Identification and Characterization of Interneurons in *Drosophila* Gustatory Circuitry

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Der Dekan
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Life is finite but we could get infinitely close to truth.

Confucius

CONTENT

1. Summary

Neural circuits transform sensory information in the external world into abstract patterns of electrical activity in the nervous system and generate optimal behaviours in response to diverse environmental cues. Research in a few model organisms have substantially contributed to our understanding neural wiring, neural coding and the contribution of individual neuronal ensembles to specific animal behaviours. We have fMRI techniques to study a large area of brain and the large-scale functional networks with the resolution of millions of neurons. On the other end of the scale, we know molecular mechanisms of synaptic transmission at single neuronal level. However we lack knowledge on the middle scale. So far our understanding of the organization of a complete neural circuit, such as a typical circuit from sensory input to motor output, is very limited. In order to fill in the gap and understand perception, motion, and memory, we need to study the components, coding and processing in a neural circuit.

In this study, we are using the *Drosophila* gustatory circuit as a model system to address a fundamental question: how does a circuit transform sensory stimuli into appropriate behavioural responses. Flies respond to attractive taste stimuli (sweet, low-salt) and reject aversive taste stimuli (bitter, high-salt). These responses are largely determined by internal nutritional state and can be modified by learning and experience. To gain genetic control of circuit elements involved in feeding behaviour, we performed a screen for Gal4 lines which elicit repetitive proboscis response upon inducible TrpA1 activation and identified a subset of neurons which are neither sensory nor motor neurons (hereafter referred as VM neurons). Inhibition of VM neurons significantly impairs the sensitivity of flies to sucrose. These results suggest that VM neurons provide fine-tuning of sweet sensitivity.

Interestingly, VM neuron arborisations lie in close proximity to the axonal projections of sweet receptor neurons and overlap with the branches of insulin-producing cells. Moreover we demonstrated that VM neuronal activity is modulated by insulin signaling and thus changes according to the circulating levels of insulin-like peptides which closely reflect and regulate internal nutritional state. This enables flies quickly adjust to physiological needs and adapt to the dynamic environment. Altogether our study contributes to further understanding interneuron elements in the gustatory circuit and how neuropeptides modulate these elements to optimise behaviours.

2. Introduction

2.1 The Assembly, Organization and Coding of Circuits

Neurons don't function in isolation. They are organized into anatomically interconnected ensembles and functionally autonomous circuits that process specific kind of information and in many cases regulate their own activity using a feedback loop. Assembling functional neural circuits in the nervous system is challenging with hundreds of different neuronal types forming at least thousands of synaptic connections. Although the organization of neural circuits varies greatly depending on the functions and components, they share some principles of connection and features that are characteristic of neuronal ensembles. The connections in neural circuits can be simplified into three basic patterns: divergence (one neuronal ensemble send information to multiple targets), convergence (axons of multiple neurons all send information to the same neuronal ensemble, allowing comparison and integration of different signals), and feedback (the neuronal ensemble send information to one or more presynaptic targets, sometimes to itself).

Neuronal identity are correlated with the connectivity within a circuit. It has been shown that the diversity, stereotypical position and connectivity of motor neurons that project to specific target muscles in the limb are established by a Hox transcriptional regulatory network (Dasen, De Camilli et al. 2008). The Hox regulatory network directs selective motor neuron connectivity with limb muscles, drives the diversification of motor neurons at a single segmental level and constrain motor pools to specific rostrocaudal levels of the spinal cord. Moreover the coordination of motor output depends critically on sensory feedback information provided by proprioceptive sensory neurons. It has been reported that transcriptional factors in sensory neurons could determine the projection pattern of sensory axons within the spinal cord and influence specificity of sensory-motor connections, partly through expression of cell surface recognition proteins (Chen, Hirabayashi et al. 2006).

In addition to assembly of circuits, it is important to understand how neurons respond to a wide variety of stimuli, how the stimulus attributes (e.g. intensity, direction) are encoded in the circuit, and how to construct models in attempt to predict responses to certain stimuli. Neurons in circuits process information by combining and integrating information from different sources. It is usually thought that neural circuit represents information in the pattern of action potentials in specific neuronal ensembles and the relationship among the action potentials of the neurons in the ensemble.

Rate coding or frequency coding is a traditional and robust coding scheme. It assumes that most information about the stimulus is contained in the firing rate of the neurons and calculates the average number of spikes per unit of time. Rate coding was first introduced based experimental results that the number of spikes recording from nerves innervating a muscle increase as the weights hung from the muscle increases (Adrian and Zotterman 1926) . Nowadays firing rate became a standard parameter to describe the properties of sensory or cortical neurons. It has led to the concept that a neuron transforms information about a single input variable (e.g. the stimulus strength) into a single continuous output variable (the firing rate). However average firing rate ignores possible information contained in the precise timing of spikes and thus quite inefficient, especially when neurons exhibit high-frequency fluctuations of firing rates. Temporal coding offers a complementary approach in these cases and employs the features not counted by rate coding, such as precise spike timing.

One type of temporal coding is called latency to first spike, which counts the time from the onset of stimulus to the first spike. It has been suggested that retina neurons encoding fast visual stimuli (Gollisch and Meister 2008). This strategy is also employed by the mitral/tufted cells in the olfactory bulb of mice that first-spike latency relative to the start of a sniffing action allows for rapid identification of an odor (Wilson 2008). The inter-spike interval could also be used to encode information, which is important when the spike rate reaches its limit. In addition, there is evidence showing that in Purkinje neurons information is not only encoded in firing but also in the timing and duration of non-firing, quiescent periods (Forrest 2014).

In reality, information is usually represented in the nervous system by a combination of spatial and temporal coding. Firing rates evoked by a stimulus or the temporal spiking patterns is determined both by the dynamics of the stimulus and by the nature of the information processed within the circuit. In the mammalian gustatory system, different basic tastes (sweet, bitter, sour, salty, umami) elicit differences in firing rates and temporal patterns may help discriminate between tastants of the same category (e.g. denatonium versus quinine) (Hallock and Di Lorenzo 2006). Similarly in learning process, synapses are modified, depending not only on spike rates but also on spike timing patterns.

2.2 The classical Sensorimotor Circuits

There are three classes of neurons that form the basic constituents of a typical sensorimotor neural circuit: nerve cells that carry information toward the central circuit are called afferent neurons and nerve cells that carry information away from the center are called efferent neurons, and nerve cells that only participate in local transmission within the circuit are called interneurons.

A simple example of a sensorimotor circuit is the so called knee-jerk reflex, that subserves the myotatic spinal reflex (Nakanishi, Nakao et al. 1965). In the reflex, the afferent neurons are sensory neurons of dorsal root ganglion in the periphery. These afferent neurons could target directly the motor neurons that control extensor muscles via excitatory connections so that activation in afferent neurons lead to contraction of extensor muscles. Alternatively afferent neurons could synapse with inhibitory interneurons that are connected to motor neurons projecting to flexor muscles. Activation of interneurons by the afferents diminishes electrical activity in motor neurons, causing the flexor muscles to become inactive. When the knee is tapped, the sensory receptors in the leg are activated. On one side, activated sensory neurons excite motor neurons and the corresponding extensor muscles, leading to contraction. On the other side, they excite the interneurons, which inhibit the motor neurons for flexor muscles and cause flexor muscles to relax. As a consequence of the two complementary pathways, the leg extends and an upright posture is maintained (Figure 1).

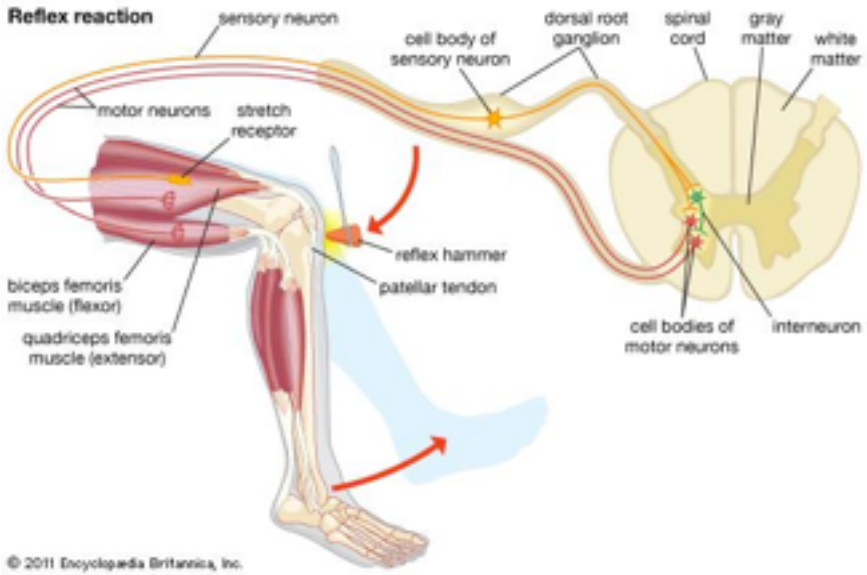

Figure 1 Schematic illustration of the 'knee-jerk' reflex from tendon tap from jerking the leg

Most sensorimotor circuits are much more complex than this reflex, with hierarchical organization and horizontal connections. An extensively-studied example is that olfactory circuitry in drosophila (Figure 2). The first point in the circuitry consists of olfactory sensory neurons (OSNs) expressing odourant receptors. OSNs form synaptic connections with the projection neurons (PNs) in the glomeruli in the antennal lobe as well as with inhibitory GABAergic local neurons (iLNs) and excitatory cholinergic local neurons (eLNs).

It has been shown that OSNs expressing the same olfactory receptor project to the same glomerulus in the antennal lobe. PNs send axons to Kenyon cells of the mushroom bodies and to the lateral horn. Most PNs innervate a single glomerulus, suggesting that they receive information from a single class of OSNs. Many imaging studies support the labelled line coding strategy that odour information is transferred directly from OSNs to PNs (Keene and Waddell 2007). Nonetheless PNs can be more broadly tuned to multiple odours than the afferent OSNs. Some iLNs and eLNs ramify widely throughout the antennal lobe and supposedly impart lateral inhibitory or excitatory effects to connections from OSNs to PNs or from PNs to PNs. Altogether in antennal lobe there is a myriad of OSNs projections, PNs dendrites and local interneurons which connect extensively to shape odour responses. Olfactory information are involved in various behavioural contexts.

