Abstract

The sense of taste plays a critical role in animal behaviour. The ability to taste prior to ingestion allows an animal to differentiate between beneficial substances, such as calorie-rich foods, from those that may be toxic and which often taste bitter. For animals to avoid harmful substances, exposure to bitter substances must not only activate bitter-sensing neurons, but also suppress the response of acceptance-mediating neurons. The ability to choose appropriately between consumption and avoidance requires functional neuronal circuits in the brain that start with the processing of taste input from the environment and end with a behavioural output.

γ-aminobutyric acid (GABA) is the primary inhibitory neurotransmitter in the insect and mammalian brain. We show evidence of functional connections between GABAergic interneurons (those that release GABA) and taste sensory neurons in the fly brain. Neurons that detect palatable substances (i.e. sugars) express high levels of a metabotropic GABA<sub>B</sub> receptor (GABA<sub>B</sub>R), whereas those that sense unpalatable substances (i.e. bitter compounds) express little to no GABA<sub>B</sub>R. Using a behavioural assay (proboscis extension reflex) and calcium imaging, I investigated how GABAergic inhibition shapes sweet neuron output and contributes to the integration of competitive taste stimuli. When GABA<sub>B</sub>R is knocked down in sugar neurons, the behavioural response to sugars is elevated. Pharmacological assays show that GABAergic activation suppresses the sweet neuron response while GABAergic blockade potentiates the response. In flies expressing GABA<sub>B</sub>R knockdown, suppression of sugar neuron activity by bitter
exposure is relieved both behaviourally, by increased acceptance of bitter mixtures, and cellulary, by increased calcium response. Our model proposes that GABA acts via GABA$_B$R in the fly taste circuit to produce ecologically relevant responses to both attractive and repellent energy sources.
Preface

This work was conducted at UBC’s Life Sciences Institute by Bonnie Chu and Dr. Michael Gordon. I performed all behavioural and calcium imaging experiments. As well, I conducted the immunohistochemical assays used to determine levels of GABA$_{B}$R expression and GRASP signal. I acquired and quantified data using Microsoft Excel and performed preliminary statistical analysis. The introductory figures in Chapter 1 (Figures 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7) were reproduced and adapted from Dr. Gordon’s CIHR grant, Figure 3.1 and Table 3.1 (from Chapter 3.1.1) were produced from data collected by Vincent Chui, and the immunolabelling of GABAergic neuron expression in Figure 3.3 (from Chapter 3.2) was performed by Dr. Gordon. All other figures were produced from experiments I conducted.

A version of Chapter 3 has been published in *Current Biology*. Bonnie Chu is the first author, followed by Vincent Chui and Kevin Mann, who are listed as second and third author respectively. Michael D. Gordon is the principal investigator. Vincent Chui performed the analysis of GABA$_{B}$R expression in taste neurons (Figure 3.1). Kevin Mann provided input that led to the successful implementation of pharmacology experiments (Figures 3.10 and 3.14). Michael D. Gordon designed and supervised the project, and wrote the manuscript with input from the co-authors.
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>3-APPA</td>
<td>3-aminopropylphosphonous acid</td>
</tr>
<tr>
<td>α</td>
<td>anti-</td>
</tr>
<tr>
<td>AHL</td>
<td>adult hemolymph-like</td>
</tr>
<tr>
<td>AP</td>
<td>action potential</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>AU</td>
<td>airy units</td>
</tr>
<tr>
<td>Ca^{2+}</td>
<td>calcium</td>
</tr>
<tr>
<td>ChR2</td>
<td>channelrhodopsin-2</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CO_{2}</td>
<td>carbon dioxide</td>
</tr>
<tr>
<td>cVA</td>
<td>11-cis vaccenyl acetate</td>
</tr>
<tr>
<td>DCSO</td>
<td>dorsal cibarial sense organ</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>GABA</td>
<td>γ-Aminobutyric acid</td>
</tr>
<tr>
<td>GABA_{A,R}</td>
<td>ionotropic γ-Aminobutyric acid (GABA) receptor</td>
</tr>
<tr>
<td>GABA_{B,R}</td>
<td>metabotropic γ-Aminobutyric acid (GABA) receptor</td>
</tr>
<tr>
<td>GAD1</td>
<td>glutamic acid decarboxylase</td>
</tr>
<tr>
<td>GECI</td>
<td>genetically-encoded calcium indicator</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>GPCR</td>
<td>G protein-coupled receptors</td>
</tr>
<tr>
<td>GR</td>
<td>gustatory receptor</td>
</tr>
<tr>
<td>GRASP</td>
<td>GFP reconstitution across synaptic partners</td>
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<td>GRN</td>
<td>gustatory receptor neurons</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>IR</td>
<td>ionotropic glutamate receptor</td>
</tr>
<tr>
<td>KCI</td>
<td>potassium chloride</td>
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<tr>
<td>K_{IR}</td>
<td>inwardly rectifying potassium channel</td>
</tr>
<tr>
<td>LH</td>
<td>lateral horn</td>
</tr>
<tr>
<td>LN</td>
<td>local interneuron</td>
</tr>
<tr>
<td>LSO</td>
<td>labral sense organ</td>
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<td>molar</td>
</tr>
<tr>
<td>MB</td>
<td>mushroom body</td>
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<td>Mg^{2+}</td>
<td>magnesium</td>
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<td>mM</td>
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<tr>
<td>mRNA</td>
<td>messenger RNA</td>
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<tr>
<td>NaChBac</td>
<td>bacterial sodium channel</td>
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<td>NaCl</td>
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<td>NaH_{2}PO_{4}</td>
<td>monosodium phosphate</td>
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<td>NaHCO_{3}</td>
<td>sodium bicarbonate</td>
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<tr>
<td>OBP</td>
<td>odorant-binding protein</td>
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<tr>
<td>OR</td>
<td>olfactory receptor</td>
</tr>
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<td>ORN</td>
<td>olfactory receptor neurons</td>
</tr>
<tr>
<td>OSN</td>
<td>olfactory sensory neurons</td>
</tr>
<tr>
<td>P_{2}X_{2}</td>
<td>purinergic receptor ligand-gated ion channel</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PER</td>
<td>proboscis extension reflex</td>
</tr>
<tr>
<td>PN</td>
<td>projection neuron</td>
</tr>
<tr>
<td>RNAi</td>
<td>ribonucleic acid (RNA) interference</td>
</tr>
<tr>
<td>s</td>
<td>seconds</td>
</tr>
<tr>
<td>SEZ</td>
<td>subesophageal zone</td>
</tr>
<tr>
<td>Shi&lt;sup&gt;ts1&lt;/sup&gt;</td>
<td>temperature-sensitive shibire</td>
</tr>
<tr>
<td>SNMP</td>
<td>Sensory Neuron Membrane Protein</td>
</tr>
<tr>
<td>TH</td>
<td>tyrosine hydroxylase</td>
</tr>
<tr>
<td>TNT</td>
<td>tetanus toxin light chain</td>
</tr>
<tr>
<td>TRiP</td>
<td>Transgenic RNAi Project</td>
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<tr>
<td>TRPM5</td>
<td>transient receptor potential cation channel subfamily M member 5</td>
</tr>
<tr>
<td>UAS</td>
<td>Upstream Activation Sequence</td>
</tr>
<tr>
<td>µM</td>
<td>micromolar</td>
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<tr>
<td>VCSO</td>
<td>ventral cibarial sense organ</td>
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Last but not least, thanks to Tony, for keeping me in one piece, most of the time.
1. Introduction

1.1 The Fly Gustatory System

Composed of just 100,000 neurons, the brain of the fruit fly *Drosophila melanogaster* presents a relatively simple and tractable system in which to study sensory processing. The display of complex and stereotyped feeding behaviours, along with the wealth of genetic tools available, make the fruit fly a model organism for the exploration of neural circuitry underlying taste. In order for animals to survive, they must use their sense of taste to guide them toward appropriate feeding decisions. To choose correctly between the ingestion or rejection of a food substance requires the coordinated effort of multiple components in the fly taste circuit, starting with the detection of gustatory cues by specialized sensory bristles and ending with consumption or avoidance behaviour executed by a motor program.

1.1.1 Anatomy of Gustatory Organs

Unlike vertebrates, flies walk directly on their food source, and have evolved to sense external stimuli through a number of taste organs distributed along the body (Figure 1.1) (Singh, 1997; Stocker, 1994). They are not only able to taste with the proboscis (the tube-like mouthpart used for feeding), but also with their legs and wings. At these positions along the body, taste is conveyed by specialized chemosensory bristles called sensilla, which may be thought of as analogous to mammalian taste buds (Stock, 1994). Female flies are further
adapted to evaluate taste quality by a specialized sensory organ, the vaginal plate, which is located at the tip of the abdomen and may contribute to selecting oviposition site (Stocker, 1994; Yang, Belawat, Hafen, Jan, & Jan, 2008). The distal end of the proboscis comprises of two labial palps that open to receive food, and each labellum houses 31 taste sensilla as well as a few simpler structures called taste pegs. The fly pharynx contains three bilaterally symmetric internal taste organs: the labral sense organ (LSO), the ventral cibarial sense organ (VCSO), and the dorsal cibarial sense organ (DCSO) (Gendre et al., 2004) (Figure 1.1). These internal mouthparts harbour additional taste sensilla, allowing the fly to evaluate food immediately following ingestion but before further digestion can take place. Flies also exhibit some sexual dimorphism with respect to number of sensilla. Males have 50 sensilla on their forelegs compared to 37 on females, most likely reflecting the importance for males to sense pheromones that dictate sexual behaviour (Bray & Amrein, 2003).

Types of taste sensilla can be further defined by length: long (l-type), intermediate (i-type) and short (s-type) (Hiroi, Marion-Poll, & Tanimura, 2002). Each sensillum is innervated by 2-4 gustatory receptor neurons (GRNs), one mechanosensory neuron, and several accessory cells (Figure 1.2). Each GRN is a bipolar cell with its cell body housed in the bristle shaft, where its dendrite extends to the bristle pore to make direct contact with the environment. Basally, each GRN sends its axon to the subesophageal zone (SEZ) in the brain. In general, there are roughly four GRNs per sensilla responsible for detecting one of four modalities: sweet, bitter, low concentrations of salt, and water (low
osmolarity) (Hiroi et al., 2002; Hiroi, Meunier, Marion-Poll, & Tanimura, 2004; Meunier, Marion-Poll, Rospars, & Tanimura, 2003).

Once a palatable (i.e. sweet) substance is encountered, the fly initiates a robust feeding behavior called the proboscis extension reflex (PER) (Dethier, 1976; Rajashekhar & Singh, 1994a). PER is a coordinated contraction of muscles driving the proboscis of the fly toward a food source. It consists of lifting the rostrum, followed by extension of the haustellum, and culminating in spreading of the labial palps (Figure 1.3). An extended proboscis will retract (and the above motor program inhibited) by the stimulation of sensory bristles with a repellent stimulus, such as a bitter compound (Rajashekhar & Singh, 1994b). This discrete behaviour is easy to observe, quickly scored by hand, and amenable to analysis.
Figure 1.1 Gustatory tissues in the adult fly.

*Drosophila* adults detect gustatory cues in the environment with specialized sensory bristles located on the proboscis, legs, wings, and internal mouthparts such as the labral and dorsal/ventral cibarial organs. (Adapted from Scott et al., 2001).

Figure 1.2 A typical taste bristle in the adult fly.

The bristle shaft is innervated by the dendrites of gustatory neurons and a mechanosensory neuron that are exposed to external cues via the bristle pore, allowing contact with the environment. Generally, each sensillum contains one sugar-sensing GRN and one bitter-sensing GRN. (Adapted from Stocker, 1994).
Figure 1.3 Appetitive behaviour in the adult fly.

(A) Diagram of adult fly head with proboscis extended. Segments of proboscis are labelled and arrows show direction prior to food consumption. When PER is initiated, the rostrum is lifted from the head, the haustellum extended, and the labial palps opened to begin ingestion. (B) Time series of behavioural assay for PER. Arrow points to sugar stimulus touched to taste sensory bristles present on the legs at 0 s. Times indicated are relative to stimulus presentation. (Adapted from Gordon and Scott, 2009).
1.1.2 Gustatory Receptors

Individual GRNs express receptors that specify ligand receptivity and define each GRN by the taste modality it detects. In the mammalian gustatory system, bitter, sweet, and umami sensation is mediated by heterodimeric G protein-coupled receptors, whereas sour and salt tastes are detected by ion channels (Chandrashekar, Hoon, Ryba, & Zuker, 2006; Yarmolinsky, Zuker, & Ryba, 2009). By looking for similar proteins in the fly, the gustatory receptor (GR) genes were first identified in 2000 from a *Drosophila* genome database that identified a putative family of seven-transmembrane domain proteins (Clyne, Warr, & Carlson, 2000). These proteins were mostly expressed in the labellum, the main gustatory organ (Scott et al., 2001). Analyses of sequence similarity and tissue expression in adults and larvae have further extended this candidate receptor family to 68 members, many of which share a common signature motif in the seventh transmembrane domain (Scott et al., 2001).

Mounting interest in the neural circuitry of fly feeding behaviour has led to a surge of studies dissecting the functional significance of GR genes. Specific GRs required for detection of distinct taste modalities have since been identified. Interestingly, the GRNs responsible for sweet sensation and those responsible for bitter sensation express non-overlapping subsets of GRs (Z. Wang, Singhvi, Kong, & Scott, 2004). For example, the receptor *Gr5a* is widely expressed in most sugar-responsive GRNs but is absent in bitter-responsive GRNs, and experiments confirmed that *Gr5a*-labelled neurons correspond to the sweet-sensing cell in taste sensilla (Thorne, Chromey, Bray, & Amrein, 2004; Z. Wang
et al., 2004). Gr5a encodes a narrowly tuned trehalose receptor; Gr5a mutants are severely defective in their response to trehalose but retain sensitivity to other sugars (Dahanukar, Foster, van der Goes van Naters, & Carlson, 2001). This prompted the search for additional sugar-activated GRs that must be co-expressed with Gr5a to respond to the repertoire of sugars a fly encounters in the wild. Indeed, GR genes more broadly tuned to sugars have since been identified, including the Gr64 gene cluster that encodes Gr64a-f (Jiao, Moon, & Montell, 2007). These 6 receptors are closely related to and co-expressed with Gr5a in some GRNs. Of these, Gr64a was found to be necessary for the sucrose, maltose, and glucose response, whereas Gr64f was required for the response to all sugars tested, except fructose (Dahanukar, Lei, Kwon, & Carlson, 2007; Jiao, Moon, Wang, Ren, & Montell, 2008). However, ectopic expression of Gr64f with Gr64a or Gr5a was incapable of reproducing a sugar response, indicating that the detection of sugar requires the combination of at least three GR subunits (Jiao et al., 2008).

