255 – 266.

Zhou C, Rao Y, Rao Y (2008) A subset of octopaminergic neurons are important for *Drosophila* aggression. Nature Neuroscience 11: 1059 – 1067.