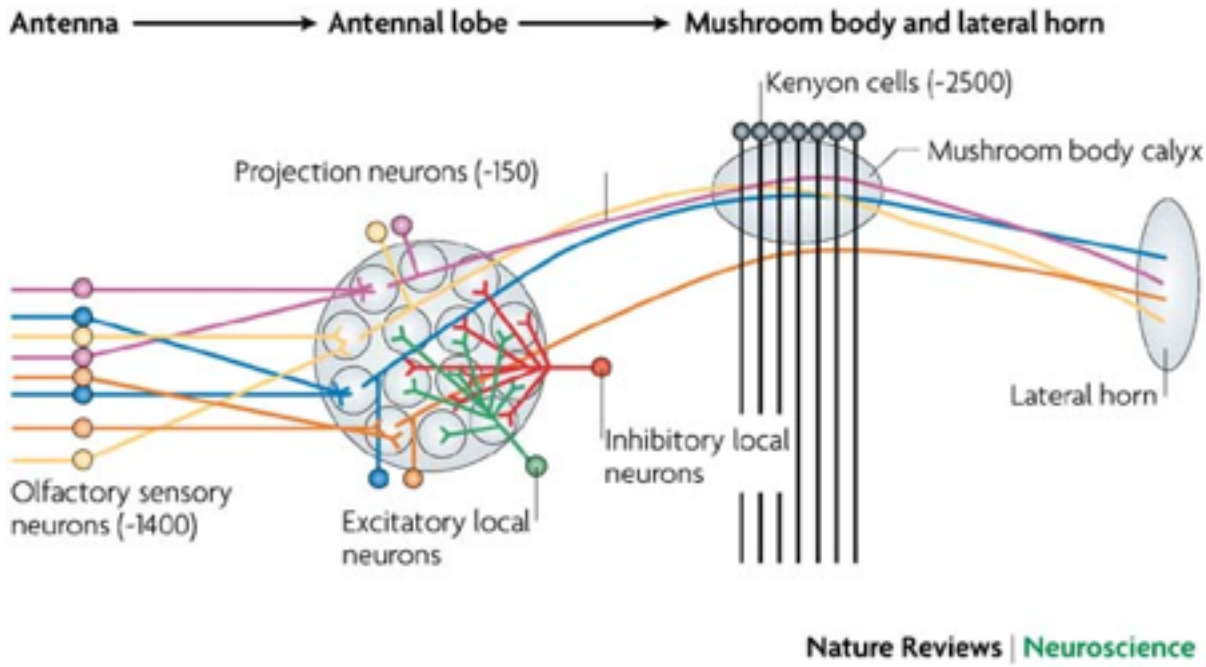

Figure 2 Schematic of the adult fly olfactory circuitry.

2.3 The Vertebrate Gustatory Circuitry.

In vertebrates, taste is the sensation produced by the chemical reaction between a taste compound and taste receptor cells. Along with olfaction, textures, temperature, taste sensation determines the flavours of taste substances.

Taste receptor cells could transform detection into perception. Sweetness is detected by a variety of G protein coupled receptors, T1R2+3 (heterodimer) and T1R3 (homodimer), which account for all sweet sensing in vertebrates. Amino acids detectors are T1R1 + T1R3. Bitterness receptors also belong to the superfamily of G-protein coupled receptors and were actually the first taste receptors ever discovered. Instead of GPCRs, sour taste receptors and salt receptors for sodium-salt taste, ENaC, are ion channels (Chandrashekar, Kuhn et al. 2010). Sour taste receptors cells host the PKD2L1 (Huang, Chen et al. 2006) receptors which could detect high concentration of hydrogen ions or acidity as well as the Car4 receptors (Chandrashekar, Yarmolinsky et al. 2009) which sense carbon dioxide.

Taste receptors cells are located in taste buds which are clustered into gustatory papillae and embedded in the surface of oral cavity, such as tongue and palate. Natural sweet substance activates the GPCR. Subsequently gustducin is released and activates the molecule adenylate cyclase, which catalyses the production of the molecule cAMP. This molecule closes the potassium ion channels, resulting in depolarisation and neurotransmitter release. Synthetic sweeteners activate different GPCRs and induce taste receptor cell depolarisation by an alternative pathway.

Taste information is sensed by the peripheral receptors and processed by the central pathways. Taste receptor cells embedded in the taste buds in the tongue, palate, epiglottis, and the oesophagus, make synaptic connections with primary sensory axons that run in the chorda tympani nerve and the facial nerve (cranial nerve VII), the glossopharyngeal nerve (cranial nerve IX), and the vagus nerve (Cranial nerve X) respectively. The primary sensory neurons in the cranial nerve ganglia send axons to the nucleus of the solitary tract in the medulla. The rostral (gustatory) part of nucleus of the solitary tract project axons to the ventral posterior complex of the thalamus and information is further relayed to several regions of the neocortex including the gustatory cortex. The primary gustatory cortex consists of the anterior insular lobe and the frontal operculum on the inferior frontal gyrus of the frontal lobe (AI/FO). Neurons in the AI/FO respond to sweetness, saltiness, bitterness, and sourness (Rolls 2005). Gustatory information is conveyed from the AI/FO to the orbitofrontal cortex,

where a small percent of neurons respond to taste stimuli and even finely tuned to particular taste stimuli (Thorpe, Rolls et al. 1983, Rolls, Yaxley et al. 1990).

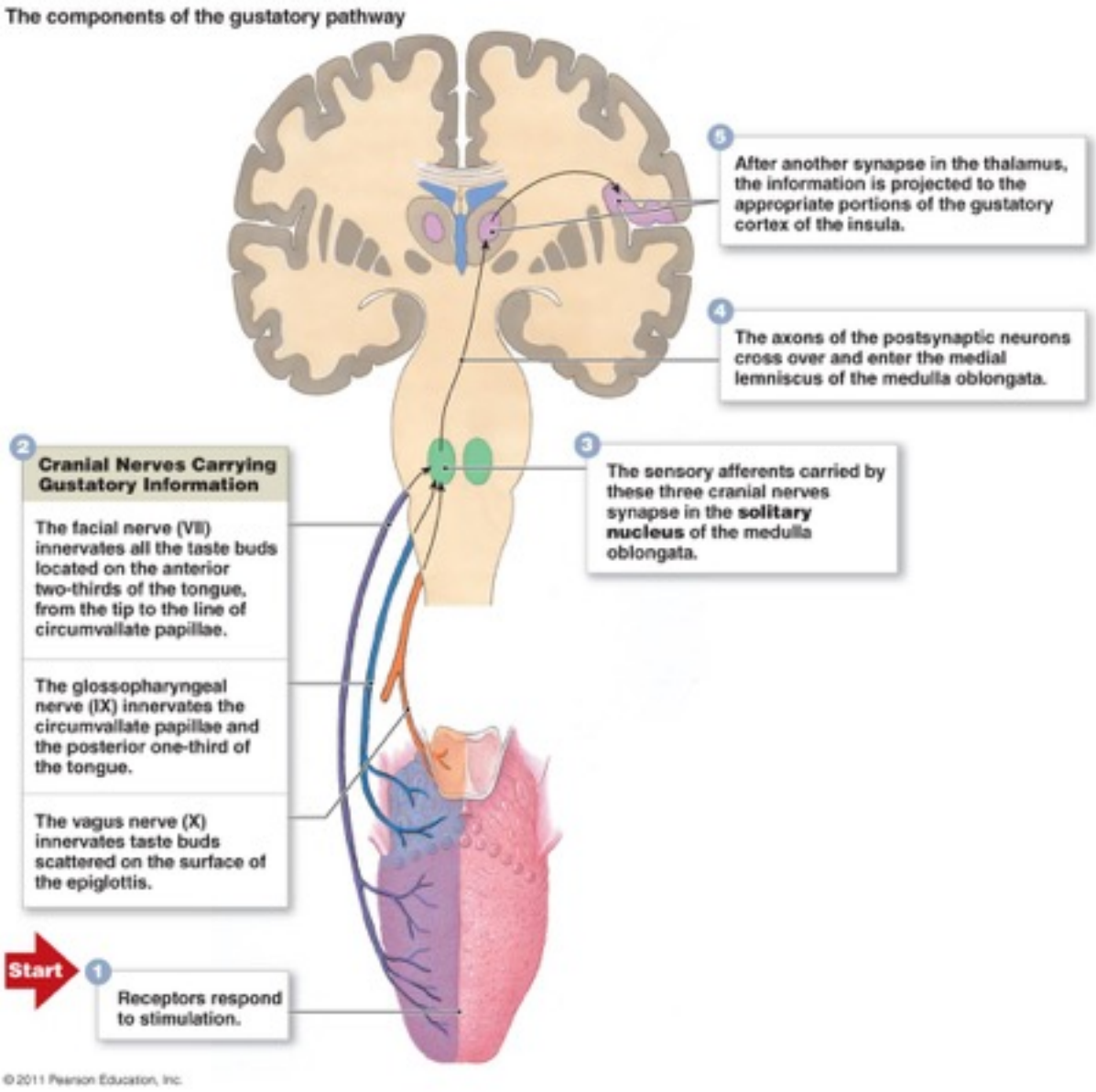

Figure 3 Schematic of the adult mammalian gustatory circuitry.

2.4 The Adult Drosophila Gustatory Circuitry.

The organization of gustatory circuitry in drosophila is very similar to it in vertebrates. Flies detect and evaluate food using gustatory receptors expressed in taste receptor cells, which are located in the taste sensilla on the labellum of the proboscis, an equivalent of the vertebrate tongue (Stocker 1994). Stimulation of the sweet receptor neurons in the labellum and forelegs triggers proboscis extension and ingestion of food (Dethier 1976). Upon ingestion, food comes into contact with the taste neurons located in the pharynx (Stocker 1994). Taste receptor neurons from the mouthparts and the pharynx send projections to distinct regions of the subesophageal ganglion zone (SEZ) . It it presumably the primary gustatory processing center housing first-order projection neurons, feeding command interneurons, GABAergic modulatory neurons and motor neurons controlling the feeding motor program (Gordon and Scott 2009, Flood, Iguchi et al. 2013, Pool, Kvello et al. 2014, Kain and Dahanukar 2015, Miyazaki, Lin et al. 2015). In addition, a confluence of neuromodulatory input from dopamine, serotonin, neuropeptide F, and short-neuropeptide F converge on the gustatory circuit to modulate food intake by altering the activity of sensory neurons or by changing status of homeostatic neurons (Inagaki, Ben-Tabou de-Leon et al. 2012, Inagaki, Panse et al. 2014, Albin, Kaun et al. 2015)(Root, Ko et al. 2011).

Large-scale calcium-imaging study on representations of different taste modalities in the adult fly brain reveals that sweet, bitter, and water sensory cells activate different cell populations throughout the SEZ (Harris, Kallman et al. 2015). Only a small percent of cells respond to more than one modality. Pathways for sweet and bitter tastes stay segregated in higher brain regions such as in the mushroom body.

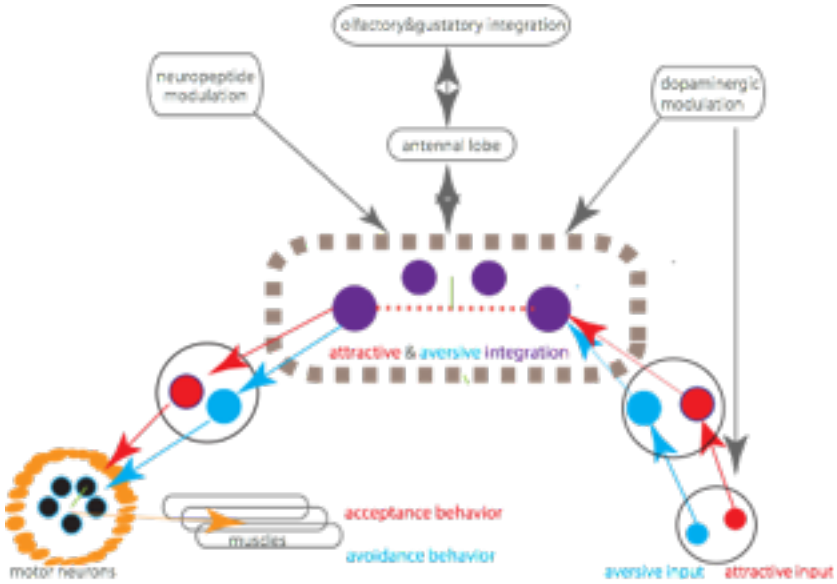

Figure 4 Schematic model of the adult drosophila gustatory circuitry.

2.5 Aim of thesis:

We use the Drosophila gustatory circuit as a model circuit to study the molecular mechanisms controlling neuronal circuit formation, the wiring diagram of the circuit and the functional relevance in feeding behaviour.