Similarly, specific receptors for other taste modalities have been identified. Bitter compounds, which are aversive to flies, are recognized by Gr66a, which is expressed in a population of cells separate from those expressing Gr5a (Thorne et al., 2004; Z. Wang et al., 2004). Gr66a and Gr93a are necessary for the characteristic avoidance of caffeine, and a third receptor, Gr33a, is required for sensing a broad range of repulsive bitter compounds including caffeine, quinine, denatonium, and berberine (Lee, Moon, & Montell, 2009; Moon, Lee, Jiao, & Montell, 2009). However, as in sugar detection, misexpression of these receptors
in sweet GRNs does not confer a bitter response, implicating the requirement of at least four receptors in bitter detection (Lee et al., 2009). For salt sensing, a member of the ionotropic glutamate receptor (IR) family, *Ir76b*, was recently shown to function in the detection of appetitive low salt concentrations and mark a GRN population distinct from the sweet and bitter neurons (Y. V. Zhang, Ni, & Montell, 2013). When *Ir76b* function is lost, flies show an aversion to low salt instead of the expected attraction. This led to a proposed model of competition between GRNs to account for the bidirectional response to salt: at low concentrations, *Ir76b*-expressing neurons dominate over high salt-sensing neurons mediating rejection, and the reverse occurs at high salt concentrations, resulting in the acceptance or rejection of salt respectively.

Although the GRs discussed so far have been responsible for detecting tastants, recent characterization of GRs has led to surprising insights. GRs are not only limited to taste perception, but also serve to alert the fly of diverse environmental stimuli. Of particular interest is a pair of GR genes, *Gr21a* and *Gr63a*, which are expressed in fly olfactory receptor neurons (ORNs) and function together to detect CO$_2$ (Jones, Cayirlioglu, Kadow, & Vosshall, 2007; Kwon, Dahanukar, Weiss, & Carlson, 2007). This is noteworthy due to the presence of homologs of these receptors in the olfactory organ of the malaria vector mosquito, *Anopheles gambiae* (Jones et al., 2007). Because the mosquito uses CO$_2$ plumes emitted by mammals as a cue for host tracking (S. L. Turner et al., 2011), the manipulation of these receptors could have important societal consequences. Another interesting function of GRs lies with *Gr43a*, which was
recently identified in the brain as a nutrient sensor for levels of dietary fructose in the hemolymph (Miyamoto, Slone, Song, & Amrein, 2012). Intriguingly, GRs may also play a role in non-chemosensory contexts. *Gr28b(D)* was shown to act as a heat sensor expressed in the arista, a feathery sensory appendage on the fly antenna (Ni et al., 2013; Thorne & Amrein, 2008). When flies lack *Gr28b(D)* function, they are impaired in their avoidance of noxious heat. Light may be another stimulus in the array of stimuli recently found to be perceived by GRs: one of the *Gr28b* proteins expressed in the larval body wall acts to guide the animal away from noxious light (Xiang et al., 2010). Novel receptor genes are currently being uncovered and progress is continually being made in the identification and characterization of GRs responsible for detecting evolutionarily significant stimuli including, but not limited to, taste.

1.1.3 Adult Neural Circuitry of Taste

GRNs distributed on different body parts of the fly all send projections to the primary taste-processing centre of the brain, the subesophageal zone (SEZ) (Figure 1.4). GRNs expressing the same GR but located on different body parts project to non-overlapping locations in the SEZ, suggesting that the same tastant may elicit different behaviours depending on where the taste was encountered (Thorne et al., 2004; Z. Wang et al., 2004). GRNs located in the internal mouthparts project to the anterior dorsal SEZ and proboscis GRNs send axons to the central SEZ, while leg GRNs target the posterior SEZ.
There is also spatial segregation in the projection patterns between GRNs responsive for different taste modalities. Sugar-responsive GRNs project to an area of the SEZ that is spatially distinct from the projections of bitter-responsive GRNs (Figure 1.5) (Thorne et al., 2004; Z. Wang et al., 2004). This presents evidence of two functionally distinct circuits for two opposing modalities: one that detects sugar and mediates downstream acceptance behaviour and a second that detects bitter compounds and mediates rejection. The projections of water GRNs, which partially overlap with sugar GRN projections, offer further support for this circuit organization since water stimulation also initiates feeding (Inoshita & Tanimura, 2006).

The spatial segregation between sugar and bitter neuron projections in the SEZ and resulting opposing outcomes suggest GRNs very likely synapse with gustatory interneurons that regulate responses. Very little has been deciphered regarding higher-order neurons linking sensory GRNs to a behavioural output. A recently identified pair of “feeding neurons” was shown to induce the complete feeding program when thermo-activated and disrupt feeding when laser ablated, but these neurons were not demonstrated to synapse with any known taste neurons (Flood et al., 2013). Thus far, the only higher-order component identified to function as part of the primary taste circuit is a pair of E49 motor neurons shown to be both necessary and sufficient for a specific subprogram of PER (Figure 1.4) (Gordon & Scott, 2009). Flies with a silenced pair of E49 motor neurons display an unusual, and specific, defect in feeding: they are unable to lift the rostrum from the head, although the other components of the PER program
are intact and function independently. However, E49 motor neurons do not appear to synapse with primary sensory neurons, thus the circuitry bridging these two populations is under investigation.
Figure 1.4 Simplified scheme of the fly gustatory circuit.

Known neurons in the taste circuit are labelled. Tastants bind to gustatory receptor neurons (GRNs) expressed in taste organs and are shown as different colours to represent different modalities sensed (only two distinct classes are shown, but at least four classes exist). GRNs project axons to the primary relay of gustatory processing, the subesophageal zone (SEZ). Unidentified second-order neurons (teal) and GABAergic interneurons (red) are also present in the SEZ and may contact GRN axon terminals. The E49 motor neuron (magenta) is necessary and sufficient for the appetitive response.
Figure 1.5 Segregation of sweet and bitter taste projections.

Binary reporter expression assay using Gal4/UAS and LexA/LexAop showing sweet and bitter GRN projections. (A) Sweet neurons are labelled by Gr5a-LexA to drive expression of LexAop-GFP (green). (B) Bitter neurons are labelled by Gr66a-Gal4 to drive expression of UAS-dsRed (magenta). (C) Merged image shows spatial segregation of sweet and bitter GRNs in the SEZ. Sweet GRN projections (green) are anterior to bitter neuron projections while bitter neuron projections (magenta) lie in a triangular web in the medial SEZ.
1.2 The Fly Olfactory System

The senses of smell and taste are intricately linked and both are indispensable to our perception of the chemical environment. Smell and taste pathways interact in higher brain centres to guide us toward feeding and social behaviours. The fly olfactory system offers a valuable comparison to taste and exhibits convenient parallels to the taste system. Olfactory receptor neurons (ORNs) are housed in various olfactory organs of the fly, primarily the antennae, and ORNs express olfactory receptors (ORs) (Vosshall, Amrein, Morozov, Rzhetsky, & Axel, 1999). The OR genes exhibit some sequence homology to the GR genes and circuit organization between the two systems are similar (Scott et al., 2001), making fly olfaction a logical starting point from which to explore taste.

1.2.1 Anatomy of Olfactory Organs

The olfactory organs of the fly are highly specialized and morphologically distinct from the mammalian nose. Flies are able to detect odours via two main structures: the pair of antenna situated on the head and the maxillary palps on the proboscis. Similar to gustation, the ORNs are housed in sensory bristles called sensilla, which decorate the olfactory organs and protect the ORNs from environmental damage (Couto, Alenius, & Dickson, 2005). There are three types of antennal sensilla (trichoid, basiconic, and coeloconic) and these types differ in size, morphology, and the odorants detected (Vosshall & Stocker, 2007). ORNs in the trichoid sensilla are activated by pheromones and are generally unresponsive to other environmental odorants (van der Goes van Naters &
Carlson, 2007). In contrast, basiconic ORNs respond to general odorants, including the stress odorant CO$_2$, but not to pheromones (Hallem, Dahanukar, & Carlson, 2006). Coeloconic ORNs, which use a distinct set of olfactory receptors, also respond to general odorants, namely amines and carboxylic acids, which represent floral and fruity compounds (Yao, Ignell, & Carlson, 2005). There is also evidence of sexual dimorphism in the number and type of olfactory sensilla expressed, which likely reflect competing evolutionary drives. Males possess slightly more pheromone-responsive trichoid sensilla than females, while females have more food-sensing basiconic sensilla than males (Stocker, 1994).

Fly ORNs, like fly GRNs, are bipolar cells. From the apical side of an ORN, a sensory dendrite extends into the sensillum shaft to interact with odorants while basally, a single axon projects to an olfactory glomerulus in the antennal lobe. Each sensillum holds 1-4 ORNs surrounded by support cells that secrete sensillum lymph and provide electrical insulation from neighbouring ORNs (Vosshall & Stocker, 2007). There are between 1,100-1,250 ORNs on each antenna, while the maxillary palps are simpler. Each palp contains only about 60 sensilla, all of which are basiconic, and each of these contains just 2 ORNs (Stocker, 2001). ORNs are further organized into about 50 distinct types, which correspond to the 50-60 OR genes expressed in the olfactory organs (Couto et al., 2005). Interestingly, ORNs from the maxillary palp project through the SEZ to terminate in the antennal lobe, possibly indicating a role for this organ in taste processing. Indeed, in the blowfly *Phormia regina*, some maxillary ORNs project to the SEZ where they overlap with labellar GRNs and establish synapses
(Maeda et al., 2014), strongly supporting an interaction between these two chemosensory inputs.

1.2.2 Olfactory Receptors

In general, most ORNs express a specific OR along with a common co-receptor Or83b (Larsson et al., 2004). An exception is the ORNs in the antenna responsible for CO₂ detection, which express GRs instead of the conventional ORs (Jones et al., 2007; Kwon et al., 2007). As well, there are ORNs in the coeloconic sensilla that express ionotropic receptors from an entirely different family of receptors (Liang & Luo, 2010). These ionotropic receptors are related to ionotropic glutamate receptors and detect volatile amines, carboxylic acids, and a few food odours. ORs and GRs are members of the same chemosensory gene family identified in 2000 and are predicted to be seven transmembrane G protein-coupled receptors (Scott et al., 2001). The gating properties of these proteins remain inconclusive, although recent evidence suggests that ORs are ligand-gated ion channels rather than metabotropic receptors (Sato et al., 2008; Wicher et al., 2008).

The ligand specificity for most of the ORs has been characterized by Carlson and colleagues using the in vivo “decoder neuron” system, in which a mutant ORN (Δab3A) lacking its native receptor genes acts as a “decoder” to express an OR of interest (Dobritsa, van der Goes van Naters, Warr, Steinbrecht, & Carlson, 2003). Because ORN tuning is entirely defined by the receptor(s) expressed, the electrophysiological responses to a panel of 10-100 pure and
natural odorants can then be directly compared to reveal the receptive properties of each OR and its cognate ORNs. This global investigation of odour coding, which included antennal (Hallem & Carlson, 2006; Hallem, Ho, & Carlson, 2004), maxillary palp (de Bruyne, Foster, & Carlson, 2001), and larval receptors (Kreher, Kwon, & Carlson, 2005), revealed several principles about OR response: a receptor can mediate either excitatory or inhibitory responses, different odorants can excite many or only a few receptors, and different ORs can respond to many odorants or just a select few. The ORNs and ORs of the olfactory system have thus been thoroughly characterized.

One of the most well-studied OR is Or67d, which selectively responds to 11-cis vaccenyl acetate (cVA) (Ha & Smith, 2006). cVA is a volatile pheromone exclusively secreted by male flies, but plays a critical role in modulating the behaviour of both sexes: it represses courtship between males while inducing receptivity in females (Kurtovic, Widmer, & Dickson, 2007). During mating, cVA can also transfer from males to females, making previously-mated females less appealing to other males. Studies of the activation of Or67d have led to some intriguing insights into the mechanisms of odour detection. Multiple counterparts in addition to Or67d are necessary for its activation by cVA, including the obligate co-receptor Or83b, a small two-transmembrane domain protein called Sensory Neuron Membrane Protein (SNMP) (Benton, Vannice, & Vosshall, 2007; Jin, Ha, & Smith, 2008), and a secreted member of the olfactory binding protein (OBP) superfamily OBP76a (or LUSH) (Ha & Smith, 2006; Xu, Atkinson, Jones, & Smith, 2005). It has been postulated that the ligand for Or67d may not be the
pheromone at all, but LUSH itself, which undergoes a conformational change upon binding cVA. However, this has recently been refuted in a study comparing unbound LUSH, cVA-bound LUSH, and LUSH bound to non-pheromone ligands that showed no difference between the structures that could account for the proposed activated state of cVA-bound LUSH (Gomez-Diaz, Reina, Cambillau, & Benton, 2013). The recognition of pheromonal signals by receptors remains to be conclusively determined, but clearly, odorant detection can occur by more complicated means than the simple binding of odorant to OR.

1.2.3 Adult Neural Circuitry of Olfaction

Different odorants can excite a few or many receptors, and receptors can be broadly or sparsely tuned to different odorants (Hallem & Carlson, 2006). The olfactory processing circuit has been under heavy investigation and many more of its components have been identified and characterized than of the taste system. However, higher-order levels of processing remain incompletely understood.