There are several advantageous properties of the gustatory circuit in drosophila. First of all, there are a limited number of taste modalities that flies could perceive and transduce, meaning sweet, bitter, sour, salt and water. These sensory pathways have different periphery receptors and distinct projection patterns in the SEZ. It has been shown that starvation increases, and satiety decreases the gustatory gain (Inagaki, Panse et al. 2014). Starvation could increase the positive reinforcement value of sucrose in learning and memory assays as well as to promote relentless food seeking behaviour (Wu, Zhao et al. 2005, Huetteroth, Perisse et al. 2015). Further when flies walk, locomotion could inhibit proboscis extension (Mann, Gordon et al. 2013). Collectively it suggests that the gustatory circuity is much more complex than a simple circuit from sensory neurons, through interneurons that integrate different taste modalities to motor neurons. Instead this circuit integrates at least inputs signaling nutritional state, motivational value, and locomotion. Many nodes in the gustatory circuit are modulated by neuropeptides. Thus it resembles a good model circuit to study how internal state modulate sensory perception and how various information summate to make behavioural

decision. Lastly the behavioural output of the circuit is much richer than simple proboscis extension or retraction. There are many interesting parameters such as the proboscis extension probability (reflecting taste sensitivity or feeding motivation), feeding frequency, feeding preference, and meal size. An array of sensitive and very reproducible behavioural assays are developed from the feeding pad which measures feeding frequency (Itskov, Moreira et al. 2014), to the Espresso assay which could measure real-time feeding in single fly at nanoliter scale (Yapici, Cohn et al. 2016).

Our first step is to identify and gain genetic control of the neuronal components that constitute the circuitry. The next step is to map the connectivity of individual components to upstream and downstream elements, to resolve how activity of these neuronal components influences feeding and how neuropeptides modulate these components to maintain homeostasis according to internal physical needs and external food availability. Eventually we want to illustrate an anatomical and functional map of gustatory processing pathway and gain insights into how homeostasis is established and maintained by concert of gustatory circuitry, neuropeptides and metabolism.

3. Results

3.1 Characterisation of Gustatory Interneurons

Manuscript

Starvation-dependent modulation of sweet taste sensitivity by Gustatory interneurons in Drosophila

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Abstract

Precise control of feeding behaviour is essential for the survival of animals and is largely determined by internal nutritional state and food palatability. While progress has been made towards the identification of gustatory circuitry, it remains largely elusive how information regarding internal nutritional state is integrated into the feeding circuitry to modulate feeding behavior. Here we identify a new class of interneurons (VM neurons) within the gustatory circuitry of drosophila that modulate sweet sensitivity of the animal in a starvation-dependent manner. VM neurons likely receive direct input from Gr5a sweet receptor neurons and VM neurites are present in closed proximity to the branches of insulin producing cells. Conditional silencing of VM neurons impairs sweet sensitivity of starved animals while artificial activation is sufficient to elicit proboscis extension. Interestingly silencing of insulin producing cells (IPCs) increases sweet sensitivity, suggesting that IPCs exert a modulatory effect on the sensitivity of the gustatory circuitry. in a manner opposite to VM neurons. Consistent with our hypothesis that VM neurons representing a direct target of IPCs in the gustatory circuit, blocking insulin signaling in VM neurons increases sweet sensitivity in fed animals. Taken together, we identified a neuromodulatory pathway that insulin signaling translates information of internal nutritional state into changes in gustatory circuit sensitivity. This mechanism enables animals rapidly adjust their feeding behaviours to maintain a homeostatic nutritional state.

Introduction

To survive and thrive in a constantly changing environment, animals are challenged to make appropriate behavioural decisions according to a confluence of sensory stimuli, internal state and previous experiences. Feeding is vital to the survival of animals. It is usually triggered by attractive taste stimuli and physical needs (Dethier 1976), and modified by learning and experience (Masek and Scott 2010, Kirkhart and Scott 2015). In Drosophila, it is has been demonstrated that starvation can increase sweet sensitivity and food searching behaviour (Dethier 1976, Inagaki, Ben-Tabou de-Leon et al. 2012, Marella, Mann et al. 2012, Inagaki, Panse et al. 2014) as well as reducing bitter sensitivity and aversion to noxious food (Wu, Zhao et al. 2005, Inagaki, Panse et al. 2014). However the circuit-level mechanisms underlying these state-dependent regulation remain to be elucidated. Feeding behavior starts with detection of attractive taste compounds by sensory bristles on the legs or proboscis and results in proboscis extension and feeding initiation (Stocker 1994, Thorne, Chromey et al. 2004, Wang, Singhvi et al. 2004, Gordon and Scott 2009). The probability that an animal initiates the proboscis extension response (PER) is influenced by the palatability of the taste compound, the energy needs of the animal and experiences (Dethier 1976, Inagaki, Ben-Tabou de-Leon et al. 2012, Marella, Mann et al. 2012, Inagaki, Panse et al. 2014).

Neuropeptides, as messengers of internal nutritional status, have been shown to modulate neuronal activity and information processing within sensor-motor circuits. Recent studies demonstrated that upon starvation dopamine is released onto sweet receptor neurons and enhance the sugar-invoked calcium responses in these neurons, thus enhancing sensitivity to sweet stimuli (Inagaki, Ben-Tabou de-Leon et al. 2012, Marella, Mann et al. 2012). In addition, it has been shown that that starvation increases odour-evoked presynaptic facilitation in olfactory receptor neurons and food search behaviour by upregulating sNPFR expression levels (Root, Ko et al. 2011). It is reasonable to propose that neuromodulation happens at multiple functional nodes within the gustatory circuitry.

The gustatory circuit of Drosophila represents an attractive model to study state-dependent neuromodulation of feeding behaviour due to the modality-specific sensory input (attractive versus aversive) and robust behavioural output (probability of proboscis extension).

Flies respond with proboscis extension to attractive taste compounds (sweet, low-salt) but reject aversive taste stimuli (bitter, high-salt). Gustatory receptor neurons from the proboscis and legs project to subesophageal ganglion zone (SEZ) of the fly brain and terminate in distinct regions de-

pending on their receptor class (Stocker 1994, Thorne, Chromey et al. 2004, Wang, Singhvi et al. 2004). The cell bodies and dendrites of motor neurons that execute the feeding program also are located in SEZ (Stocker 1994, Gordon and Scott 2009). Therefore the SEZ very likely represent a primary gustatory center. The absence of direct connectivity between gustatory receptor neurons and motor neurons suggests the existence of a local circuit controlling proboscis extension (Gordon and Scott 2009).

Four classes of gustatory interneurons have been identified so far. The first class are the feeding command neurons (Fdg) that can be acutely activated by sucrose in starved animals and that are sufficient to elicit the entire feeding program (Flood, Iguchi et al. 2013). Suppression and ablation of these neurons eliminate sugar-invoked feeding response. Interestingly Fdg neurons are not in direct contact with sweet receptor neurons or the motor neurons. The second class of interneurons are four GABAergic interneurons which set a central feeding threshold by imparting an inhibitory control over feeding (Pool, Kvello et al. 2014). Inactivation of these neurons causes ravenous and indiscriminate overconsumption of all compounds regardless of metabolic state. These GABAergic neurons show no response to gustatory cues or changes in metabolic state and they act upstream of motor neurons for multiple feeding subprograms. Notably a subset of serotonergic neurons with expression in SEZ were recently identified which provide a stimulatory effect on feeding and thus likely lower the feeding threshold (Albin, Kaun et al. 2015). Acute activation of these serotonergic neurons induces feeding in sated flies. The third class are the first-relay interneurons that receive direct input from gustatory sensory neurons. These neurons include gustatory projection neurons (GPNs) that send projections to a brain region called the antennal mechanosensory and motor center (Kain and Dahanukar 2015). GPNs are activated by sweet tastants and are sufficient to elicit feeding or proboscis extension. A different study identified a bilateral pair of gustatory second-order neurons (G2Ns) (Miyazaki, Lin et al. 2015). These neurons project to a distinct region outside the primary gustatory center, different from the AMMC region. The precise role of G2Ns within the gustatory role remains elusive. The fourth class are a group of cholinergic interneurons (IN neurons) that connected to the afferent projections of the sweet pharyngeal taste receptors neurons labeled by Gr43a and Gr64e but not to Gr5a sweet taste neurons on the labellum (LeDue, Chen et al. 2015, Yapici, Cohn et al. 2016). Inhibition of IN neurons led to reduced total food intake. IN neurons are activated when starved animals ingest high concentration sucrose and the activity gradually reduces as animas get satiated. In addition many neuropeptidergic cells either have extensive projections or are directly located in SEZ. These neuropeptides with the corresponding neuropeptider-

gic signalling regulate feeding behaviour by either promoting food intake such as Neuropeptide Y, small Neuropeptide F and dopamine (Lee, You et al. 2004, Wu, Zhao et al. 2005, Inagaki, Ben-Tabou de-Leon et al. 2012, Marella, Mann et al. 2012), or inhibiting specific aspects of feeding exemplified by allatostatin, hugin, leukokinin, drosulfakinin and MIP (Melcher and Pankratz 2005, Al-Anzi, Armand et al. 2010, Hergarden, Tayler et al. 2012, Soderberg, Carlsson et al. 2012, Min, Chae et al. 2016).

Here, we performed a Gal4-based screen to genetically tag gustatory interneurons in the SEZ. We identified a new class of interneurons which have their cell bodies located in the ventral medial SEZ (hereafter referred to as VM neurons). VM neuronal arborizations are present in close proximity to the axonal projections of sweet receptor neurons, suggesting that they receive direct gustatory input. Silencing VM neurons significantly impairs the sensitivity of flies to sucrose indicating these neurons are required for the fine-tuning of sweet sensitivity.

Interestingly, VM neurons are in juxtaposition to neurites of Insulin producing cells (IPCs) in the brain. There are a set of 14 IPCs embedded in a cluster of median neurosecretory cells (MNCs), which produce insulin-like peptide Dilp2, 3 and 5 (Brogiolo, Stocker et al. 2001, Ikeya, Galic et al. 2002). IPCs are located in pars intercerebralis (PI), a brain region analogous to the mammalian hypothalamus. They extend neurites near their cell bodies and into the SEZ adjacent to VM neurons. IPCs project axons to the corpora cardiaca (CC), aorta, proventriculus (PV), and the crop, where they release insulin-like peptides (ILPs) into circulation for systemic regulation.

Insulin-like peptides (ILPs) and the corresponding insulin signaling (IIS) pathway are evolutionarily conserved regulators of metabolism, growth, reproduction and lifespan across phyla (Nassel, Kubrak et al. 2013, Nassel, Liu et al. 2015). Several studies indicate that IPCs and Insulin-like peptides (ILPs) could link changes in nutritional state to changes in feeding behaviour (Ikeya, Galic et al. 2002, Geminard, Rulifson et al. 2009). Adult IPCs have glucose-sensing capacity (Kreneisz, Chen et al. 2010), possibly through type-1 glucose transporter Glut1(Park, Alfa et al. 2014). Silencing or ablating IPCs leads to increased feeding (Cognigni, Bailey et al. 2011) and the levels of circulating insulin-like peptide likely reflects the internal nutritional state. It has been shown that the levels of circulating insulin-like peptide peak quickly after feeding (Kim and Rulifson 2004, Geminard, Rulifson et al. 2009). This is, at least in part, triggered by leptin-like peptide Upd2 that is released from the fat body upon availability of nutrients (Rajan and Perrimon 2012). Correspondingly, the levels of circulating insulin-like peptide decrease in response to starvation (Geminard, Rulifson

et al. 2009, Park, Alfa et al. 2014). Levels of circulating insulin-like peptide in nutrient-deprived flies are considerably lower than that in flies raised on rich food (Geminard, Rulifson et al. 2009).