ORNs that express the same OR project their axons to the antennal lobe, the insect analog of the mammalian olfactory bulb. The antennal lobe is organized into glomeruli, and ORNs expressing the same OR converge onto the same glomerulus (Gao, Yuan, & Chess, 2000; Vosshall, Wong, & Axel, 2000) (Figure 1.6). Most ORs have now been mapped to their corresponding glomeruli in the brain (Couto et al., 2005; Fishilevich & Vosshall, 2005). There are 43 glomeruli in each of two antennal lobes (one per hemisphere) (Laissue et al.,
1999), and within these sites ORNs contact both local interneurons (LN)s as well as projection neurons (PN)s, the second-order olfactory neurons (Liang & Luo, 2010) (Figure 1.6). PN$s are predominantly cholinergic; they release acetylcholine from their axons to higher brain regions and from dendrites to their respective ORNs (Kazama & Wilson, 2009; Ng et al., 2002). PN$s excite sister PN$s, as well as LN$s and ORNs in the same glomeruli, allowing reciprocal interactions between these three neuron types. Each PN synapses with a single glomerulus and each glomerulus contains the dendrites of several sister PN$s that show highly correlated patterns of activity (Kazama & Wilson, 2009; Tanaka, Awasaki, Shimada, & Ito, 2004). Since each antennal lobe encompasses ~150 PN$s, the average glomerulus provides input to ~3 PN$s.

Although PN$s receive the majority of their excitation from cognate ORNs, minimal resemblance has been observed between the odour responses of ORNs and their postsynaptic PN$s. Several important differences have been revealed thus far in the representation of odours from ORNs to PN$s: importantly, PN$s show less variability in their responses to the same stimulus compared to ORNs (Bhandawat, Olsen, Gouwens, Schlief, & Wilson, 2007), and PN$s are generally more broadly tuned than their ORN partners (Olsen & Wilson, 2008; Wilson, Turner, & Laurent, 2004). The reduced variability is likely due to high convergence of ORN inputs onto PN$s. PN$s contact all the ORN axons that reside in its cognate glomerulus, resulting in the summation of multiple ORN inputs and improved reliability (Kazama & Wilson, 2009). Using electrophysiology, the transformation of the odour code from ORN to PN has been compared for
different OR classes. In one study, two narrowly tuned ORs and their corresponding PN responses were examined: *Or67d*, the receptor for mating pheromone cVA, and *Or82a*, the receptor for the fruit odorant geranyl acetate (Schlief & Wilson, 2007). The PNs in the pheromone-sensing glomerulus were as narrowly tuned as their cognate ORNs whereas the PNs postsynaptic to the fruit-sensing ORNs were much more broadly tuned. This suggests a difference in processing logic between these two channels (Schlief & Wilson, 2007) and the existence of modulatory mechanisms between different odorant classes at the glomerular level.

The axons of PNs terminate at the mushroom body (MB) and lateral horn (LH) (Jefferis et al., 2007). Many studies have implicated the MB in learning and memory (Heisenberg, 2003; Keene & Waddell, 2007), and each MB neuron receives input from about 10 PNs in the MB calyx (G. C. Turner, Bazhenov, & Laurent, 2008). Contrary to prior relays in the olfactory pathway, the MB neuron responses to odours are highly selective and sparse. This sparse coding may serve to decrease overlap between odour representations, thus allowing flies to more finely discriminate various odorants (Lin, Bygrave, de Calignon, Lee, & Miesenbock, 2014). Recording from MB neurons shows a lack of stereotypy between flies (Murthy, Fiete, & Laurent, 2008), presenting further evidence for the role of the MB in learning and memory by encoding an individual animal’s experience to a particular odour.

LH neurons that receive input from PNs are believed to be associated with innate behaviours in the fly, in part because abolishing MB neurons disrupts
olfactory learning but leaves innate olfactory behaviours intact (de Belle & Heisenberg, 1994; Kido & Ito, 2002). The LH also appears to support labelled lines for pheromone processing (Ruta et al., 2010). LH neurons that connect to fruit odour-responsive PNs project to a different part of the brain than those connected to pheromone-responsive PNs (Jefferis et al., 2007). At even higher-order levels of processing past the LH and MB, the neural network becomes increasingly complex and the flow of information unclear. However, there has been one simple neural circuit mapped, consisting of 4 neurons connected by 3 synapses, starting from the activation of cVA-responsive sensory neurons to the excitation of motor neurons in the ventral nerve cord (Ruta et al., 2010). The identification of this minimal neural circuit is a testament to the numerical simplicity of the fly brain and the powerful tools available for its manipulation. Future studies will eventually uncover more pathways by which olfactory information is transformed into motor outputs.
Figure 1.6 Simplified scheme of the fly olfactory circuit.

Known neurons in the olfactory circuit are labelled. Odorants bind to olfactory receptor neurons (ORNs) expressed in the antenna and maxillary palp and are shown as different shades of blue to represent different receptors expressed. In the primary relay of olfactory processing in the antennal lobe, gray circles represent glomeruli that receive axons from ORNs expressing the same receptor. Projection neurons (PNs; different shades of green) and local neurons (LNs; red) also contact ORN axon terminals in glomeruli and can be excitatory or inhibitory. PNs send processes to the mushroom body and the lateral horn for higher-order processing.
1.2.4 GABAergic Inhibition in the Antennal Lobe

LN innervation is global within the antennal lobe: most individual LNs project to most or all glomeruli, although some LNs exist that contact relatively few glomeruli (Chou et al., 2010; Wilson & Laurent, 2005). Most LNs are also broadly responsive to odours (Chou et al., 2010). The degree of complexity and variation in LN interactions and physiological properties support a model of inhibitory networks linking glomeruli and modulating olfactory output.

LNs in the antennal lobe lack axons and provide interglomerular inhibition or excitation by releasing neurotransmitters from their dendrites onto their targets. Although the majority of LNs are GABAergic, some are cholinergic and excitatory (Shang, Claridge-Chang, Sjulson, Pypaert, & Miesenbock, 2007), while others are glutamatergic (Chou et al., 2010). By releasing GABA, LNs are spatially poised to exert lateral inhibition and further regulate synaptic transmission between ORNs and PNs in the glomerulus. As well, the spatial pattern of GABA release from inhibitory LNs depends on the stimulus (Ng et al., 2002), offering support for a model in which ORN input to a specific subset of glomeruli could recruit lateral inhibition by LNs to other subsets.

ORN classes are heterogeneous in their expression of GABA receptors, namely of the slow metabotropic GABA$_B$ receptor (Root et al., 2008), suggesting that glomeruli receive non-uniform levels of GABAergic inhibition. Indeed, two studies have identified presynaptic inhibition of ORNs by GABA as a mechanism of gain control for some neuron classes but not others (Olsen & Wilson, 2008; Root et al., 2008). CO$_2$-responsive ORNs, which mediate aversion to the stress
odour CO₂, do not express GABA$_B$ receptor and are not subject to GABAergic inhibition. Interestingly, pheromone-responsive ORNs exhibit high levels of GABA$_B$ receptor at synapses with GABAergic LNs, and GABAergic inhibition significantly blunts the response of these ORNs, resulting in a wider dynamic range of these neurons. This channel-specific processing reflects the evolutionary significance of these two odour modalities: pheromones are best detected over a wide concentration range, while it is advantageous to avoid CO₂ at even trace amounts in the environment.

Although the functional importance of presynaptic inhibition at ORN axons is the best characterized, ORNs are not the sole targets of GABAergic inhibition by LNs. PNs, and even LNs themselves, are hyperpolarized by iontophoretic GABA (Wilson & Laurent, 2005). Lateral inhibition clearly plays a critical role in the response to different odorants with very different behavioural demands. How inhibition and excitation interplay to transform olfactory information from ORNs to PNs remains to be fully appreciated.

### 1.3 Structure and Function of GABA Receptors

As the main inhibitory neurotransmitter in the insect and mammalian brain, γ-aminobutyric acid (GABA) acts by hyperpolarizing neurons and decreasing neuronal excitability. This is accomplished through GABA binding to two general classes of GABA receptors. The first is GABA$_A$R, the fast ionotropic receptor, that allows the flow of negatively-charged chloride ions across the cell
membrane. The second is GABA\(_B\)R, the slow metabotropic receptor (Manev & Dzitoyeva, 2010) belonging to the family of G protein-coupled receptors.

The presynaptic activation of metabotropic GABA\(_B\)R inhibits voltage-dependent Ca\(^{2+}\) channels via the βγ G protein complex, whereas postsynaptic GABA\(_B\)R activation hyperpolarizes the membrane by activating inwardly rectifying potassium channels (K\(_{\text{IRS}}\)) (Mezler, Muller, & Raming, 2001). In the mammalian olfactory bulb, GABA\(_B\)R presynaptically regulates primary olfactory sensory neurons (OSNs) (Wachowiak et al., 2005), and this feedback inhibition by GABA is proposed to mediate habituation during repeated sniffing and filtration of new olfactory information from old input (McGann, 2013). It was believed that *Drosophila* did not express GABA\(_B\)R because baclofen, the classical agonist for GABA\(_B\)R, did not produce any biochemical effects in insects. However, this was disproved by the cloning of three GABA\(_B\)R units isolated from *Drosophila* (Mezler et al., 2001). Two of these, GABA\(_B\)R1 and GABA\(_B\)R2, are both required to constitute a functional receptor and assemble as heterodimers (Mezler et al., 2001), while the third, GABA\(_B\)R3, overlaps with the other two subunits but exhibits a distinct expression pattern (Okada, Awasaki, & Ito, 2009). Since the characterization of GABA\(_B\)Rs in *Drosophila*, selective ligands have been designed and used to investigate the behavioural mechanisms of alcohol, addiction, and pain (Dimitrijevic et al., 2005; Dzitoyeva, Dimitrijevic, & Manev, 2003; Manev & Dimitrijevic, 2004). In *Drosophila* chemosensation, both GABA\(_A\)R and GABA\(_B\)R are expressed in the antennal lobe where they modulate olfactory processing (Wilson & Laurent, 2005).
1.4 Interaction Between Gustatory Cues in Animals

In the wild, animals encounter an array of competing sensory cues and must decide which is the most salient before acting accordingly. In the search for food, aversive chemicals, which signal toxicity, not only initiate avoidance behaviour but also inhibit the response of attraction-mediating cells. This relationship between opposing gustatory stimuli (termed mixture suppression) has been observed across the animal kingdom (Glendinning, 2007). In hamsters, the response to sucrose is inhibited by quinine, a bitter compound (Formaker, MacKinnon, Hettinger, & Frank, 1997), while in catfish, the appetitive gustatory response to several amino acids is suppressed by the addition of quinine (Ogawa, Marui, & Caprio, 1997). Flies avoid quinine (Tompkins, Cardosa, White, & Sanders, 1979) as well as a diverse array of other bitter compounds, including berberine, caffeine, lobeline, nicotine, and denatonium (Meunier et al., 2003).

In fly gustation, sweet GRNs are directly inhibited by bitter compounds (Jeong et al., 2013; Meunier et al., 2003). Suppression of the sweet response occurs even in sensilla lacking a bitter-sensitive neuron (Meunier et al., 2003), so bitter compounds themselves must interact with sweet GRNs independent of circuitry. This mechanism was recently shown to require the odorant-binding protein Obp49a, which is secreted from accessory cells that insulate sweet GRNs (Jeong et al., 2013). Obp49a is released in response to bitter stimulation and attenuates the sweet neuron response. It is unclear whether Obp49a exerts its action by bringing bitter chemicals to sweet GRNs and changing their affinity
state from high to low, or if Obp49a undergoes a conformation change itself to inhibit sweet GRNs.

Despite identification of *Obp49a* as a cell-autonomous mechanism for mixture suppression in the fly, multiple lines of evidence suggest the existence of an additional mechanism involving downstream taste circuits. Artificial activation of bitter neurons alone in the absence of any bitter compound is sufficient to blunt the appetitive response to sugars (Hiroi, Tanimura, & Marion-Poll, 2008; Marella et al., 2006). Further, appetitive behaviour to sugar stimulation on one leg is suppressed by bitter stimulation on the contralateral leg (Meunier et al., 2003). As well, although mutants lacking *OBP49a* displayed significantly decreased suppression of the sugar response by bitter compounds, some inhibition remained at higher concentrations of bitter tastants (Jeong et al., 2013). These studies implicate the role of a neural-circuit based mechanism for mixture suppression in fly taste.

Support for a model of mixture suppression mediated by inhibitory circuitry is present in fly olfaction. Glomeruli are linked by inhibitory networks of LNs, and the odour responses of a PN can be suppressed by recruiting the activity of additional glomeruli (Wilson, 2013). The addition of new odours to an odour mixture typically produces either direct suppression, or a response smaller than the sum of the responses generated by each odour alone (i.e. sublinear summation) (Olsen, Bhandawat, & Wilson, 2010; Silbering & Galizia, 2007). Importantly, these mixture effects can be blocked by a combination of GABA<sub>A</sub> and GABA<sub>B</sub> receptor antagonists. Different glomeruli can be linked to opposing
behavioural outcomes (Semmelhack & Wang, 2009), so a mechanism for mixture suppression involving the activation of select glomeruli would have important behavioural consequences. It has been shown that one class of ORN is responsible for the majority of the attraction response to an odorant, while the recruitment of an additional class is necessary and sufficient to switch behaviour from attraction to repulsion (Semmelhack & Wang, 2009). This model indicates that two opposing behavioural outcomes are mediated by distinct processing channels. If applicable to mixtures, this duality of processing would support a role for antennal lobe GABAergic LNs in transforming the ORN code in response to ecologically-relevant concentrations of odorants or mixtures.

Mixture suppression also occurs at both the receptor and circuit levels in mammalian taste coding. Sugars activate GPCRs, which ultimately activate the TRPM5 channel (Y. Zhang et al., 2003). Stimulating the tongue with quinine dramatically inhibits TRPM5, suppressing the cellular response to sugars (Talavera et al., 2008). Moreover, mixtures of attractive and aversive stimuli often result in responses of gustatory cortex neurons that are similar to or suppressed compared to the response to the most effective component of the mixture alone (Maier & Katz, 2013). Despite ample evidence for mixture suppression in animals, no neural-circuit based mechanism has yet been identified.
1.5 Comparison Between Insect and Mammalian Chemosensation

Despite hundreds of millions of years of evolution and specialization into dramatically different ecological niches, flies and mice display striking similarities in the fundamental principles of their respective chemosensory systems.