Here, we show that silencing IPCs significantly increases sweet sensitivity to a broad range of sucrose concentration. We demonstrate that blocking insulin signaling in VM neurons increased sweet sensitivity in fed animals while constitutive activation significantly reduced sweet sensitivity in starved animals. Our study provides evidence that a novel class of gustatory interneurons, which integrates input from sweet receptor neurons and information of nutritional state conveyed by IPCs and insulin signaling, modulates sweet sensitivity, thereby offering insights into to how neuropeptides regulate feeding behaviour to maintain energy homeostasis.

Results:

In Drosophila, proboscis extension response (PER) can be elicited by stimulating the taste receptor neurons on legs or proboscis with an sweet taste compound . In wild-type flies, the probability of flies extending proboscis to a sweet taste compound, sucrose in this case, increases as its concentration gets larger and starvation time gets longer (Figure 1A), consistent with previous reports (Dethier 1976, Inagaki, Ben-Tabou de-Leon et al. 2012, Marella, Mann et al. 2012, Inagaki, Panse et al. 2014). Thus the probability of PER, measured by percentage of extenders to a sweet compound, could reflect animals' sweet sensitivity to the stimulus and their internal drive to feed. To identify gustatory interneurons involved in the primary gustatory circuit contributing to feeding behaviour, we screened a large number of Gal4 lines from the Rubin-collection (Jenett, Rubin et al. 2012) and the VT-library (Dickson and Stark). We selected lines expressing Gal4 in a sparse number of neurons largely within SEZ with no efferent or afferent projections. Then we examined the effect on sweet taste behaviour via PER assay after silencing the Gal4 line with an inwardly rectifying potassium channel, Kir2.1 (Baines, Uhler et al. 2001). The expression of Kir2.1 was suppressed during development by a ubiquitously expressed, temperature-sensitive Gal4 repressor $Gal80^{ts}$ (McGuire, Le et al. 2003). From this screen, we found that after silencing line R40117 at adult stage, sensitivity to sucrose was significantly impaired in intermediately-starved (Figure 1C) and highly-starved (Figure 1D) animals in comparison to little or no changes in slightly-starved animals (Figure 1B). The decrease only occurs in response to sucrose stimuli within 50mM and 200mM (Figure 1E), which matches the sucrose content present at natural fruits. Normal proboscis extension response to sucrose is not abolished after silencing line R40117, in contrast to no response (to 100mM sucrose after 24h starvation) after silencing Fdg neurons (Flood, Iguchi et al. 2013), suggesting that neurons expressing in line R40117 are not a main switch in the gustatory circuit but rather fine-tune the information flow. Neither the profile of sucrose response curve nor the starvation-induced increase in sweet sensitivity is changed. After silencing line R40117, 24h-starved animals behave like 12h-starved control animals (Figure 1F), suggesting that under physiological condition neurons expressed in line R40117 serve as a gain in sweet sensitivity. In addition, PER response probability to bitter substance (caffeine) was not affected by silencing line R40117 (SupFigure 1A), indicating that these neurons mediate sweet but not bitter taste behaviour. Acutely silencing neurons in R40117-Gal4 with UAS-$shibire^{ts}$ also reduces spontaneous proboscis extensions at restrictive temperature compared to permissive temperature in intermediately-starved (SupFigure 1B) and highly-

starved (SupFigure 1C) animals. Taken together, neurons in line R40117 could increase sensitivity to sweet stimuli within naturally existent concentrations in starved animals.

We visualised the expression pattern of R40117-Gal4 with UAS-mCD8::GFP. A small number (around 12-16) of neurons with very similar morphology are labeled in the brain (Figure 2A). These neurons locate cell bodies at the ventral medial SEZ (hereafter referred as VM neurons), from where they project upwards to form broad arborisations at the dorsal medial SEZ around tritocerebrum (Figure 2B1) and end with terminal processes at the dorsal medial brain around protocerebrum (Figure 2B2). When line R40117-Gal4 was combined with Cha-Gal80, all the Gal4-driven GFP expression in brain was eliminated, indicating that these neurons are cholinergic (Figure 2C). We used a flip-out method to restrict Gal4 expression to a small subset of neurons in R40117 with a heat-sensitive Gal80 (tub-FRT-Gal80-FRT) that can be stochastically excised out upon heat-shock (Gordon and Scott 2009, Bohm, Welch et al. 2010). Visualisation of the mosaic clones revealed that a single VM neuron contains both middle arborisations at the dorsal medial SEZ and upper processes at the dorsal medial brain (Figure 2D). Labelling with the presynaptic synaptotagmin-GFP marker (Zhang, Rodesch et al. 2002) and the postsynaptic DenMark marker (Nicolai, Ramaekers et al. 2010) indicated that postsynaptic sites are found at both middle arborisations and upper processes (Figure 2E), whereas presynaptic sites exist only at middle arborisations (Figure 2F). R40117 did not label any taste sensory neurons in labellar, pharyngeal, or tarsal tissues. The anatomy of these neurons suggests that they are involved in the central circuit for gustatory information processing.

We next examined the possibility of connection between VM neurons and projections from the sweet gustatory receptor neurons using the GRASP (GFP reconstitution across synaptic partners) method, which was first developed in Caenorhabditis elegans (Feinberg, Vanhoven et al. 2008) and later adapted to flies (Gordon and Scott 2009). One fragment of the split-GFP GRASP reporter (lexAop-spGFP11::CD4) was expressed under control of R40117-Gal4 line while the expression of the complementary fragment UAS-spGFP1-10::CD4 was driven by Gr5a-lexA. If the two membrane-attached halves of GFP come close enough to the distance of a synaptic cleft (100nm), they could reconstitute and exhibit fluorescence. In addition, UAS-tdTomato and LexAop-HRP were expressed under control of line R40117-Gal4 and Gr5a-lexA respectively, to visualise VM interneurons (Figure 2I1) and Gr5a sensory neurons (Figure 2I2). Remarkably We detected endogenous GFP fluorescence in unfixed brains in the region where VM interneuron aborisations overlap with Gr5a axonal projections (Figure 2G,H,I3), indicating direct cell-cell contacts and potential synaptic

connection. Therefore, the neurites of VM interneurons lie in close proximity to the axonal termini of Gr5a sensory neurons.

We further examined whether activation of line R40117 is sufficient to elicit proboscis extension response using the temperature-gated cation channel dTrpA1 (Hamada, Rosenzweig et al. 2008). At restrictive temperature (30℃), animals expressing Gal4 in line R40117 lines repetitively extended proboscis (Figure 3A), at a frequency similar to that when activating the Gr5a sweet receptor neurons (Figure 3B). Due to the spontaneous proboscis extensions after artificial activation of line R40117, we were not able to test sweet sensitivity by scoring percentage of extenders to sucrose stimuli. Instead we increased temperature in a step-wise manner from the onset restrictive temperature (26℃) to activate line R40117-Gal4 > UAS-dTrpA1, which could presumably excite neurons to a further or full extent. The result showed that the percentage of extenders increased along with the temperature and peaked at 32℃, demonstrating that activity level in VM neurons is positively correlated with probability of proboscis extension (Figure 3C). Interestingly the temperature course of proboscis extension probability shows a similar trend when activating Gr5a-Gal4 > UAS-dTrpA1 (SupFigure 2A), whereas control flies rarely extend proboscis at all tested temperatures (SupFigure 2B,C). Additionally in our Flip-out experiments, both UAS-TrpA1 and UAS-mCD8GFP were randomly expressed in a subset of VM neurons in R40117 line. (SupFigure 3A). These flies were tested for proboscis extension at restricted temperature and the labelled neurons were visualised. We categorised the obtained mosaic clones into extender and non-extender groups (SupFigure 3B) and analysed them according to the number of labeled neurons. The summarised analysis indicates that at least three VM neurons are required to elicit proboscis extension upon TrpA1-dependent activation (SupFigure 3C). This result further supports that the combined activity level in VM neurons increase probability of proboscis extension and suggests a threshold for activating the primary gustatory circuit set by VM neurons.

We noticed that insulin producing cells (IPCs) extend branches to the tritocerebrum just above SEZ, close to arborisations of VM neurons (Figure 4A). As described before, VM neurons project up towards the pars intercerebralis (PI) and end with presynaptic termini in protocerebrum, where cell bodies of insulin producing cells (IPCs) are located (Figure 4B). When VM neurons and IPCs were visualised by GFP and RFP in the same brain, it clearly shows that VM neuronal arborisations overlap with IPCs branches (SupFigure 4C). To address the possible contacts between them, we used dilp2-Gal4 and R40117-LexA to drive expression of both fragments of the split-GFP GRASP re-

porters. Distinct endogenous GRASP signals were observed (Figure 4C,E), at the middle neuronal arborisation (Figure 4D2) as well as the upper neuronal termini (Figure 4D1). In comparison, GRASP signal wasn't detected in single fragment driven by neither dilp2-Gal4 (SupFigure 4A) nor R40117-LexA alone (SupFigure 4B). Altogether VM neurons are in close proximity of IPCs in both the protocerebrum and the SEZ regions. Based on the localisation of VM neurons in the primary gustatory center and their close proximity to Gr5a receptor neurons and IPCs, they are well situated to integrate sensory input and internal nutritional state to modulate taste behaviours.

Considering the potential communication between IPCs and VM neurons, we next investigated whether direct manipulation of IPCs could change sweet sensitivity. As Dilp2 is a principal circulating insulin in flies (Gronke, Clarke et al. 2010), we decided to examine IPCs labeled by Dilp2-Gal4. The inward-rectifying potassium channel Kir2.1 has been used to silence electrical activity of IPCs (Kim and Rulifson 2004, Park, Alfa et al. 2014), which resulted in increased accumulation of Dilp2 in IPCs (Geminard, Rulifson et al. 2009) and reduced circulatory Dilp2 in the hemolymph (Park, Alfa et al. 2014). We expressed Kir2.1 in adulthood in combination with the ubiquitous temperature-sensitive Gal4 repressor $Gal80^{ts}$ (McGuire, Le et al. 2003), and then tested responses to sucrose across the concentration range in animals under different starvation time. We found that in slightly-starved animals, inhibition of IPCs led to a significant increase in sweet sensitivity across all tested concentrations (Figure 5A, 5D). The increase is more prominent at low concentrations with an approximately two-fold increase at 50mM and 100mM in comparison to about one fourth increase at 500mM and 1M stimuli (Figure 5A). In intermediately-starved animals, there is a moderate increase after IPCs inhibition in response to stimuli between 50mM and 200mM (Figure 5B). In highly-starved animals, there is a slight increase which is still significant at low concentrations (Figure 5C). It has been shown circulatory insulin-like peptide is abundant in animals raised in rich-nutrition and scarce in animals raised in poor-nutrition (Geminard, Rulifson et al. 2009), indicating that its level positively correlates with nutritional state. Thus expression of Kir2.1 in IPCs can lead to a large decrease in circulatory insulin-like peptide level in slightly-starved animals, a moderate decrease in intermediately-starved animals and a negligible decrease in highly-starved animals. This offers an explanation for our observations of the starvation-dependent decrease in sweet sensitivity. Previous studies pointed that insulin release is gated by membrane depolarisation (Kreneisz, Chen et al. 2010, Park, Alfa et al. 2014). We also tried to activate IPCs with a temperature-sensitive cation channel dTrpA1 (Hamada, Rosenzweig et al. 2008). Concomitantly in highly-starved animals we observed significant increase in sweet sensitivity under restrictive temperature versus per-

missive temperature (SupFigure 5A), but relatively no change in intermediately-starved (SupFigure 5B) and slightly-starved animals (SupFigure 5C). Taken together, activity of IPCs could modulate sweet sensitivity, presumably by affecting circulatory insulin-like peptide level that signals the internal nutrition state of animals.

We reasoned that there could be potential downstream neurons within the gustatory circuit which could respond to circulatory insulin-like peptide and modulate sweet sensitivity. Considering that VM neurons could alter sucrose-response curve in a starvation-dependent manner without abolishing starvation-induced increase in sucrose response, we inferred that VM neurons receive input of internal nutritional state but don't encode the information themselves. Further VM neurons are in close proximity to IPCs at middle neurite arborisations where Dilp2 insulin-like peptide is abundant (SupFigure 5D).

Given the propinquity of VM neurons and IPCs and their opposite roles in modulating sweet sensitivity, we examined the possibility that IPCs modulate sweet sensitivity via insulin signaling in VM neurons. In drosophila only one type of insulin receptor (dInR) that has been detected so far (Nassel, Kubrak et al. 2013). We were not able to resolve whether expression of InR exists in VM neurons as the currently available antibody does't yield specific signal in central brain.

We tested the taste responses after activating or blocking insulin signaling in VM neurons by expressing a constitutively active InR (InR-CA) and a dominant negative insulin receptor InR (InR-DN) respectively at adult stage (Nassel, Kubrak et al. 2013). We found that blocking insulin signaling with a dominant negative InR in VM neurons increased sweet sensitivity in slightly-starved animals (Fig. 6A, D), in comparison to a small increase in intermediately-starved animals (Fig. 6B, E) and no effect in highly-starved animals (Fig. 6C, F). In contrast, constitutive activation of insulin signaling with a constitutively active InR in VM neurons significantly reduced sweet sensitivity in intermediately-starved (Fig. 6B, E) and highly-starved animals (Fig. 6C, F) whereas we observed no decrease in slightly-starved animals (Fig. 6A, D). All control lines showed very similar response curves to a series of sucrose concentration under different starvation time. When insulin signalling were manipulated in the Gr5a sweet receptor neurons with the same contracts InR(DN) and InR(CA), we didn't observe changes in sweet sensitivity under all starvation conditions (SupFigure 6). Further knocking down insulin signalling with UAS-InR RNAi (V992) in VM neurons led to a significant increase in sweet sensitivity comparing to controls in slightly-starved animals (SupFigure 7A) and no effect in intermediately-starved animals (SupFigure 7B) or highly starved animals

(SupFigure 7C). In both directions, modulation of insulin signaling affected sensitivity to a restricted range of sucrose concentration (50mM to 200mM sucrose). Expression of UAS-InR-DN and UAS-InR-RNAi both significantly increased sweet sensitivity in fed animals, indicating that under physiological conditions insulin signaling in VM neurons could down-regulate its activity and animals' sweet sensitivity in satiated instead of starved state. This is consistent with the finding that the levels of circulatory insulin-like peptide peak in well-fed animals and are largely depleted in fasted animals (Geminard, Rulifson et al. 2009). We noted that the increase in sweet sensitivity resulted from inhibition of IPCs is larger than the effect caused by blocking insulin signaling in VM neurons. It suggests there are additional downstream insulin signaling targets modulating sweet sensitivity, including the formerly reported ORNs (Root, Ko et al. 2011) and NPFR neurons (Wu, Zhao et al. 2005).

Discussion:

In this study, we identified a new type of interneurons (VM neurons) that modulate sweet taste behavior, Silencing of VM neurons impairs sweet sensitivity and TrpA1-induced activation of VM neurons results in spontaneous repetitive proboscis extension. Our results indicate that VM neurons are active in starved animals and are required to increase the response to sweet stimuli within the gustatory circuit. In contrast, in fed animals VM neurons are inactive and do not contribute significantly to signal processing within the circuitry. We provide evidence that insulin-like peptides , released by IPCs in the brain likely confer the satiety signal for VM neurons and reduce VM neuronal activity. In addition to the previously reported dopaminergic modulation of Gr5a sweet receptor neurons that increases sucrose sensitivity in the first 6 hours after starvation, VM neurons represent an interneuron node for neuromodulation and can promote increase in sucrose sensitivity after prolonged (more than 12 hours) starvation. The gustatory circuit could dynamically adjust the gain to limit various input to output within a defined range (proboscis extension probability 0 - 100%). The identification of gain-control elements indicates that there are likely neuronal ensembles with roles similar to relay, switch, amplifier, attenuator, transistor and other electrical circuit elements to ensure that animals adeptly adjust sensory sensitivity to satisfy internal physical needs.

In humans, the taste receptors of primary gustatory neurons are embedded in taste buds in the tongue, palate, pharynx and oesophagus. Taste information is sensed by receptors and transmitted via cranial nerves to the gustatory nucleus of solitary tract complex in the medulla, which further project to the ventral posterior complex of the thalamus and subsequently the gustatory cortex. The general principles of gustatory circuit organization is consistent between humans and drosophila. Importantly, modulation of feeding circuit by neuropeptides signaling metabolic needs is widely applied in mammals. The best elucidated example might be the NPY/AgRP expressing neurons in hypothalamus, which are critical regulators of feeding behaviour. Acute ablation of NPY/AgRP neurons results in life-threatening anorexia (Luquet, Perez et al. 2005), whereas activation of these neurons leads to voracious food seeking and feeding behaviours (Aponte, Atasoy et al. 2011). NPY/AgRP expressing neurons are activated by the orexigenic hormone ghrelin signalling energy deficit and inhibited by the anorexigenic hormones leptin and insulin signalling energy surfeit (Yang, Atasoy et al. 2011). However NPY/AgRP neurons are heterogeneous populations and have widespread projections in the CNS system. The VM-neuron system represents a similar modulatory system in the gustatory circuit with the advantage of drastically reduced number and homogeneity.

In this study we demonstrate that IPCs can directly modulate sweet sensitivity in addition to the previously defined roles in regulating food intake and metabolic homeostasis. It is worth noting that IPCs also express a satiety-inducing peptide named drosulfakinin (Soderberg, Carlsson et al. 2012). Thus IPCs-mediated modulation of sweet sensitivity might be a combined effect of insulin-like peptides and drosulfakinin. Besides IPCs, some other neuropeptidergic neurons that regulate feeding behaviour form branches in the pars intercerebralis (where upper terminals of VM neurons are) and/or in the tritocerebrum (where the middle arborisations of VM neurons are). These include corazonin (CRZ)/sNPF-expressing DLP neurons which regulate food intake and activate IPCs cells (Kapan, Lushchak et al. 2012), the hugin expressing neurons which are responsible for controlling feeding initiation (Melcher and Pankratz 2005), and the tachykinin expression neurons which control feeding termination (Al-Anzi, Armand et al. 2010). In the future it will be of high interest to explore the potential modulations on VM neurons by these peptides to further unravel potential anatomical and functional connections between VM neurons and neuropeptidergic neurons.

To ensure metabolic homeostasis and normal development, insulin release is precisely controlled. It has been demonstrated in rodents that there is an early phase of insulin release immediately after tasting sweet food (within 2 minutes) before any increase in plasma glucose level could happen, known as the cephalic phase insulin release (CPIR) (Berthoud, Bereiter et al. 1981) (Glendinning, Stano et al. 2015). Similarly in humans, applying sucrose to the tongue without swallowing elicits CPIR (Just, Pau et al. 2008). The mechanisms for CPIR remain elusive so far. Bilateral section of one gustatory curve, the chorda tympani nerve, abolishes CPIR in rat (Tonosaki, Hori et al. 2007), suggesting gustatory information is essential for eliciting CPIR. Absence of CPIR can lead to impaired glucose tolerance (Berthoud, Bereiter et al. 1981), by presumably effecting liver cells and halting glucose production (Cherrington, Sindelar et al. 2002). In drosophila, a fast increase in circulatory levels of insulin-like peptides was observed as well (Park, Alfa et al. 2014). It has been well documented that insulin release can be triggered directly, or indirectly via neuropeptides in response to increases in circulatory levels of nutrients (glucose, triglycerides etc). However there is a latency between taste sensation and changes in circulatory levels of nutrients. How does IPCs get the instant signal of taste stimulation to start the early release? On the basis of our findings that VM neuronal activity encodes sweet taste information and that the presynaptic terminals of VM neurons are in close proximity to cell bodies of IPCs (Figure 4), we hypothesise that VM neurons might potentially forward the instant taste information from Gr5a sweet receptor neurons to IPCs, in addition to being themselves modulated by the insulin-like peptides. Unfortunately, with the current genetic

and technical tools we are unable to directly investigate whether VM neuronal activity could play a role not only in modulating sweet sensitivity but also in regulating insulin release. This tantalizing hypothesis awaits future investigation.

Figures:

Figure 1

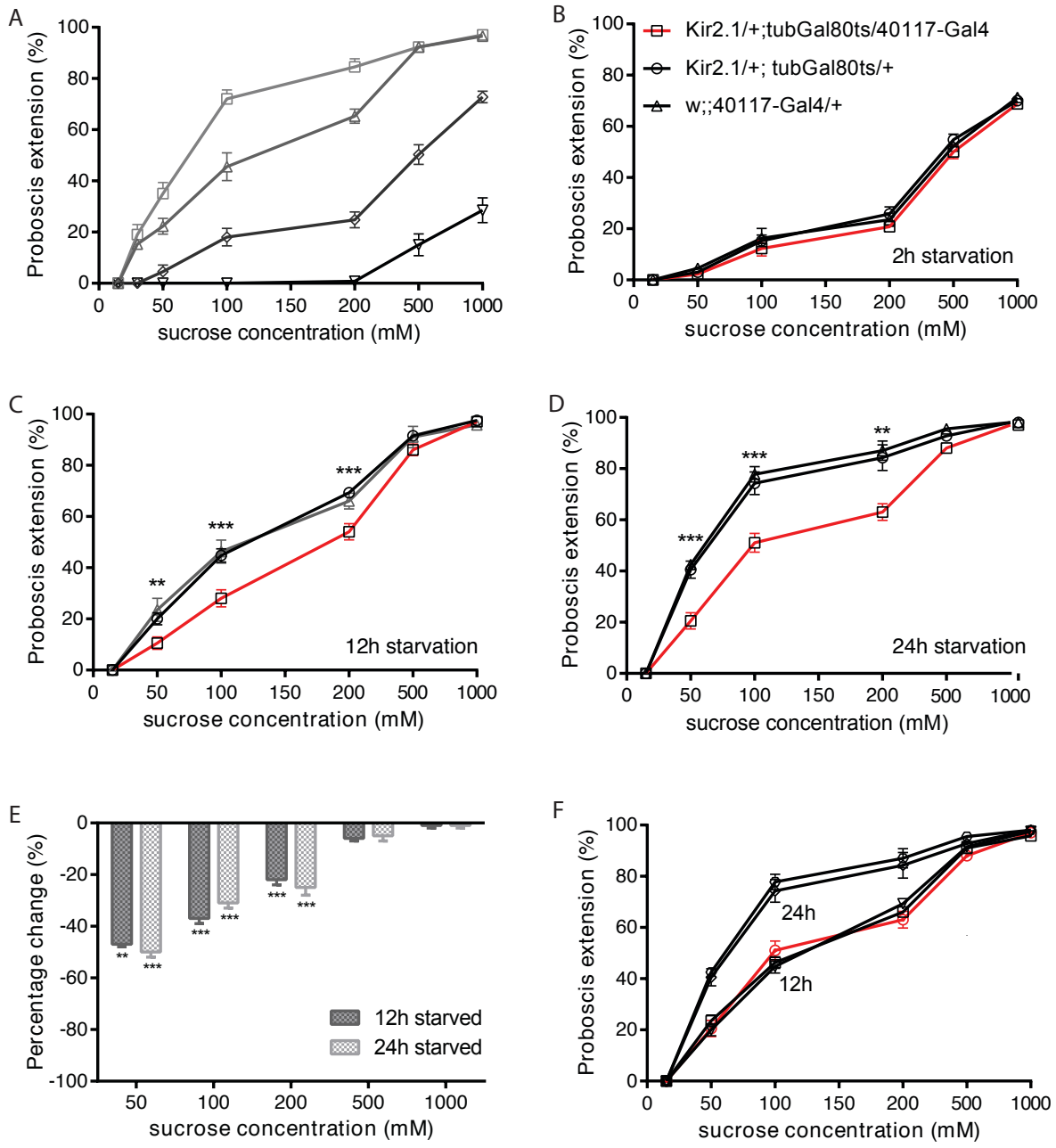


Figure 1. Silencing line R40117 impairs sucrose sensitivity in starved animals.