Mammals detect smells via OSNs expressed in sensory hairs located in the nasal cavity (Su, Menuz, & Carlson, 2009), which is analogous to the expression of ORNs in sensilla on the fly antennae. In both cases, ORNs express one or a few ORs, and ORNs expressing the same OR converge on the same glomerulus in the antennal lobe (in flies) or olfactory bulb (in mammals) (Strausfeld & Reisenman, 2009; Zou, Chesler, & Firestein, 2009). In glomeruli, projection neurons (called mitral and tufted cells in mammalian olfaction) and local interneurons also synapse with ORNs in both flies and mammals (Vassar et al., 1994; Vosshall et al., 2000). Higher-order olfactory processing appears to be mirrored in both systems. Odour information is encoded by the pattern of activation in glomeruli in both species. As well, odour representations in higher brain centres, such as the mushroom body in flies and the piriform cortex in mammals, are sparsely encoded (Wilson & Mainen, 2006). This comparable logic in glomerular organization and consequently similar processing at the primary olfactory relay establishes the fly as a model for understanding sensory processing in mammals.

Mammals detect sweet, bitter, salty, sour, and umami, and the behavioural relevance of these modalities is shared with flies. In both phyla, sweet taste is attractive, and activation of taste cells responsible for sweet detection is sufficient
to induce appetitive behaviour (Gordon & Scott, 2009; Marella et al., 2006; Zhao et al., 2003). Similarly, both mice and flies avoid bitter tastants, and activation of bitter-responsive cells elicits avoidance behaviour (Dethier, 1976; Marella et al., 2006; Yarmolinsky et al., 2009). Consistent with a common drive to avoid potential toxins, both flies and mice express more taste receptors dedicated to detecting bitter tastants than sweet ones, and bitter cells express many more receptors than sweet cells (Vosshall & Stocker, 2007; Yarmolinsky et al., 2009). This allows both flies and mice to detect and respond to a wide repertoire of bitter compounds, but potentially at the expense of the ability to discriminate between them.

Parallels between fly and mammalian gustation exist not only at the periphery, but also in the taste-processing centre of the brain. In both the SEZ of flies and primary gustatory cortex of mice, there is spatial segregation of taste information (Chen, Gabitto, Peng, Ryba, & Zuker, 2011; Z. Wang et al., 2004). Different taste modalities are represented in stereotyped, non-overlapping regions of the brain, supporting a labelled line model in both organisms.

The mammalian taste bud, which functions analogously to the fly sensillum, is also functionally organized in a way that provides possible mechanisms for mixture suppression. Contrary to popular belief, there is no “tongue map” in which taste modality is segregated on the tongue. Rather, every taste bud contains all types of taste receptor cells, and this may allow for intra-taste bud regulation (Yarmolinsky et al., 2009). Interestingly, sour-sensing mammalian taste cells release serotonin and GABA in an activity-dependent
manner (Cao, Zhao, Kolli, Hivley, & Herness, 2009; Huang et al., 2005). This release could potentially modulate the activity of neighbouring taste cells upon exposure to taste mixes containing both attractive and repulsive substances.

Although functionally alike, the taste receptor cells of flies and mammals are anatomically different. While *bona fide* neurons detect taste information at the periphery in flies, taste cells in mammals are specialized epithelial cells that transmit gustatory signals to afferent nerve fibres (Yarmolinsky et al., 2009). As well, the receptor gene families expressed in the two phyla are non-homologous (Benton, Sachse, Michnick, & Vosshall, 2006). However, like GRNs in flies, each taste receptor cell in mammals is selectively tuned to a taste modality and mediates a hardwired response (Marella et al., 2006; Thorne et al., 2004; Y. Zhang et al., 2003). These fundamental similarities, along with the genetic toolbox available in the fly, permit the mapping of neural circuitry and understanding of chemosensory processing that is otherwise highly limited in mammalian brain studies.

### 1.6 Technical Details

A number of technical strategies were employed to investigate the role of GABA in modulating GRN response. The Gal4/UAS system is specific to *Drosophila* genetics, and GCaMP imaging is broadly used but has been adapted for use in flies.
1.6.1 Gal4/UAS System

The development of the Gal4/UAS system in flies profoundly advanced *Drosophila* genetics and allowed for unprecedented precision in the study of behaviour, neuroscience, and development. This system is comprised of two transgenic fly lines: first, the yeast transcription factor Gal4 that is expressed under the control of an enhancer, which labels a subset of cells; second, the UAS (or upstream activating sequence), which acts as the binding site for Gal4, is cloned upstream of an effector gene of interest (Brand & Perrimon, 1993; White & Peabody, 2009) (Figure 1.7). Any transgene of interest can be coupled to UAS, allowing for protein expression under control of Gal4. In the study of neuronal function, these transgenes can manipulate specific cell populations by exciting or suppressing neuronal activity.

UAS-driven suppressors can be constitutive, such as *UAS-TNT* (tetanus toxin light chain) (Mochida et al., 1990), or conditional, such as *UAS-Shi\textsuperscript{ts1}* which encodes a temperature-sensitive mutant of *shibire* and blocks synaptic transmission by inhibiting synaptic vesicle recycling (Kitamoto, 2001). *UAS-Shi\textsuperscript{ts1}* can be induced by a simple temperature shift, allowing the experimenter temporal control over when silencing occurs. Similarly, there are constitutive activators (e.g. NaChBac) as well as conditional ones (e.g. ChR2, P\textsubscript{2}X\textsubscript{2}) that are activated under experimental control, depolarizing relevant neurons (White & Peabody, 2009). Further, spatial control can be exerted by expression of Gal80, a repressor of Gal4-mediated transcription. UAS expression can be made even more specific with Gal80\textsuperscript{ts}, the temperature-sensitive form of Gal80, which
represses Gal4 activity at lower temperatures but not at higher ones (McGuire, Mao, & Davis, 2004).

In addition to targeted gene expression, the Gal4/UAS system has been adapted to express RNAis to knock down proteins of interest. A 300-400 base pair inverted repeat to a sequence of interest is cloned downstream of UAS, which when expressed under the control of a Gal4 line, results in siRNAs that drive degradation of target mRNAs in specific cells or tissues (Ahlquist, 2002). The success of Gal4/UAS-driven gene expression led to the generation of a second independent binary expression system for Drosophila, the LexA/LexAop system. LexA binds to and activates the LexA operator (LexAop), in the same way Gal4 activates UAS reporter expression, allowing two in vivo manipulations of gene expression simultaneously (Lai & Lee, 2006). The LexA/LexAop system can be used alone or with Gal4/UAS, and is ideal for high levels of expression since the LexA domain harbours one of the strongest DNA-binding domains known (del Valle Rodriguez, Didiano, & Desplan, 2012). Thus, Gal4/UAS and LexA/LexAop constitute a powerful dual binary expression system that can be adapted to express activators, silencers, reporters, and more, in specific cellular subsets and with pre-determined spatiotemporal parameters.
A genomic enhancer lies upstream of a transposable element carrying the transcription factor Gal4, causing Gal4 expression in specific cells. The transcribed Gal4 protein binds to a UAS line and drives expression of downstream effector/reporter genes in the same fly. Gal4 activity is blocked by co-expression of Gal80, which labels another subset of specific cells and can be temperature-sensitive. Thus, precise spatiotemporal control can be exerted.
1.6.2 GCaMP Imaging

Changes in intracellular Ca\(^{2+}\) concentration are widely used as correlates for neuronal activity, since membrane depolarizations typically lead to Ca\(^{2+}\) influx through voltage-gated Ca\(^{2+}\) channels (Berridge, 1998). As well, neurotransmitter release and synaptic input at dendrites are both associated with Ca\(^{2+}\) influx (Berridge, 1998). Many genetically-encoded Ca\(^{2+}\) indicators (GECIs), including GCaMP, have been developed that provide advantages over electrophysiology and synthetic calcium indicators (Miyawaki et al., 1997). In *Drosophila*, GECIs can easily and non-invasively be targeted to specific cell-types using the Gal4/UAS system and allow the visualization of many neurons simultaneously.

All GECIs are modified fluorescent proteins derived from green fluorescent protein (GFP) or its variants. In the case of GCaMP, a GFP molecule is circularly permuted such that the C- and N- termini have been fused to produce a new terminus in the middle of the protein (Nakai, Ohkura, & Imoto, 2001). The new terminus is fused to the M13 domain of a myosin light chain kinase and calmodulin, which has 4 binding sites for calcium ions. When calcium is present as a result of neuronal activity, calmodulin undergoes a conformational change, resulting in the increase of GFP fluorescence.

In the context of sensory processing, Ca\(^{2+}\) imaging has been instrumental in answering key questions. Using Ca\(^{2+}\) imaging, the logic of odour representation was demonstrated as the combinatorial activity of glomeruli in the antennal lobe (J. W. Wang, Wong, Flores, Vosshall, & Axel, 2003). In taste, the spatial segregation of gustatory inputs in the SEZ of the fly brain was validated.
using Ca\textsuperscript{2+} imaging (Marella et al., 2006). Despite obvious advantages over some approaches, the spatial and temporal resolution of Ca\textsuperscript{2+} imaging can limit its application (Riemensperger, Pech, Dipt, & Fiala, 2012). To this end, recent improvements to the original GCaMP have resulted in GCaMP3, which was used in all the functional imaging experiments performed here. GCaMP3 exhibits an increased baseline fluorescence, wider dynamic range, and higher affinity for calcium than GCaMP2 (Tian et al., 2009). The signal-to-noise ratio and photostability of GCaMP3 are also significantly improved compared to GCaMP2.
1.7 Hypothesis

Due to promising similarities between fly gustatory and olfactory processing at the primary relays as described above, I hypothesize that GABA in the SEZ of the gustatory system functions in a similar way as GABAergic LNs of the olfactory system, where GABA_{B}R expressed on ORNs mediate gain control. I will also explore the contribution of GABA acting through GABA_{B}R to intermodal inhibition and mixture suppression. The main question investigated will be:

*How does GABA act via GABA_{B}Rs to modulate taste neuron function and output?*

To address this question, experiments were designed to test the following:

1. Do different taste neuron classes express different levels of GABA_{B} receptor?
2. How does manipulating GABA activity change the behavioural response to tastants?
3. How does manipulating GABA activity change the cellular taste neuron response?

To address point 1, immunohistochemical analyses were performed using both taste receptor markers and a GABA_{B}R marker to drive fluorescent reporters using the binary expression systems Gal4/UAS and LexA/LexAop.

To address points 2 and 3, genetic knockdown and pharmacological blockade of GABA_{B}R were employed, and the resulting cellular and behavioural effects to relevant stimuli were observed.
2. Materials and Methods

Fly stocks

Flies were raised on standard cornmeal fly food at 25°C and 70% relative humidity. The following fly lines were used: UAS-CD8::dsRed (Ye et al., 2007); GABA$_B$R2-Gal4 and UAS-GABA$_B$R2(RNAi)#2 (Root et al., 2008); LexAop-CD2::GFP (Lai & Lee, 2006); Gr64f-LexA::VP16 (Miyamoto et al., 2012); Gr66a-LexA::VP16, ppk28-LexA::VP16, and UAS-CD8::tdTomato (Thistle, Cameron, Ghorayshi, Dennison, & Scott, 2012); Gr64f-Gal4 (Dahanukar et al., 2007); Gr66a-Gal4 (Z. Wang et al., 2004); Gr5a-LexA::VP16, UAS-CD4::spGFP1-10, and LexAop-CD4::spGFP11 (Gordon & Scott, 2009); GAD1-Gal4; UAS-GCaMP3 (Tian et al., 2009); w$^{118}$, UAS-wntD(RNAi), UAS-Ccap(RNAi), UAS-GABA$_B$R2(RNAi) (TRiP collection, Bloomington Stock Center).

Tastants

The sugars D-sucrose (Fisher BioReagents) and D-glucose (Sigma) were kept as 1 M stocks in water and diluted to the desired concentration(s) for PER and calcium imaging experiments. The bitter compounds L-canavanine (from Canavalia ensiformis, jack bean) and denatonium benzoate were both obtained from Sigma-Aldrich and kept as 1 M stocks dissolved in water. L-canavanine was stored at 4°C.
**GABA$_B$R2 quantification**

To quantify GABA$_B$R2 expression in Gr64f axon terminals, flies expressing *UAS-GABA$_B$R2(RNAi)* under the control of *Gr64f-Gal4* were compared to flies expressing *UAS-wntD(RNAi)* (an isogenic control RNAi line against a gene not expressed in taste neurons) under the same driver. *UAS-GCaMP3* was also expressed in both groups driven by *Gr64f-Gal4*, allowing the visual identification of a Region of Interest (ROI) around the densest observable cluster of Gr64f synaptic boutons (based on GFP immunofluorescence) in individual optical slices of 0.5 µm. The mean pixel intensity value of GABA$_B$R2 immunofluorescence was recorded for this ROI representing Gr64f axon terminals. To control for overall immunofluorescence intensity, a second ROI of comparable area was drawn around a distinct region adjacent to the Gr64f projections and the mean intensity values recorded. The relative immunofluorescence was measured as the experimental ROI intensity divided by the control ROI intensity. This value was averaged across 10-15 brains and compared between brains expressing *UAS-GABA$_B$R2(RNAi)* and those expressing the control *UAS-WntD(RNAi)*.

**Immunohistochemistry**

Antibody staining on brains and proboscis labella was carried out as described (Z. Wang et al., 2004). The following primary antibodies were used: mouse α-GFP (1:100; Sigma #G6539), rabbit α-GABA$_B$R2 (Dick Nassel; 1:100) [the specificity of this α-GABA$_B$R2 has been described by Hamasaka et al. (2005) and Enell et al. (2007)], and rabbit α-DsRed (1:500; Clontech #632496). The
secondary antibodies used were: goat α-mouse Alexa 488 (1:100; Invitrogen #A11029), and goat α-rabbit Alexa 568 (1:100; Invitrogen #A11036). Brains were mounted in SlowFade (Invitrogen) using small cover slips as spacers. Confocal z-stacks were acquired using a Leica SP5 II confocal microscope with 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 2048 × 2048 pixels.