A) PER response curves of wild-type flies in response to a series of sucrose concentration under different starvation time. Error bars represent SEM.

B-D) PER response curves of R40117-Gal4 flies expressing UAS-Kir2.1 at adult stage and indicated controls to a series of sucrose concentration after 2h starvation (B), 12h starvation (C) and 24h starvation (D). Flies were raised at 18°C and shifted to 31°C experiments for 3 days prior to behaviour. Experiments were performed at 31°C. Each data point represents n = 4 trials of 15 flies each. Two-way ANOVA followed by post hoc t-test with Bonferroni correction at each sugar concentration. $*p < 0.05$; $**p < 0.005$, $***p < 0.001$. Error bars indicate SEM.

E) Plot of changes in decrease of sweet sensitivity calculated as (silencing lineR40117 - control)/ control under different starvation time. Controls Kir2.1/+; tub $Gal80^{ts}$/+ were used for quantification.

F) An overlay of PER response curve of R40117-Gal4 flies expressing UAS-Kir2.1 at adult stage after 24h starvation on the PER response curves of control flies at 2h, 12h and 24h starvation time to different concentrations of sucrose.

Figure 2

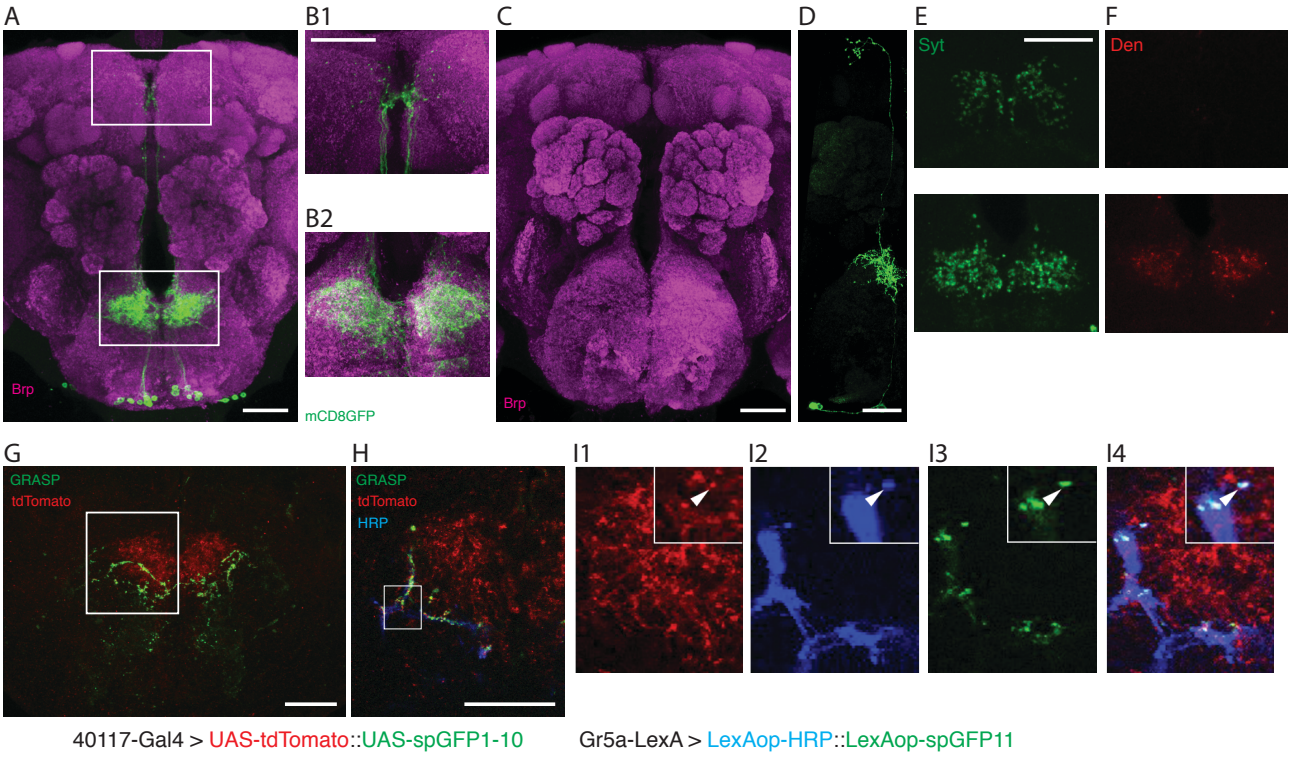

A
B1
B2
C
D
E
F
Brp
mCD8GFP
Brp
Syt
Den
G
H
I1
I2
I3
I4
GRASP
tdTomato
GRASP
tdTomato
HRP
40117-Gal4 > UAS-tdTomato::UAS-spGFP1-10
Gr5a-LexA > LexAop-HRP::LexAop-spGFP11

Figure 2. R40117-Gal4 line expresses in VM neurons which are in close proximity to projections of Gr5a sweet receptor neurons.

A) Whole brain image of line R40117-Gal4 visualised with mCD8::GFP. The neuropil is stained with Brp in magenta.

B) VM neurons in the central brain have broad aborisations B1) in the dorsal medial SEZ and terminal processes B2) in the dorsal medial brain.

C) Whole brain image of line R40117-Gal4 combined with Cha-Gal80. All Gal4 expression visualised with mCD8::GFP is eliminated.

D) A mosaic clone depicting one single VM neuron expressing mCD8-GFP. R40117-Gal4 line expressing the presynaptic marker UAS-Syt-GFP (E) and the postsynaptic marker UAS-DenMark (F). Images E and F are the same regions as the rectangles show in (A), indicating the broad aborisations in the middle and terminal processes in the dorsal brain.

G) Endogenous GRASP signal (in green) between 40117-Gal4 (labeled by tdTomato in red) and Gr5a-lexA line.

H) A single lane of the confocal stack depicting the part shown by the inset in image (G) from flies of genotype (Gr5a-LexA; UAS-tdTomato::UAS-spGFP1-10; LexAop-HRP::LexAop-spGFP11/40117-Gal4). VM neurons are labeled by tdTomato in red and Gr5a neuronal projections are labeled by HRP in blue. A single punctum expressing tdTomato, HRP and GRASP (I1,2,3,4) is indicated by an arrowhead and represents the putative synaptic connection. Scale bars represent 20 μm in all images.

Figure 3

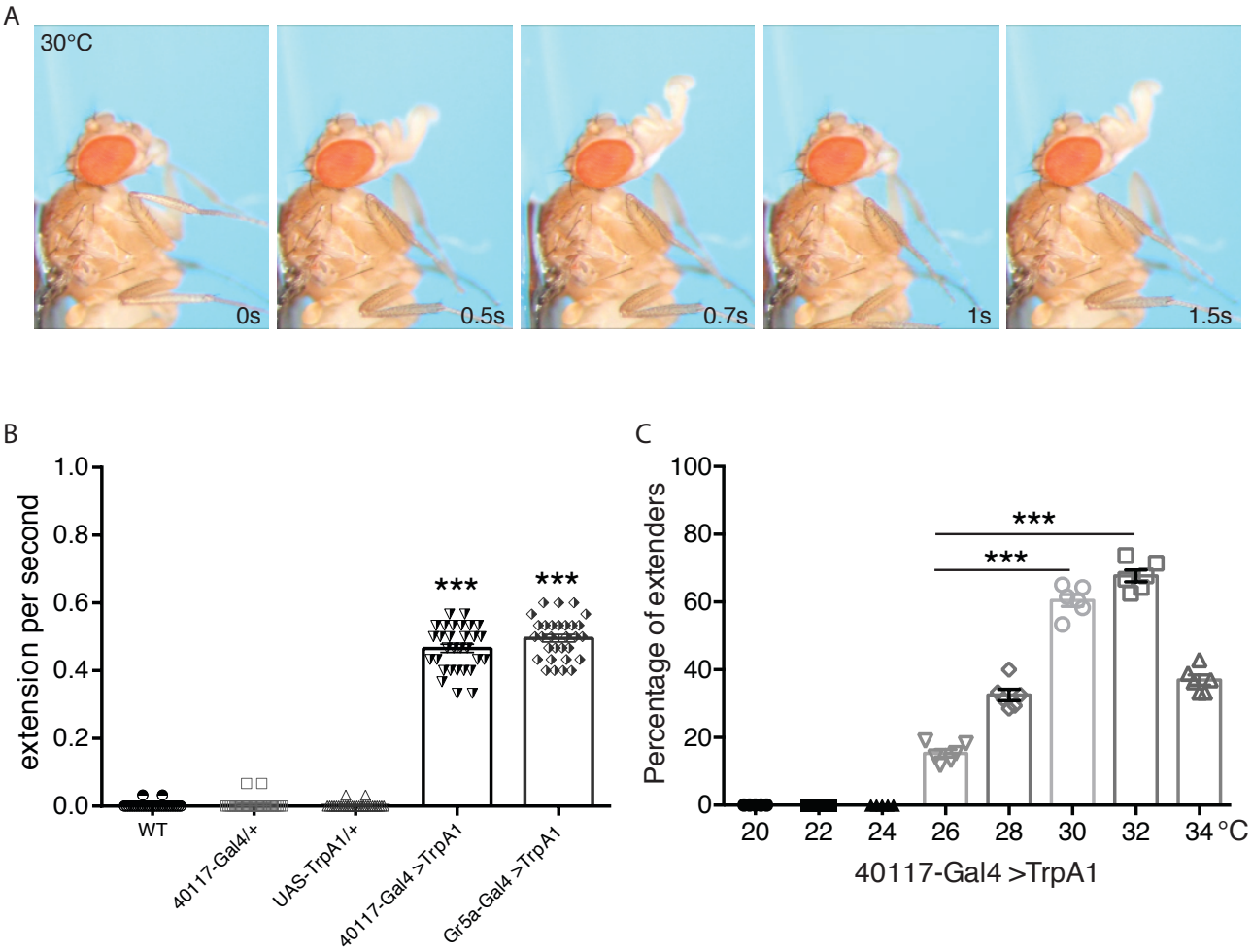


Figure 3. TrpA1-dependent activation of R40117-Gal4 Neurons Triggers Repetitive Proboscis Extension

A) Snapshots of UAS-dTRPA1; R40117-Gal4 flies showing proboscis extension at restrictive temperature (30°C).

B) Proboscis extension frequency per second, each bar represents the denoted genotype, n=30 for each genotype. The proboscis extensions of saturated flies in the first 30s were counted one by one after 2minutes in the heating chamber. ***$p < 0.001$, one way ANOVA followed by Tukey's HSD test. Error bars indicate SEM.

C) Percentage of extenders in R40117-Gal4 > UAS-TrpA1 flies at a series of temperatures. Each bar represents 5 trials, n=15 ~ 20 flies per trial. ***$p < 0.001$, one way ANOVA followed by Tukey's HSD test. Error bars indicate SEM.

Figure 4

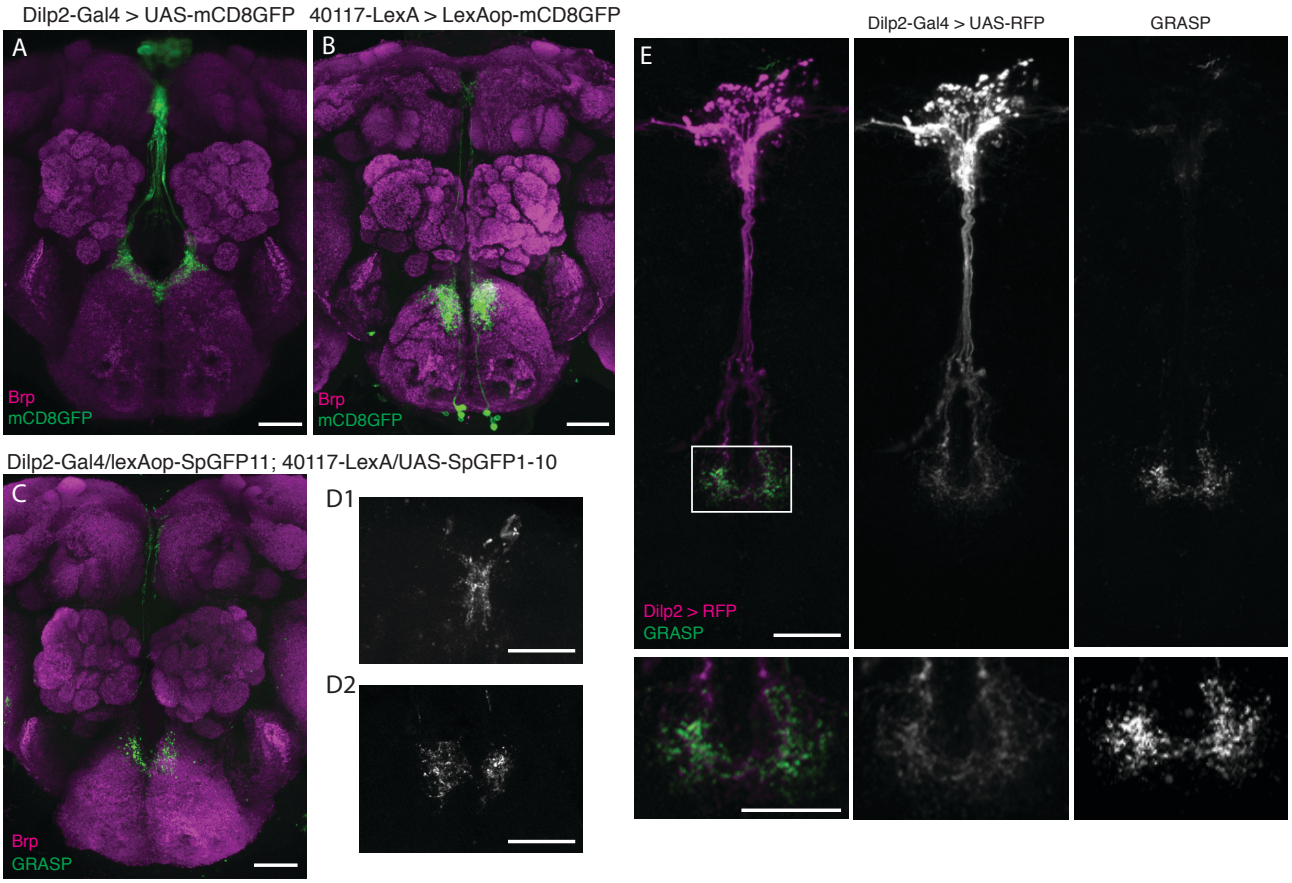


Figure 4. VM neurons are in close proximity to insulin-producing cells (IPCs).

A) Whole brain image of line Dilp2-Gal4 showing insulin producing cells that produce Dilp2 peptide. The neuropil is stained with Brp in magenta.

B) Whole brain image of line 40117-LexA which expresses in 8~12 VM neurons and largely recapitulate expression pattern of 40117-Gal4 line.

C) Endogenous GRASP signal detected (genotype: Dilp2-Gal4/lexAop-spGFP11; 40117-LexA/UAS-spGFP1-10) at both protocerebrum (D1) and dorsal medial SEZ region (D2). In unfixed brains, endogenous GRASP signals are found to overlap with RFP signals labelling Dilp2 cells (E). Scale bars represent 20 μm in all images.

Figure 5

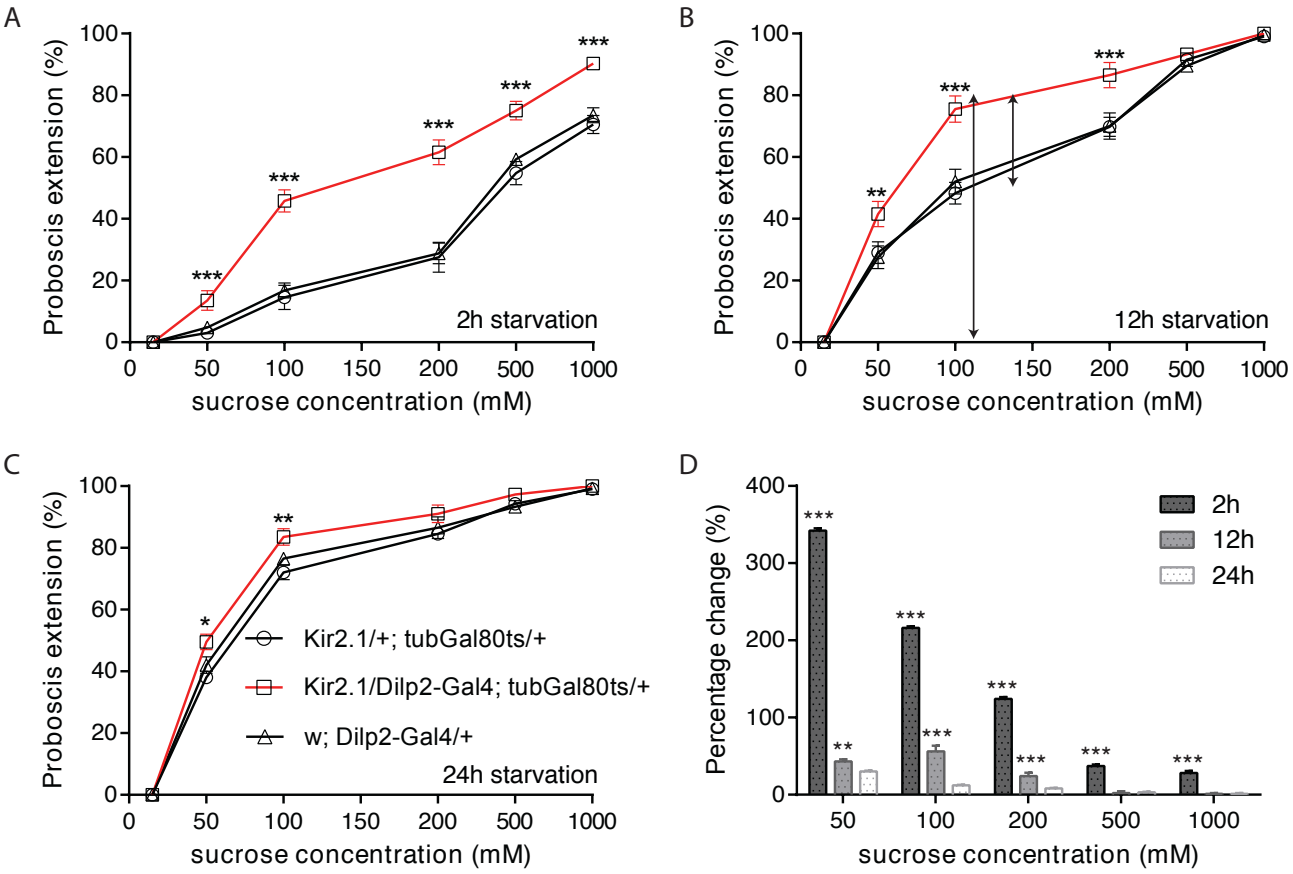


Figure 5. Silencing of IPCs increases sweet sensitivity in a starvation-dependent manner

A) PER response curves of Dilp2-Gal4 flies expressing UAS-Kir2.1 at adult stage and indicated controls to a series of sucrose concentrations after 2h starvation, 12h starvation (B), and 24h starvation (C). Flies were raised at 18°C and shifted to 31°C after eclosion for 3 days prior to behavioural experiments. Experiments were performed at 31°C. Each data point represents n = 4 trials of 15 flies each. Two-way ANOVA followed by post hoc t-test with Bonferroni correction at each sugar concentration. *$p < 0.05$; **$p < 0.005$, ***$p < 0.001$. Error bars indicate SEM.

D) Plot of changes in decrease of sweet sensitivity calculated as (silencing IPCs - control)/control under different starvation time. Controls Kir2.1/+; tub Gal80^{ts}/+ were used for quantification. The remarkable decrease is observed in response to 50mM (~3.5 fold) and 100mM (~2.2 fold) in slightly-starved animals.