**Behavioural assays**

For PER assays, adult female flies aged 3-10 days were used. Flies were starved on water-saturated Kimwipes at 25°C for 22-24 hours before testing. After mounting flies on strips of myristic acid, they were allowed to recover in a humidified chamber for 1-2 hours. Before testing, each fly was stimulated with water on the distal tarsi of the foreleg and allowed to drink *ad libitum*. Testing commenced once none of the flies responded to water after two consecutive foreleg stimulations. For each concentration curve, each fly was stimulated on the foreleg for 500 ms with the indicated tastant, which was removed before the proboscis could make contact. Water was offered between stimulations to wash tarsi and keep flies water-satiated. Each tastant was tested twice consecutively and the number of extensions recorded. PER extension as a percentage was quantified as the number of extensions divided by total stimulations. Flies were offered 1 M sucrose at the end of each experiment to confirm viability; flies that did not respond were discarded from the data set. Control animals for RNAi experiments consisted of *Gr64f-Gal4* or *Gr66a-Gal4* crossed to one of two
isogenic RNAi lines from the same RNAi collection targeting genes known to lack expression in taste neurons: \textit{UAS-Ccap(RNAi)} or \textit{UAS-wntD(RNAi)}. The two control RNAi lines gave indistinguishable results.

\textbf{GCaMP imaging}

Female flies aged 2-12 days were used for calcium imaging. To prepare flies for imaging, they were briefly anesthetized and all legs were clipped to allow unobstructed access to the proboscis. Using a customized 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 (108 mM NaCl, 5 mM KCl, 4 mM NaHCO$_3$, 1 mM NaH$_2$PO$_4$, 5 mM HEPES, 15 mM ribose, 2 mM Ca$^{2+}$, and 8.2 mM Mg$^{2+}$) was immediately injected into the preparation to cover the exposed brain. The esophagus was clipped to allow clear imaging of the SEZ. A coverslip was inserted into the chamber to keep the proboscis dry and separate from the preparation. For pharmacology experiments, freshly sharpened forceps were used to make a tear in the glial sheath to allow drug access. The sheath was pinched near the antennal lobe to prevent damage to the SEZ. After pinching, forceps were quickly tugged away from the brain to produce a small hole, allowing diffusion of drugs.
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 8. Images were acquired at a speed of 8000 lines per second with a line average of 4, resulting in a collection time of 131 ms per frame at a resolution of 512 × 512 pixels. The pinhole was opened to 2.68-4 AU. For each taste stimulation, images were acquired for 10 s prior to stimulation, 1 s during stimulation, and at least 9 s following stimulation.

Prior to recording, a pipette tip filled with 1-2 µL of taste solution was positioned a few microns away from the proboscis labellum. Around frame 76 (10 s), the pipette made contact with the proboscis using a manually-controlled micromanipulator. Taste solutions were dissolved in distilled water and used at the indicated concentrations. Between taste stimulations, the pipette tip was rinsed with water.

The maximum change in fluorescence (Δf/f) was calculated as the peak intensity change divided by the average intensity prior to stimulation. Quantification of fluorescence changes was performed on Microsoft Excel.

**Pharmacology**

The following pharmacological agents and their respective final concentrations were used: CGP54626 hydrochloride (Tocris) was dissolved as a 50 mM stock in DMSO and used at a final concentration of 25 µM. SKF97541 (Tocris) was kept as a 100 mM stock in 100 mM NaCl and used at a final
concentration of 20 µM. GABA (Sigma) was prepared fresh, kept on ice, and used at a final concentration of 20 µM. The appropriate volume of each drug was first diluted in AHL buffer to make a 4×-5× solution and then added to the preparation to achieve the final concentration. The time between drug addition and stimulation was between 1-5 minutes, depending on number of flies in the chamber.

Statistical analyses

For PER analyses, the 95% binomial confidence interval was calculated using JavaStat (http://statpages.org/confint.html). Fisher’s exact tests were calculated using Graphpad QuickCalcs (http://www.graphpad.com/quickcalcs/). All other statistical tests were performed using GraphPad Prism 6 software.
3. Results

3.1 GABA\textsubscript{B}R Expression in Taste Neurons

First, we wanted to establish whether different taste neuron classes express GABA\textsubscript{B}R, as those expressing GABA\textsubscript{B}R would be possible targets of GABAergic inhibition. In fly olfaction, odour-evoked GABA\textsubscript{B}R-mediated inhibition is non-uniform across glomeruli and between odours (Root et al., 2008). Pheromone-sensing ORNs express high levels of GABA\textsubscript{B}R and are presynaptically inhibited by GABA whereas CO\textsubscript{2}-sensing ORNs do not express GABA\textsubscript{B}R. Using LexA lines to label taste receptors and a Gal4 line to co-label GABA\textsubscript{B}R2-expressing neurons, we drove fluorescent reporters and quantified expression of GABA\textsubscript{B}R2 in each taste neuron class.

3.1.1 Sweet, but not bitter, neurons express GABA\textsubscript{B}R

A binary reporter expression assay was used to determine whether different taste neuron classes express different levels of GABA\textsubscript{B}R. We used LexA taste receptor markers for sweet (Gr64f-LexA), bitter (Gr66a-LexA), and water (Ppk28-LexA) to drive LexAop-GFP in 3 of the known taste neuron classes, and GABA\textsubscript{B}R2-Gal4 driving UAS-CD8::dsRed to report GABA\textsubscript{B}R expression. Since GABA\textsubscript{B}R is an obligate heterodimer of GABA\textsubscript{B}R1 and GABA\textsubscript{B}R2, the GABA\textsubscript{B}R2 reporter provides a good proxy for GABA\textsubscript{B}R expression (Mezler et al., 2001; Root et al., 2008). Using a transgenic fly line that contains the fusion Gal4 DNA with the genomic DNA immediately upstream of the open reading frame of the
GABA<sub>B</sub>R2 gene, we could successfully recapitulate expression of GABA<sub>B</sub>R2 protein (Root et al., 2008). In ORNs, the faithfulness of this Gal4 line was confirmed by RT-PCR and antibody expression which both show GABA<sub>B</sub>R2 expression in the axon terminals of ORNs, corroborating reporter expression (Enell, Hamasaka, Kolodziejczyk, & Nassel, 2007; Root et al., 2008). By co-labelling with GABA<sub>B</sub>R2-Gal4 and each of the 3 taste neuron markers individually, we visualized GABA<sub>B</sub>R2 expression in each GRN class (Figure 3.1). Quantification of GFP and tdTomato signal showed expression of GABA<sub>B</sub>R2 in most sweet neurons. Conversely, bitter and water receptor expression did not overlap with GABA<sub>B</sub>R2 expression, suggesting these are separate, but neighbouring, cell populations. Quantification of co-expression data showed 88% of sweet neurons express GABA<sub>B</sub>R2 whereas only 6% of bitter and 2% of water neurons express GABA<sub>B</sub>R2. While we did not have a Gal4-independent reporter for ppk23 to label the pheromone-responsive population, we did drive UAS-CD8::tdTomato under the control of both ppk23-Gal4 and GABA<sub>B</sub>R2-Gal4 to examine expression pattern. We saw the same number of positively-labelled GRNs per palp for GABA<sub>B</sub>R2-Gal4 expression alone and when coupled with ppk23-Gal4 expression, demonstrating that most, if not all, ppk23-Gal4 neurons co-express GABA<sub>B</sub>R2-Gal4. Also, ppk23-Gal4 is expressed in 22 +/- 2 GRNs per palp, which corresponds exactly to the number of GABA<sub>B</sub>R2-positive cells that are not labelled by Gr64f-LexA (Table 3.1). This suggests that the pheromone (ppk23-Gal4) and sweet (Gr64f-LexA) GRN classes can account for almost all of the GABA<sub>B</sub>R2-positive cells.
We found that similar to ORNs, GRNs also express different levels of GABA$_B$R (Figure 3.1). Sweet neurons express high GABA$_B$R and drive the appetitive response, whereas bitter neurons express little to no GABA$_B$R and mediate avoidance. Therefore, we decided to pursue the role of presynaptic inhibition by GABA in these two taste neuron populations that drive opposing behavioural outcomes.
Figure 3.1 Sweet, but not bitter, neurons express GABA_bR2

(A-D) Single labial palp (A) or representative individual sensilla (B-D) from flies expressing CD8::tdTomato (magenta) under the control of GABA_bR2-Gal4. Each sensillum contains 1-2 GABA_bR2-positive neurons. Flies also express LexAop-CD2::GFP (green) under control of Gr64f-LexA::VP16 (B'; sweet), Gr66a-LexA::VP16 (C'; bitter), or ppk28-LexA::VP16 (D'; water) to label distinct GRN classes. (B’’-D’’). Merged images show overlap in expression between Gal4- and LexA- driven fluorescent reporters in sweet (B’’) neurons but not bitter (C’’) or water (D’’) neurons. Scale bars = 20 mm.
Table 3.1 Sweet, but not bitter, neurons express GABA<sub>B</sub>R2

<table>
<thead>
<tr>
<th>GRN type</th>
<th>GRN marker+ GABA&lt;sub&gt;B&lt;/sub&gt;R2+</th>
<th>GRN marker+ GABA&lt;sub&gt;B&lt;/sub&gt;R2-</th>
<th>GRN marker- GABA&lt;sub&gt;B&lt;/sub&gt;R2+</th>
<th>Proportion of GRN type that expresses GABA&lt;sub&gt;B&lt;/sub&gt;R2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sweet</td>
<td>26 +/- 1</td>
<td>4 +/- 1</td>
<td>22 +/- 2</td>
<td>88% +/- 2%</td>
</tr>
<tr>
<td>Bitter</td>
<td>1 +/- 1</td>
<td>19 +/- 1</td>
<td>48 +/- 2</td>
<td>6% +/- 1%</td>
</tr>
<tr>
<td>Water</td>
<td>0.3 +/- 0.2</td>
<td>15 +/- 1</td>
<td>46 +/- 3</td>
<td>2% +/- 0.5%</td>
</tr>
</tbody>
</table>

Number of GRNs co-expressing GABA<sub>B</sub>R2-Gal4 and each GRN marker (GRN marker<sup>+</sup> GABA<sub>B</sub>R2<sup>+</sup>), number of GRNs expressing each GRN marker alone (GRN marker<sup>+</sup> GABA<sub>B</sub>R2<sup>-</sup>), number of GRNs expressing GABA<sub>B</sub>R2-Gal4 alone (GRN marker<sup>-</sup> GABA<sub>B</sub>R2<sup>+</sup>), and percentage value of each GRN class observed to co-express GABA<sub>B</sub>R2-Gal4. Values represent mean +/- SEM, n = 6 palps per genotype.
3.1.2 \textit{GABA}_B^{b}R2(RNAi)\textit{ expressed in sweet neurons sufficiently knocks down GABA}_B^{b}R2\textit{ expression}

To confirm GABA\textsubscript{B}R\textsubscript{2} expression in sweet neurons and test the efficacy of RNAi knockdown for subsequent behavioural experiments, we used a \textit{GABA}_B^{b}R2(RNAi)\textit{ line from the \textit{Drosophila} Transgenic RNAi Project (TRiP) collection obtained from the Bloomington Drosophila Stock Center (BDSC). For controls, we obtained RNAi lines from the TRiP collection for genes that are not expressed in GRNs [\textit{UAS-wntD(RNAi)} and \textit{UAS-Ccap(RNAi)}]. GABA\textsubscript{B}R\textsubscript{2} or control RNAi was co-expressed with GCaMP3 in sweet GRNs under control of \textit{Gr64f-Gal4}, and protein levels were detected with immunolabelling using primary antibodies against GABA\textsubscript{B}R\textsubscript{2} and GFP. GABA\textsubscript{B}R\textsubscript{2} expression levels were quantified in regions corresponding to the highest GRN bouton intensity based on GFP expression. To control for overall immunofluorescence due to the widespread expression of GABA\textsubscript{B}R in the brain, the GABA\textsubscript{B}R\textsubscript{2} immunofluorescence in a region adjacent to the Gr64f projections was quantified. The relative immunofluorescence was then measured as the Gr64f intensity divided by the control region intensity. This analysis demonstrated that RNAi against \textit{GABA}_B^{b}R2 results in a significant reduction in GABA\textsubscript{B}R\textsubscript{2} expression in sweet GRNs (Figure 3.2). However, while GABA\textsubscript{B}R\textsubscript{2} expression was quantified in the plane of highest Gr64f synaptic bouton density, signal in these areas likely comes from non-GRN neurons as well, so the absolute level of knockdown in sweet neurons alone may be underestimated. For subsequent experiments, this
RNAi line was used to effectively knock down GABA\textsubscript{B}R2 protein levels in taste neurons.
Figure 3.2 GABA\textsubscript{B}R2 is sufficiently knocked down in flies expressing GABA\textsubscript{B}R2(RNAi) in sweet neurons

(A-B) Single 1 \(\mu\)m optical slices of flies expressing GCaMP3 alone (A) or with GABA\textsubscript{B}R2(RNAi) (B) under control of Gr64f-Gal4. Brains are immunostained against GFP (A,B) and GABA\textsubscript{B}R2 (A',B') antisera. Dotted yellow lines in A' and B' outline sweet GRN axon terminals as shown by GFP labelling. (C) Quantification of GABA\textsubscript{B}R2 immunofluorescence in regions of sweet GRN axon terminals, showing reduction of expression following RNAi in sweet GRNs. Values are normalized to GABA\textsubscript{B}R2 levels outside target area, and scaled to make controls = 1. Graph represents mean +/- SEM. Asterisks indicate significant difference from control: *** \(p < 0.001\). Scale bars = 20 \(\mu\)m.
3.2 Anatomy and Activity of GABAergic neurons in the SEZ

Many different olfactory systems have shown evidence for the role of GABAergic interneurons in mediating lateral inhibition between glomeruli, from the antennal lobe of the honeybee *Apis mellifera* (Sachse & Galizia, 2002) to the olfactory bulb of the rat (Isaacson & Strowbridge, 1998) and mouse (Mori, Nagao, & Yoshihara, 1999). As shown by detailed studies in insects, the olfactory system is densely populated with GABAergic interneurons. For example, cockroach ORNs are connected to PNs both monosynaptically as well as polysynaptically through GABAergic interneurons (Distler & Boeckh, 1997). Similarly in the fly brain, an antiserum to the enzyme glutamic acid decarboxylase (GAD1), which is required for GABA production, and a GAD1 Gal4 line driving GFP both show widespread GABA transmission in all major neuropils of the brain, including the SEZ (Enell et al., 2007). Having established differential expression of GABA_B_R2 in taste neuron classes (Figure 3.1), we next wanted to confirm that GABA-releasing interneurons were indeed present in the taste region and spatially poised to contact GRN axon terminals. We investigated the expression pattern of GABAergic interneurons in the brain by using GAD1-Gal4 to drive expression of GCaMP3. Although GAD1-Gal4-driven expression is somewhat more widespread than GABA expression pattern (Enell et al., 2007), partially due to the expression of GAD1 in glutamatergic and other non-GABAergic neurons (Hamasaka, Wegener, & Nassel, 2005), GAD1-Gal4-driven GFP closely matches that of GAD1 immunolabelling in the brain (Enell et al., 2007). In the olfactory system, use of GAD1-Gal4 successfully labels GABAergic interneurons in known synaptic
target fields or ORNs, PNs, and LNs (Ng et al., 2002). We observed widespread GFP immunofluorescence throughout all major brain regions, as expected by previous characterization of GABA signaling (Enell et al., 2007). We focused our attention to the taste-responsive region of the SEZ, where several hundred GABAergic neurons can be seen located dorsally and laterally relative to the SEZ (Figure 3.3).
Figure 3.3 GABAergic neurons are present in the region of the SEZ

(A-A") A dissected brain showing GCAMP3 expression in GABAergic neurons under the control of GAD1-Gal4 shows immunofluorescence of GFP (green) and nc82 (neuropil, magenta) in the SEZ. In the bottom panel, images are reproduced in grayscale to better visualize expression pattern. Images are projections through 5 µm optical sections in the anterior half of the SEZ. Sections are spaced 5 µm apart and labels indicate relative position of each. Arrows indicate visible GABAergic processes in the region of GRN axons in A' and A".

SEZ = subesophageal zone, AL = antennal lobe, e = esophagus. Scale bars = 50 µm.
3.2.1 GABAergic neurons lie within synaptic distance of taste neurons

To address whether GABA$_\text{R}2$-expressing GRNs may make synaptic contact with GABAergic interneurons, we employed GFP reconstitution across synaptic partners (GRASP). GRASP detects possible synaptic connections by expressing two halves of a split-GFP on two distinct neuronal populations (Feinberg et al., 2008). Neither half of GFP is sufficient to fluoresce alone but when the two halves come into close proximity, GFP is reconstituted and produces a strong fluorescence signal. To adapt this technique to flies, one membrane-tethered split-GFP fragment was placed under control of Gal4 (UAS-CD4::spGFP1-10) and the other under control of the LexA promoter (LexAop-CD4::spGFP11), allowing two different cell populations to be marked by specific Gal4 and LexA lines respectively (Gordon & Scott, 2009).

GRASP experiments performed between GABAergic interneurons (GAD1-Gal4) and either sweet (Gr5a-LexA) or bitter (Gr66a-LexA) neurons both produced a strong GFP signal at sites of contact (Figure 3.4). Note that reconstituted GFP signal co-localizes with known patterns of Gr5a and Gr66a projections (Marella et al., 2006), suggesting that the axon terminals of taste neurons may excite GABAergic interneurons and/or receive GABAergic modulation.
Figure 3.4 GABAergic neurons lie within synaptic distance of taste neurons

GRASP (GFP reconstitution across synaptic partners) reveals contact between GABAergic interneurons and sweet (Gr5a, left) or bitter (Gr66a, right) GRN axon terminals. GABAergic interneurons are labeled by GAD1-Gal4 driving expression of both CD8:dsRed (magenta) and CD4::splitGFP1-10, one non-functional half of GFP. Under control of Gr5a-LexA::VP16 (left, sweet GRNs) or Gr66a-LexA::VP16 (right, bitter GRNs), relevant GRNs express CD4::splitGFP11, the second non-functional half of GFP. Images are reproduced in grayscale in the bottom panel to better visualize GRASP signal. Reconstituted GFP (green signal) as a result of close contact between the GFP halves expressed by GABAergic interneurons and the indicated GRN class show possible synaptic connectivity between both sweet or bitter GRNs and GAD1-expressing interneurons. Dotted lines signify edges of brains.
3.2.2 GABAergic neurons respond to tastants

We have demonstrated that GRNs express GABA\textsubscript{B}R2 and lie in close proximity to GABAergic interneurons in the SEZ. Next, we wanted to establish that these GABAergic interneurons are responsive to tastes as well, suggesting the release of GABA upon taste stimulation. In fly olfaction, calcium imaging has been used to record odour-evoked spatiotemporal activity patterns in LNs, ORNs, and PNs (Silbering, Okada, Ito, & Galizia, 2008).

To investigate taste-evoked responses in the fly SEZ, the response profile of GABAergic interneurons to a tastant series was generated using calcium imaging (Figure 3.5). GCaMP3 was expressed in GAD1-expressing neurons and a plane of interest was imaged in the SEZ in the region of contact between GAD1-expressing neurons and GRN axons. Individual flies were stimulated on the labellum with a panel of sweet and bitter substances and the corresponding GABAergic neuron activity was recorded. We identified both GABAergic processes as well as cell bodies that responded in a taste-dependent manner. Importantly, responses were consistently recorded in GABAergic processes in the SEZ to taste stimulations, in conjunction with cell body responses. Most identified cell bodies (23 of 36) responded to both sugar and bitter compounds, supporting GRASP data which showed GABAergic neurons to contact both sweet and bitter GRN populations. A heat map of responding cells was generated, which showed taste profiles of some cells tuned to every taste modality, some cells responsive to sugar and bitter compounds but not water, as well as other cells tuned to bitter but not sugar and vice versa. The broad tuning
of GABAergic neurons to tastants resembles that of GABAergic LNs in the antennal lobe, and like olfaction, GABAergic interneurons in the SEZ likely contact GRNs of more than one type. GRASP data shown in Figure 3.4 also corroborates synaptic communication between GRNs and GABAergic interneurons. We pursued this mechanism further by manipulating the activity of GABA_β_R and observing its cellular and behavioural effects on GRN output.
Figure 3.5 GABAergic neurons respond to tastants

(A) Baseline fluorescence (grayscale) in the brain expressing GCaMP3 in GABAergic interneurons. Visible clusters of GABAergic cell bodies lateral to the SEZ can be seen prior to stimulation. (B) Heat map showing change in GCaMP fluorescence following stimulation with the bitter compound L-canavanine at 100 mM. Arrow marks single cell body observed to respond, whose processes may be among those activated within the SEZ. (C) Responses of 36 GABAergic neurons from 20 different flies to stimulation with water, sweet (100 mM sucrose) and bitter (100 mM L-canavanine). Cells are grouped by receptive field, with each response category colour-coded.
3.3 Behavioural Phenotypes of GABA_B R Knockdown

Having confirmed GABA_B R2 expression in sweet neurons and the responsiveness of GABAergic interneurons to a variety of tastants, we next wanted to ask how genetic knockdown of GABA_B R2 may affect GRN activity and ultimately, behaviour. Root and colleagues used RNAi to knock down GABA_B R2 expression in ORNs, which caused a disruption in the ability of male flies to locate a receptive female (Root et al., 2008). RNAi-expressing flies showed a greater response to stimuli than control flies, resulting in saturation at lower concentrations and a narrower dynamic range. Presynaptic inhibition by GABA thus exerts gain control on pheromone-sensing ORNs in the olfactory system, and we set out to determine if GABA performs a similar function in GRNs of the taste system using the verified RNAi construct (Figure 3.2).

3.3.1 GABA_B R knockdown in sweet neurons elevates the behavioural response to sucrose

An assay for proboscis extension reflex (PER) was used to score the appetitive behaviour of flies to increasing concentrations of sucrose. In flies expressing GABA_B R2(RNAi) in sweet neurons, PER was elevated at all concentrations compared to controls, indicating an increased acceptance of sugar (Figure 3.6A). This difference is statistically significant at concentrations of 1 mM and 10 mM sucrose. The activity of GABAergic modulation at moderate concentrations of sugar is consistent with the role of GABA_B R in expanding dynamic range. Gain control by presynaptic inhibition blunts the response to
sucrose, allowing discrimination over a wider range before saturation of the response. Similar to olfaction, GABA$_{B}$R in taste acts to exert gain control, expanding the range of stimulus concentrations between minimum detection threshold and saturation. By knocking down GABA$_{B}$R2 expression and consequently, presynaptic inhibition by GABA, flies expressing GABA$_{B}$R2 knockdown show an impaired ability to discriminate between different sugar concentrations and saturate their response at lower concentrations.

The same experiment was then performed with glucose to determine whether this mechanism applied to other nutritionally relevant sugars as well (Figure 3.6B). The same effect was observed with a glucose concentration series, in which GABA$_{B}$R2 knockdown increased PER response at all concentrations, indicating a role for GABA$_{B}$R in shaping the behavioural response to various sugars encountered in the wild. To verify our results, we used a second UAS-RNAi construct directed at a different region of GABA$_{B}$R2 and saw the same effect on PER to tarsal stimulation with a concentration range of sucrose (Figure 3.6C).
Figure 3.6 GABA$_B$R2 knockdown in sweet neurons elevates the behavioural response to sucrose

(A,B) PER responses of flies with GABA$_B$R2 knocked down in sweet GRNs under control of Gr64f-Gal4 (blue bars) compared to Gr64f-Gal4 crossed to an isogenic control RNAi line (gray bars). Two nutritionally-relevant sugars, sucrose (A) and glucose (B) were tested. For glucose, a higher concentration series was used due to the lower palatability (i.e. sweetness) of glucose compared to sucrose.

(C) Sucrose PER responses following knockdown of GABA$_B$R2 in Gr64f neurons using an RNAi construct distinct from the one shown in A and B. Graphs represent percentage of stimulations resulting in a positive response +/- 95% binominal confidence interval, n = 168-172 flies for A, n = 72 flies for B and n = 109-179 flies for C. Asterisks indicate significant change from control: * p < 0.05, ** p < 0.01, *** p < 0.001 by Fisher’s exact test.
3.3.2 GABA$_B$R knockdown in sweet neurons suppresses the behavioural aversion to bitter

The second component of GABAergic modulation we explored was that of GABA$_B$R in intermodal inhibition. In olfaction, suppression produced by odour mixtures can be blocked by GABA$_A$ and GABA$_B$ receptor antagonists (Olsen & Wilson, 2008) and odorants activate diverse patterns in glomeruli containing GABAergic interneurons (Wilson, 2013). Since these interneurons are poised to contact multiple glomeruli and are broadly activated by odours, GABAergic inhibition is an attractive candidate for the integration of opposing sensory cues. In taste, sweet and bitter stimuli elicit mutually exclusive behavioural outcomes, thus presenting an ideal scheme to test intermodal inhibition mediated by GABA.

We hypothesized that activation of GABA$_B$R elicited by bitter neuron stimulation would release GABA onto sweet GRNs, thus inhibiting their output and blunting the subsequent behavioural response to sweet compounds in the presence of bitter. To test this, PER frequency to increasing concentrations of denatonium, a highly bitter compound, was compared between control flies and those expressing GABA$_B$R2 knockdown. Flies expressing GABA$_B$R2 knockdown in sweet neurons exhibited significantly increased PER at 0.5 mM denatonium compared to control flies, with somewhat increased (but statistically insignificant) responses at other concentrations (Figure 3.7A). The partial effect of RNAi in this experiment is likely due to the confounding effect of direct inhibition by bitter substances on sweet neurons mediated by OBP49a (Jeong et al., 2013). Since this may mask the effects of GABA$_B$R2 knockdown, we excluded this cell-
autonomous effect by repeating the experiment with the bitter compound L-canavanine, which activates bitter neurons by an OBP49a-independent mechanism that does not directly suppress sweet neurons (Jeong et al., 2013). When $GABA_B R2$ knockdown flies were stimulated with increasing concentrations of L-canavanine, they exhibited a significant impairment in avoiding the bitter compared to control flies (Figure 3.7B). Control flies showed a dose-dependent decrease in PER in response to increasing L-canavanine, indicating a robust rejection of the toxin as bitter concentration increased. Remarkably, flies expressing $GABA_B R2$ knockdown attempted to consume the sucrose and L-canavanine mixtures at consistently high PER rates, regardless of bitter content.
Figure 3.7  $GABA_{B2}$ knockdown suppresses the behavioural aversion to bitter

PER inhibition by denatonium (A) or L-canavanine (B) in flies with $GABA_{B2}$ knocked down in sweet GRNs under control of $Gr64f-Gal4$ (blue bars) compared to each Gal4 line crossed to an isogenic control RNAi line (gray bars). All stimuli contain the indicated concentration of denatonium or L-canavanine added to 100 mM sucrose in order to induce PER. Chosen concentration series reflect the relative bitterness of each compound, with denatonium being highly bitter and abolishing all taste responses at around 5 mM. Graphs represent percentage of stimulations resulting in an appetitive response +/- 95% binomial confidence interval (n = 76 flies for A, n = 94 flies for B). Asterisks indicate significant change from control: * p < 0.05, ** p < 0.01, *** p < 0.001 by Fisher's exact test.
3.3.3 GABA\textsubscript{B}R knockdown in bitter neurons has no effect on the response to tastants

We saw that bitter neurons express little to no GABA\textsubscript{B}R2 (3.1.1), making them unlikely targets of GABAergic inhibition. Evidence in olfaction shows that the V glomerulus, which contains Gr21a-expressing ORNs that respond to aversive CO\textsubscript{2}, is not sensitive to pharmacological agents affecting GABA\textsubscript{B}R activity (Root et al., 2008). Thus, we suspected a similar outcome in bitter taste neurons. To confirm that GABAergic activity has no effect on bitter neuron output, \textit{GABA\textsubscript{B}R2} was knocked down in bitter neurons and the resulting PER responses to tastants recorded (Figure 3.8). A concentration series of both sucrose and denatonium were tested. There was no statistically significant difference in behaviour to either taste modality at any concentration between control flies and those expressing \textit{GABA\textsubscript{B}R2} knockdown in bitter neurons. Both groups exhibited a dose-dependent increase in PER to sucrose, and a dose-dependent decrease in PER to denatonium. These experiments demonstrate the specificity of the effect observed following \textit{GABA\textsubscript{B}R2} knockdown in sweet neurons.
Figure 3.8 $GABA_B^2$ knockdown in bitter neurons has no effect on the response to tastants

(A) Sucrose or denatonium (B) PER responses of flies with $GABA_B^2R2$ knocked down in bitter GRNs under control of Gr66a-Gal4 (green bars) compared to Gr66a-Gal4 crossed to an isogenic control RNAi line (gray bars). Indicated concentration of denatonium contains 100 mM sucrose to induce PER. Graphs represent percentage of stimulations resulting in an appetitive response +/- 95% binomial confidence interval (n = 126-130 flies for A, n = 60 flies for B). Asterisks indicate significant change from control: * p < 0.05, ** p < 0.01, *** p < 0.001 by Fisher’s exact test.
3.4 Functional Imaging of GABA$_{B}$R Manipulation

The identification of a behavioural phenotype associated with GABA$_{B}$R2 knockdown led us to question whether this would be reflected on the cellular level as a change in output. We hypothesized that if reducing GABA$_{B}$R activity in sweet neurons was sufficient to elevate the behavioural response to sugar, the neuronal response may similarly be potentiated by GABA$_{B}$R2 knockdown. To test this, we manipulated GABA$_{B}$R activity both genetically, using RNAi, as well as pharmacologically, using SKF97541 (a GABA$_{B}$R agonist) and CGP54626 (a GABA$_{B}$R antagonist). Calcium imaging was used to record the calcium response in GRN axon terminals in vivo.

3.4.1 GABA$_{B}$R knockdown does not significantly increase the sweet neuron response to sugar

To examine the neurophysiological effect of GABA$_{B}$R2 knockdown in sweet neurons, GCaMP3 was co-expressed with GABA$_{B}$R2(RNAi) in sweet neurons under the control of Gr64f-Gal4. The optical plane showing the densest observable cluster of Gr64f synaptic boutons (based on GCaMP3 baseline fluorescence) was selected for imaging in each brain. The calcium responses of sweet neurons were recorded following stimulation of the proboscis with the same concentration series of sucrose used for behaviour. At all concentrations, the responses of sweet neurons in control and GABA$_{B}$R2 knockdown flies were not statistically significant. One possible explanation for this negative result is the relatively high variability observed with GCaMP imaging. Another possible cause
for the discrepancy between behaviour and imaging is the temporal kinetics of 
GCaMP3. A change in taste neuron output may occur during a small critical time 
window that is masked by the large, slow change in GCaMP3 fluorescence (Tian 
et al., 2009).

In this experiment, we used unstarved flies for imaging because we 
speculated that starvation might affect GABA_\text{B}R signaling by downregulating the 
amount of GABA released. Such an effect would be ecologically adaptive – if an 
animal is starved, disinhibition of sweet neurons would result in increased 
appetitive response to more food sources, potentially including bitter compounds. 
In these circumstances, eating suboptimal food sources is preferential to not 
eating at all. With this in mind, we decided to image flies with normal access to 
food. Despite observing consistently higher responses of unstarved flies 
expressing \textit{GABA_\text{B}R2(rnai)} compared to control flies, none of these differences 
were statistically significant (Figure 3.9). Ultimately, the unavoidable degree of 
fly-to-fly variability and low throughput of calcium imaging made it difficult to 
accumulate enough responses to distinguish differences between groups. We 
decided to pursue pharmacological, rather than genetic, inhibition of GABA_\text{B}R 
with the expectation that the robust effects of drug application would reduce 
noise and produce higher GABA_\text{B}R blockade than RNAi.
Figure 3.9 GABA$_B$R2 knockdown by RNAi in sweet neurons does not significantly increase the calcium response to sugar

Average GCaMP3 peak fluorescence changes in Gr64f axon terminals in unstarved flies following stimulation with increasing concentrations of sucrose. Responses are compared between flies expressing GABA$_B$R2 knockdown in sweet GRNs under control of Gr64f-Gal4 (purple bars) compared to Gr64f-Gal4 crossed to an isogenic control RNAi line (black bars). Graphs represent mean +/- SEM (n = 20 per concentration).
3.4.2 Pharmacological blockade of GABA<sub>B</sub>R activity elevates sweet neuron output

Despite evidence of a robust behavioural phenotype, we were unable to observe an effect on GRN output by driving GABA<sub>B2(RNAi)</sub> in sweet neurons. Thus, we turned to pharmacological blockade using CGP54626 hydrochloride, a silent competitive GABA<sub>B</sub>R antagonist (i.e. binds to GABA<sub>B</sub>Rs at the same active site as the endogenous ligand, and has zero intrinsic activity for activating the receptor). CGP54626 has been used extensively to characterize rat CNS receptors (Brugger, Wicki, Olpe, Froestl, & Mickel, 1993) as well as GABA<sub>B</sub>Rs in non-mammalian vertebrates such as the bullfrog (*Lithobates catesbeianus*) (Asay & Boyd, 2006). In *Drosophila*, CGP54626 has been shown to effectively block GABA<sub>B</sub>Rs both in heterologous cells (Mezler et al., 2001) as well as in cultured circadian clock neurons (Hamasaka et al., 2005).

We recorded the calcium responses of sweet neurons to sucrose stimulation after application of CGP54626 or an equal volume of vehicle alone (DMSO) to AHL solution bathing the live brain (Figure 3.10). Before drug application, a tear was made in the glial sheath to facilitate the diffusion of drugs into the brain. At all sucrose concentrations tested, the addition of CGP54626 resulted in significantly higher sweet neuron responses to sucrose compared to stimulation in the presence of vehicle alone. This suggests that GABA<sub>B</sub>R function is necessary for modulating the output of sweet GRNs; however, effects from other, non-synaptic partners of GRNs cannot be excluded due to the global effect of CGP54626 on all GABA<sub>B</sub>R-expressing neurons in the brain.
CGP54626 was also added to bitter neurons and the response to
denatonium recorded. No difference was observed between CGP54626 and
DMSO alone, confirming that sweet, but not bitter, neuron response is modulated
by GABAergic activity. These results corroborate the behavioural data shown in
Figure 3.6 and show that activation of GABABR signalling is necessary for the
appropriate magnitude of sweet neuron output.
**Figure 3.10 Pharmacological blockade of GABA<sub>B</sub>R activity elevates sweet neuron output**

(A-B) Average GCaMP3 peak fluorescence changes in sweet (A) or bitter (B) GRN axon terminals following stimulation with increasing concentrations of sucrose (A) or denatonium (B) in the presence of the GABA<sub>B</sub>R antagonist CGP54626 (red) or vehicle alone (black). Graphs represent mean +/- SEM (n = 13-15 flies for each concentration). Calcium responses to sucrose in sweet GRNs are consistently higher in the presence of the GABA<sub>B</sub>R antagonist CGP54626, but CGP54626 has no effect on the bitter GRN response to denatonium. P-value indicates significant difference between CGP54626 and vehicle alone by two-way ANOVA.
3.4.3 Pharmacological activation of GABA\textsubscript{B}R activity inhibits sweet neuron output

To artificially activate GABA\textsubscript{B}R signaling, we recorded calcium responses of sweet neurons following application of GABA or SKF97541, a selective GABA\textsubscript{B}R agonist. SKF97541 mimics GABA\textsubscript{B}R activation and is widely used as a sedative in drug addiction research (Frankowska, Nowak, & Filip, 2009). SKF97541 is at least 10-fold more potent in rat brain slices than either of the other two classical GABA\textsubscript{B}R agonists 3-APPA and baclofen (Froestl et al., 1995; Seabrook, Howson, & Lacey, 1990). In Drosophila, SKF97541 has been used to investigate modulation of ORNs (Ignell et al., 2009) and was found to significantly reduce calcium transients in ORN terminals (Root et al., 2008).

We recorded the calcium responses of sweet neurons to sucrose stimulation after application of SKF97541 or an equal volume of vehicle alone (NaCl) to the AHL solution bathing the live brain (Figure 3.11B,C). The taste-evoked calcium response of sweet neurons to 100 mM sucrose was significantly decreased in the presence of SKF97541 compared to stimulation in vehicle alone. Quantification of average fluorescence changes showed a decrease of 30% as a result of SKF97541 application compared to a decrease of <5% with vehicle alone. This demonstrates that GABA\textsubscript{B}R activation is sufficient to inhibit sweet neuron output.
Figure 3.11 Pharmacological activation of GABA\textsubscript{B}R activity inhibits sweet neuron output

(A) Fluorescence changes of GCaMP3-expressing sweet GRN axon terminals in the SEZ before (top) and after (bottom, heat map) labellar stimulation with 100 mM sucrose. Scale bar = 25 µm. (B) Representative traces of fluorescence changes in Gr64f axon terminals to a 100 mM sucrose stimulation before (black) and after application of the GABA\textsubscript{B}R agonist SKF97541 (magenta) or vehicle alone (grey). A marked decrease in calcium response to sucrose is seen in the presence of SKF97541. (C) Decrease in peak fluorescence change in response to 100 mM sucrose stimulation in the absence or presence of SKF97541. Graph represents mean +/- SEM (n = 17-19 flies) and asterisks indicate significance by student’s t-test: ** p < 0.01.
3.4.4 The response of sweet neurons is suppressed by L-canavanine

The mechanism by which bitter substances inhibit sweet neuron activity in flies was recently revealed to require an odorant-binding protein (OBP) termed OBP49a (Jeong et al., 2013). In electrophysiological recordings of L-type sensilla, sweet and bitter mixtures suppress action potentials induced by sucrose alone, but this effect was abolished in mutant flies lacking OBP49a expression. The only exception to mixture suppression by OBP49a was L-canavanine, a bitter and highly deleterious insecticide produced by certain plants, the avoidance of which did not depend on any of the Obp mutants tested (Jeong et al., 2013).

The anomaly seen with L-canavanine detection provides a unique solution to the problem of dual inhibition by bitter compounds in our investigation. By using L-canavanine, we can effectively eliminate the cell-autonomous effect of direct bitter suppression, which would otherwise confound all our experiments involving bitter and sweet mixtures. Thus, any inhibition observed by L-canavanine would be attributed to a neural circuit based mechanism.

Electrophysiology measures the action potential (AP) firing of a single channel, but with calcium imaging, both AP firing of GRNs and synaptic modulation can be integrated and observed. To probe the activity of all Gr64f-expressing sweet GRNs to L-canavanine, sucrose and increasing concentrations of L-canavanine were used to stimulate the proboscis and the resulting calcium responses recorded (Figure 3.12). Despite the lack of suppression by L-canavanine on spike rate as assessed by electrophysiology (Jeong et al., 2013),
live imaging showed a robust and dose-dependent inhibition by L-canavanine on sweet GRN terminals. Since L-canavanine avoidance does not depend on OBP49a, this implicates the role of bitter neuron-mediated circuitry to contribute to the integration of competitive taste cues. We hypothesized this mechanism to be mediated by GABA\textsubscript{B}Rs expressed on sweet GRNs. Since GABAergic interneurons connect to both sweet and bitter GRNS (Figure 3.4) and show taste-evoked activity to bitter compounds (Figure 3.5), they present an ideal candidate to mediate sensory integration.
Figure 3.12 The response of sweet neurons is suppressed by L-canavanine

(A) Representative traces of fluorescence changes over time for sweet GRNs expressing GCaMP3 under control of Gr64f-Gal4 and stimulated on the proboscis with 100 mM sucrose alone (black trace), 100 mM sucrose + 50 mM L-canavanine (pink trace), and 100 mM sucrose + 100 mM L-canavanine (red trace). Note dose-dependent suppression of response by the addition of L-canavanine. (B) Quantification of fluorescence changes in sweet GRNs to 100 mM sucrose plus indicated increasing concentrations of the bitter L-canavanine. Graph represents mean +/- SEM (n = 12 flies for each concentration) and asterisks indicate significance by one-way Anova: * p < 0.05, ** p < 0.01, *** p < 0.001.
3.4.5 Genetic knockdown of GABA$_B$R activity relieves the suppression of sweet neuron response by L-canavanine

We saw that behavioural avoidance to L-canavanine was impaired in flies expressing $GABA_B R2(RNAi)$ in sweet neurons (Figure 3.7B) and stimulation of sweet neurons with mixtures of sucrose and L-canavanine led to significant suppression of the sweet GRN calcium response (Figure 3.12). To examine if this is reflected in the cellular response and as further investigation of GABA$_B$R as the mediator of sweet and bitter integration, we genetically reduced $GABA_B R2$ expression in sweet neurons using RNAi and activated bitter neurons by L-canavanine stimulation (Figure 3.13). The calcium response of sweet neurons was visualized to examine if suppression of sweet neurons by L-canavanine was dependent on GABA$_B$R2 function. We observed a significant decrease in suppression in $GABA_B R2$ knockdown flies compared to control flies in response to stimulation with 100 mM sucrose mixed with 50 mM L-canavanine. This suggests that normal GABA$_B$R expression and function is required for the appropriate inhibition of sweet neuron output by a bitter compound.
Figure 3.13 Genetic knockdown of GABA_B activity relieves the suppression of sweet neuron response by L-canavanine

Decrease in GCaMP fluorescence (i.e. inhibition by L-canavanine) in sweet GRN axon terminals in response to 100 mM sucrose + 50 mM L-canavanine after prior stimulation with 100 mM sucrose alone. The difference in fluorescence was compared between control flies (gray) and flies expressing GABA_B(RNAi) in sweet GRNs (blue). 50 mM L-canavanine was chosen for this experiment because of the more robust behavioural effects seen in Figure 3.7. Graph represents mean +/- SEM (n = 11 flies). Asterisks indicate significance by student’s t-test: * p < 0.05.
3.4.6 Pharmacological blockade of GABA<sub>B</sub>R activity relieves the suppression of sweet neuron response by L-canavanine

We saw that stimulation of sweet neurons with sucrose and L-canavanine led to significant suppression of the sweet GRN response (Figure 3.12), and this was partially relieved by genetic knockdown of GABA<sub>B</sub>R<sub>2</sub> (Figure 3.13). Since the efficiency of RNAi is incomplete, we also decided to use pharmacological blockade of GABA<sub>B</sub>R to maximize knockdown of GABAergic activity. We recorded the calcium responses of sweet neurons to a mixture of sucrose and L-canavanine in the presence or absence of CGP54626, the selective GABA<sub>B</sub>R antagonist (Figure 3.14). Similar to genetic reduction, pharmacological blockade of GABA<sub>B</sub>R completely abolished suppression by L-canavanine. This confirmed that activation by GABA<sub>B</sub>R is required to modulate sweet neuron output in response to sweet compounds laced with bitter substances.
**Figure 3.14** Pharmacological blockade of GABA$_B$R activity relieves the suppression of sweet neuron response by L-canavanine

Decrease in GCaMP3 fluorescence of sweet GRNs to 100 mM sucrose + 20 mM L-canavanine after prior stimulation with 100 mM sucrose alone. The difference in fluorescence was compared after addition of the GABA$_B$R antagonist CGP54626 (red) or vehicle alone (black). Note the addition of CGP54626 (red) produced an increase in sweet GRN response to stimulation with a mixture of sucrose + L-canavanine, as opposed to inhibition as observed in previously-described behavioural and pharmacologic controls. Graph represents mean +/- SEM (n = 11 flies). Asterisks indicate significance by student’s t-test: * p < 0.05.
3.5 $GABA_B$R activity mediates gain control of sweet GRNs and suppresses sweet GRNs by bitter GRN activation

We have shown that sweet, but not bitter, neurons express $GABA_B$R2 (Section 3.1.1) and GABAergic interneurons in the SEZ contact both sweet and bitter neurons (Section 3.2.1). Further, GABAergic interneurons in the region of primary sensory neurons show taste-evoked activity to a panel of tastants (Section 3.2.2). Our model proposes that activation of sweet GRNs by sugars elicits feedback inhibition mediated by GABA release by GABAergic interneurons onto $GABA_B$R2-expressing sweet neurons. We have demonstrated through calcium imaging and behaviour that this serves as a mechanism for gain control and expansion of dynamic range: when $GABA_B$R function is disrupted, sweet neuron activity is elevated both behaviourally and cellularly, which narrows the concentration range over which these flies are able to respond with a change in output.

A second, novel function of $GABA_B$R in the fly taste circuit is that of mixture suppression. In our model, activation of bitter GRNs triggers GABA release onto $GABA_B$Rs expressed on sweet neurons, which results in decreased sweet neuron output and consequently, a reduced behavioural response to sugars mixed with bitter compounds. Flies expressing $GABA_B$R2 knockdown show decreased suppression of sweet neuron activity by bitter exposure both behaviourally, by increased acceptance of bitter mixtures, and cellularly, by increased calcium response of sweet neurons. Gain control and intermodal
inhibition, both mediated by GABA\(_B\)R, thus provide a newfound layer of complexity in the neural process of deciding what, and how much, to consume.
Figure 3.15 GABA$_B$R activity mediates gain control on sweet GRNs and suppresses sweet GRN output by intermodal inhibition

Model for the role of GABA$_B$R in fly taste processing and integration. GABAergic interneurons contact $GABA_B$R2-expressing sweet GRNs to mediate gain control by modulating sweet GRN output to a wide range of sugar concentrations. A dual role for GABA$_B$R lies in suppression of sweet GRNs to sweet-bitter mixtures by inhibiting their output when bitter GRNs are activated. This leads to the successful inhibition of sweet GRN output, and subsequent avoidance of food, when a fly encounters suboptimal food sources. GRN = gustatory receptor neuron
4. Discussion

The evidence presented here has shown that (1) GABA$_{\beta}$R2 expression is non-uniform between taste neuron classes, (2) sweet and bitter stimuli excite GABAergic interneurons in the region of GRN axons, and (3) GABA$_{\beta}$R activity presynaptically inhibits the output of sweet neurons to expand their dynamic range as well as suppress their response to sweet-bitter mixtures. Thus, both the olfactory and taste systems of the fly appear to employ the same strategy of presynaptic gain control to modulate behaviour according to ecological need. Sugars must be sensed over a wide concentration range to guide feeding decisions, whereas bitter substances must be detected, and avoided, at even trace concentrations. Inhibitory interactions have been postulated to underlie mixture suppression in animals, and indeed GABAergic interneurons exert widespread inhibition among fly ORNs. We have found a role for GABA signaling in the fly taste system that demonstrates the first evidence in any animal for a circuit-based mechanism mediating sweet and bitter integration.

4.1 GABA$_{\beta}$R-mediated gain control

In fly olfaction, ORNs responsible for detecting attractive pheromones express high levels of GABA$_{\beta}$R whereas those activated by CO$_2$ do not express GABA$_{\beta}$R. Also, the activity of glomeruli (the site of ORN axons) that are responsive to fruit odours is potentiated by the addition of a GABA$_{\beta}$R antagonist, and consequently these glomeruli saturate at a lower concentration of odorant.
When $GABA_{B}R2$ was knocked down in pheromone-sensing ORNs, male flies were impaired in their ability to locate a virgin female. In this case, $GABA_{B}R$-mediated gain control allows the animal to discriminate between fine changes in pheromone concentration in order to locate a receptive female. GABAergic activity may thus alter behaviour dependent on internal and external contexts. Factors such as whether the female has recently mated, or whether the male has more pressing concerns (such as food) may dictate the level of GABAergic interneuron activity in the modulation of pheromone-sensing ORNs.

Similarly, intramodal $GABA_{B}R$-mediated gain control appears to function in the same manner in taste as in smell. GABA acts presynaptically on sweet GRNs, which express $GABA_{B}R2$, to expand their dynamic range. Conversely, bitter GRNs, which express little to no $GABA_{B}R2$, are not subject to presynaptic inhibition. Different taste neuron classes could express different levels of $GABA_{B}R$ as a reflection of ecological relevance. It would be evolutionarily advantageous for an animal to be able to discriminate between different food sources and choose the optimal food in an environment where it may be limited, thus reserving resources for other needs such as mating and courtship. There is evidence of such a role in the behavioural experiments presented here: when $GABA_{B}R2$ was knocked down in sweet neurons, flies responded at higher rates to lower concentrations of sugar (their responses saturated earlier). When $GABA_{B}R2$ was knocked down in bitter neurons, no effect was seen, consistent with the evidence that bitter neurons do not express $GABA_{B}R2$, thus retaining maximal sensitivity to bitter and ensuring reliable avoidance of potential toxins. It
would be interesting to explore how internal cues, such as satiety, and external cues, such as food availability or mating pressure, may change global modulation of GABA\textsubscript{B}R expression or GABA release. For example, in OSNs of mice and rats, olfactory sensory deprivation downregulates expression of both tyrosine hydroxylase (TH) and the GAD67 isoform of GAD, the rate-limiting enzymes in dopamine and GABA synthesis, respectively (McGann, 2013).

We saw evidence that most, if not all, pheromone-responsive GRNs also express GABA\textsubscript{B}R2. Since GABA\textsubscript{B}R in pheromone-sensing ORNs (located in the antennae) is necessary for mate localization, to what extent do pheromone-responsive GRNs contribute to pheromone processing? Since these GRNs are expressed in tactile appendages like the leg and wing, disruption of GABA\textsubscript{B}R2 in pheromone-responsive GRNs may affect the initiation, duration, or termination of copulation itself.

The study by Root et al. (2008) used calcium imaging to show that metabotropic GABA\textsubscript{B} receptors, but not ionotropic GABA\textsubscript{A} receptors, mediate presynaptic inhibition. However, previous whole-cell recordings of PNs in response to ORN stimulation suggest that both GABA\textsubscript{A}R and GABA\textsubscript{B}R are expressed on the same ORN axon terminals, and that GABA\textsubscript{A}R activity facilitates the early portion of inhibition while GABA\textsubscript{B}R mediates the late phase. Further molecular experiments will be required to determine whether GRNs express GABA\textsubscript{A}R as well as GABA\textsubscript{B}R, and tools more temporally sensitive, such as electrophysiology, will be needed to determine whether GABA\textsubscript{A}Rs contribute to presynaptic gain control.
We have identified GABAergic interneurons as a modulatory component of GRNs in the primary relay of fly taste processing. However, identification of second-order neurons that transform the GRN code to higher-processing channels, and eventually to a behavioural output, remains elusive. How different neuronal populations in the SEZ interact and respond to GABA signalling will be a critical question pending a more complete picture of taste circuit wiring.

**4.2 GABA$_B$R-mediated mixture suppression**

To eliminate the cell-autonomous effect of direct suppression by bitter compounds on sweet GRNs, we used L-canavanine, which does not trigger OBP49a-mediated inhibition. Interestingly, the addition of L-canavanine to sucrose caused a decrease in the calcium response of sweet GRNs, despite no change in firing. This effect was blocked both cellurally, by a GABA$_B$R antagonist, and behaviourally, by genetic knockdown of $GABA_B$R2 in sweet neurons. Although interglomerular inhibition has been proposed to underlie mixture suppression in olfaction, no mechanism has been definitively shown for taste mixture suppression in any animal.

This work shows that flies react appropriately to sweet-bitter mixtures in part by encoding a reduced attraction to sugar at the primary synapse. However, both the inhibition of sweet neuron output and the excitation of downstream bitter circuits could contribute to the avoidance of energy sources laced with potentially harmful substances. What proportion of these different pathways drive aversive behaviour, and how does the relative contribution of each change as a result of
internal state, or as the mixture composition varies? Ultimately, the intermediate circuitry linking taste detection to motor output will need to be revealed to answer these questions.

Carboxylic acids, which are present in overripe or rotting fruit, are often associated with unpalatable foods (Charlu, Wisotsky, Medina, & Dahanukar, 2013). Using behaviour and electrophysiology, acids were shown to activate a subset of bitter neurons in the labellum and inhibit the sweet neuron response in a dose-dependent manner. Intermodal inhibition by GABA may also mediate the aversion to acid, perhaps directly by the acid-bound OBP complex interacting with GABAergic interneurons, or indirectly by the activation of bitter neurons causing GABA release onto sweet neurons. Manipulating GABA signalling in the presence of acids may reveal whether GABAgR-mediated inhibition is a general principle of gustatory integration.

Responses recorded from labellar stimulation with acids showed aversion behaviour (Charlu et al., 2013), but the same stimulus sensed by two distinct taste organs can trigger distinct behavioural outcomes. For example, female flies are repulsed by lobeline detected by the legs but the same stimulus promotes egg-laying when sensed by the internal mouthparts (Joseph & Heberlein, 2012). In our work, we performed Ca$^{2+}$ imaging on labellar projections only, but these results recapitulated behavioural assays performed on tarsi, suggesting L-canavanine ingestion to be generally detrimental regardless of on which body part it was encountered. However, there is evidence that the aversion to noxious compounds is complex in flies, with some bitter and acid substances more
aversive than others. A more thorough picture of how flies respond to the diverse repertoire of cues encountered in their environment will be needed to parse apart the processes underlying feeding decisions.

In the olfactory system, pheromone ORNs express GABA$_B$R and receive high levels of presynaptic inhibition. We observed the same phenomenon in GRNs, where pheromone-responsive GRNs labelled by *ppk23-Gal4* were identified as the other major *GABA$_B$R2*-expressing class of taste neurons. Investigating the conservation between gustatory and olfactory processing of pheromones may reveal principles underlying courtship and aggression, and how different chemosensory inputs (i.e. smell and taste) are integrated in higher brain centres. Whether pheromone processing is prone to suppression by other olfactory inputs would be an interesting question to address. For example, would activation of the CO$_2$-responsive V glomerulus cause the inhibition of pheromone ORN output to PNs in the DA1 glomerulus? Similarly in taste, the activation of bitter neurons may result in GABAergic release onto the axons of pheromone-sensing neurons, decreasing the fly’s desire to mate in unsuitable environments and increasing the viability of offspring. Investigating these relationships between pheromone and other sensory processing may reveal further insights into how animals make decisions in the context of competing drives.
4.3 Other functions of presynaptic inhibition by GABA$_B$R

In the OSN terminals of the mammalian olfactory bulb, GABA$_B$R-mediated presynaptic inhibition comprises both a tonic and feedback component. It has been suggested that GABAergic interneurons receiving direct OSN input are responsible for feedback inhibition, whereas GABAergic interneurons receiving input from higher-order neurons mediate constant, tonic inhibition (Shao, Puche, Kiyokage, Szabo, & Shipley, 2009). In the avian auditory system, GABA$_B$R-mediated presynaptic inhibition acts to protect neurotransmitter release from depletion, thus preserving resources (Brenowitz, David, & Trussell, 1998). Presynaptic inhibition by GABA could also act as a substrate for tuning perceptual filters, and this could be a role for tonic inhibition. In glomeruli of the mammalian olfactory bulb, some glomeruli exhibit stronger levels of tonic inhibition than others, shaping the overall pattern of OSN activation (McGann, 2013). In this system, global tuning of GABAergic inhibition could result in two different primary representations of the same odorant under different neural or behavioural states. Although Ca$^{2+}$ imaging was insufficient for the purposes of distinguishing between tonic and feedback inhibition in this work, distinct phases of GABAergic inhibition could facilitate multiple mechanisms of taste processing. Future work with more sensitive molecular tools will be needed to decipher additional roles of GABA in modulating the primary sensory representation of taste.
5. Conclusion

This study set out to uncover the role of GABA in modulating sweet neuron output, and has revealed two novel, non-mutually exclusive functions. Activation of GABA\textsubscript{B}Rs expands the dynamic range of sweet neurons, conferring the discrimination of food sources over a wide concentration range. The presence of bitter compounds in otherwise attractive stimuli also activates GABA\textsubscript{B}Rs to suppress the sweet neuron response to sweet and bitter cocktails. Although much remains to be uncovered in the phenomenon of taste processing, this work reveals two critical events at the primary synapse in the fly brain. Presynaptic inhibition by GABA thus provides the first neural circuit-based mechanism by which animals integrate dynamic and competing gustatory cues in their natural environment.

Whether these principles can be applied to components of analogous circuits in higher organisms will lead to important insights into how animals make feeding decisions. \textit{Anopheles gambiae}, the mosquito largely responsible for the transmission of malaria, displays high genetic conservation with \textit{Drosophila} and studies confirm similar peripheral organizations of taste between the two species. Mosquito-borne diseases kill more than 600,000 people per year (WHO, 2013), threaten half of the world's population, and result in the loss of billions of dollars annually, making the mosquito arguably one of the world's deadliest animals. Therefore, understanding the circuitry underlying host-seeking in mosquitoes, a product of chemosensory processing and integration between sensory cues, will be pertinent in facing the changing economic and environmental climate.
6. References