Figure 6

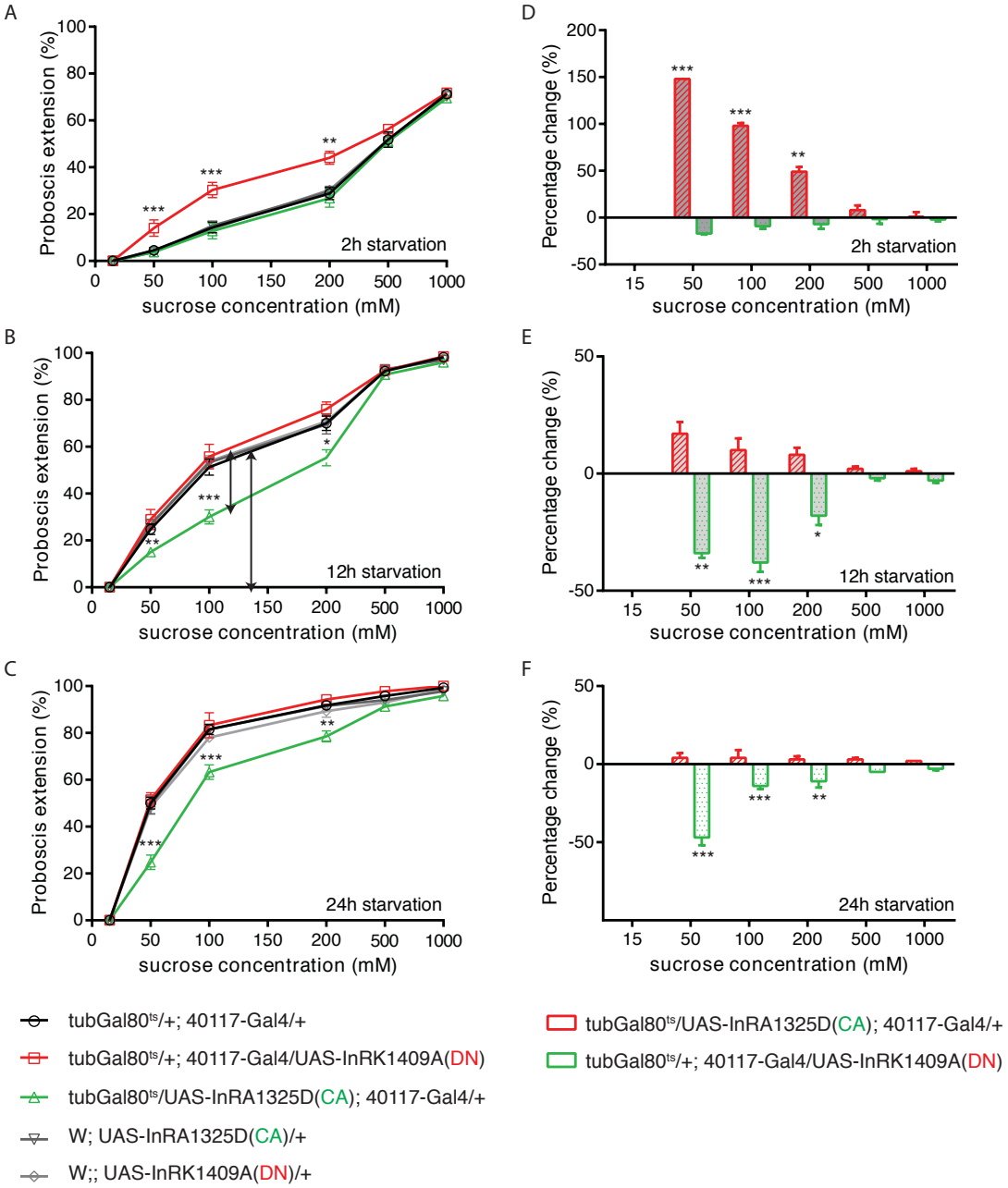

A
Proboscis extension (%)
sucrose concentration (mM)
2h starvation
B
12h starvation
C
24h starvation
D
Percentage change (%)
E
F
tubGal80ts/+; 40117-Gal4/+
tubGal80ts/+; 40117-Gal4/UAS-InRK1409A(DN)
tubGal80ts/UAS-InRA1325D(CA); 40117-Gal4/+
W; UAS-InRA1325D(CA)/+
W;; UAS-InRK1409A(DN)/+

Figure 6. Blocking insulin signaling in VM neurons increases sweet sensitivity especially in slightly starved animals.

A-C) PER response curves of tub- Gal80^{ts}; 40117-Gal4 flies expressing either UAS-InR.K1409A (DN) shown in red or UAS-InR.A1325D (CA) shown in green and indicated controls to a series of sucrose concentrations after 2h starvation (A), 12h starvation (B), and 24h starvation (C). Flies were raised at 18°C and shifted to 31°C after eclosion for 3 days prior to behavioural experiments. Experiments were performed at 31°C. Each each point represents n = 4 trials of 12 ~15 flies each. Two-way ANOVA followed by post hoc t-test with Bonferroni correction at each sugar concentration. *$p < 0.05$; **$p < 0.005$, ***$p < 0.001$. Error bars indicate SEM.

D) Plot of changes in sweet sensitivity calculated as (UAS-InR.DN - control)/control or (UAS-InR.CA - control)/control under 2h starvation, 12h starvation (E), and 24h starvation (F). Controls tub- Gal80^{ts}/+; 40117-Gal4/+ were used for quantification. Remarkable increases in sweet sensitivity are observed after expressing UAS-InR.DN in slightly-starved animals and decreases after expressing UAS-InR.CA in intermediately-starved and highly-starved animals.

Figure 7

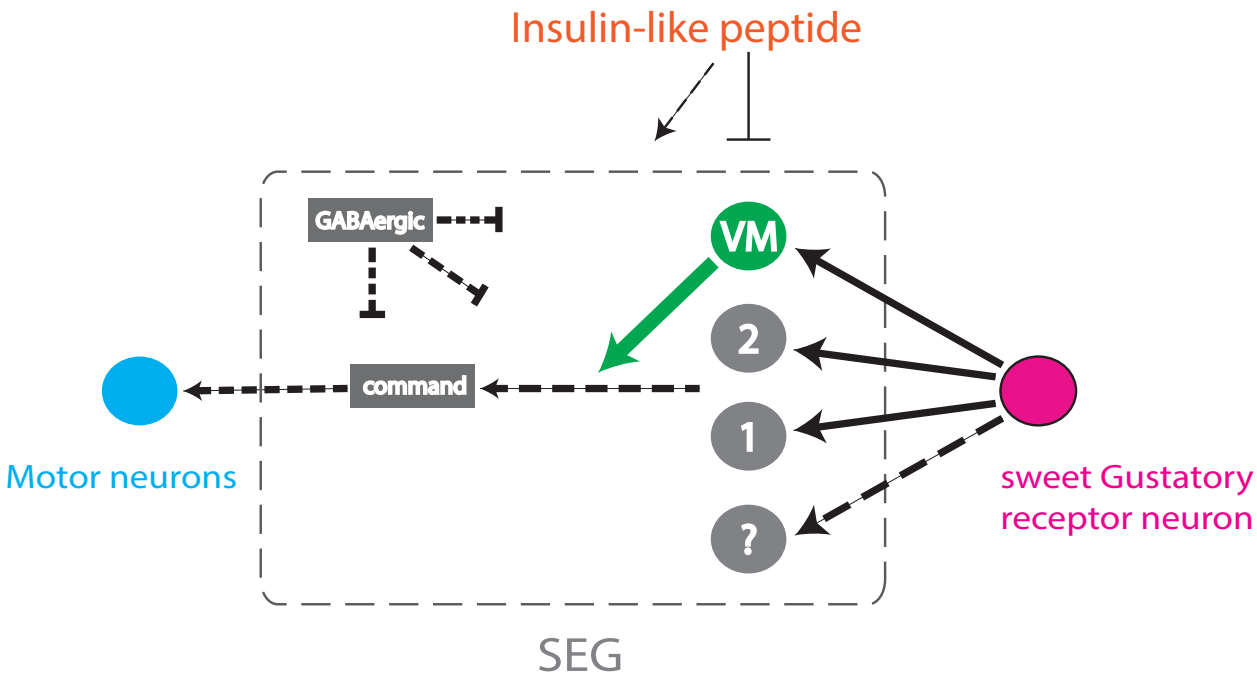


Figure 7. Schematic model of gustatory circuit from sweet receptor neurons to motor neurons. In addition to the previously identified first-order gustatory relay neurons, feeding command interneurons and GABAergic modulatory neurons, VM neurons represent a new class of gustatory neurons receiving input from sweet receptor neurons and modulated by insulin-like peptides.

Supplementary Figures

Supplementary Figure 1

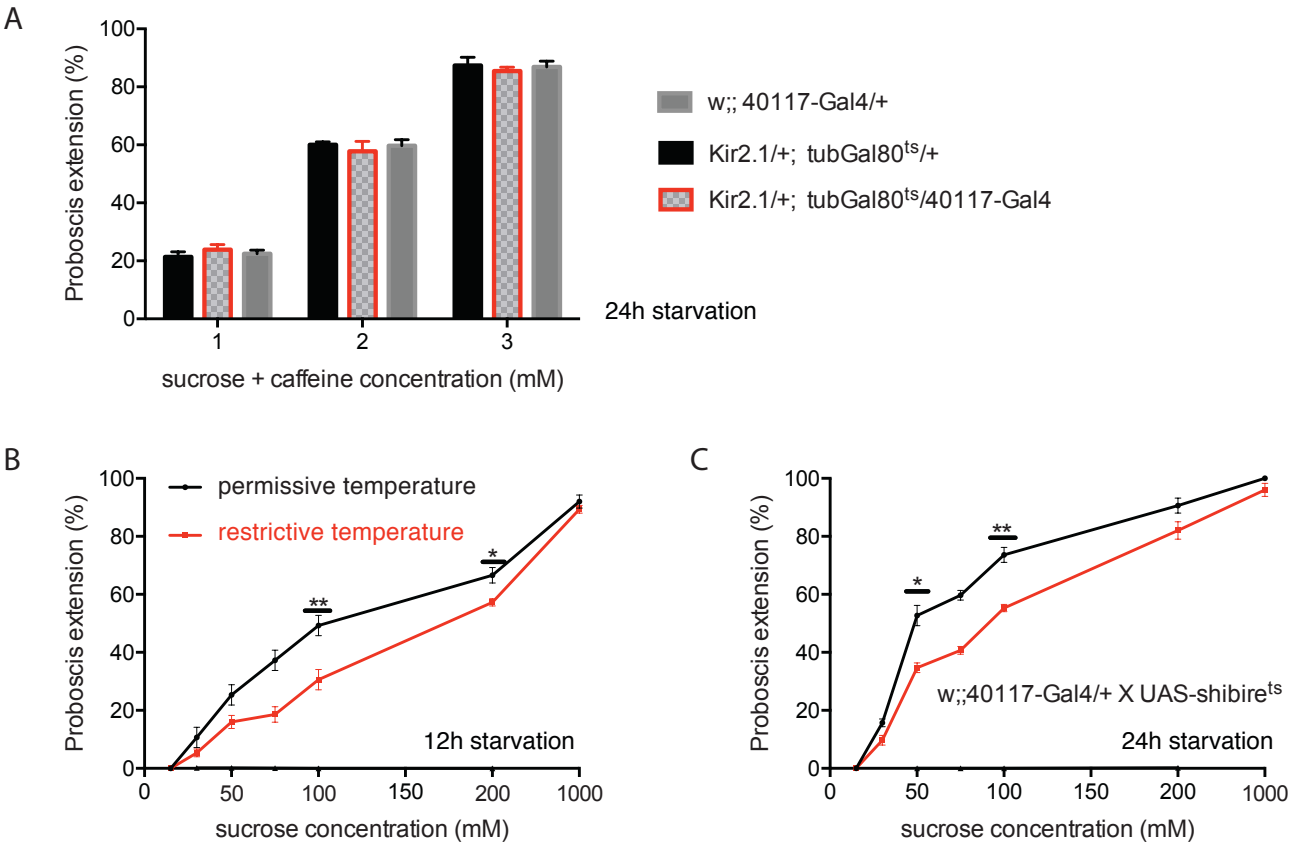


Figure 1S Chronic silencing of line R40117 did not affect bitter sensitivity and acute silencing of line R40117 reduced sweet sensitivity.

A) PER response curves of R40117-Gal4 flies expressing UAS-Kir2.1 at adult stage and indicated controls to a mixture of sucrose and different concentrations of caffeine after 24h starvation. Flies were raised at 18°C and shifted to 31°C experiments for 3 days prior to behaviour. Experiments were performed at 31°C. Each data point represents n = 3 trials of 15 flies each. Two-way ANOVA followed by post hoc t-test with Bonferroni correction at each sugar concentration. Error bars indicate SEM.

B-C) PER response curves of R40117-Gal4 flies expressing UAS-shibire^{ts} at permissive temperature (in black) and restrictive temperature (in red) to a series of sucrose concentration after 12h starvation (B) and 24h starvation (C). Flies were first tested at 22°C and then left at 30°C for 30minutes before repeated testing. Each data point represents 3 replicates of 24 flies. Students' paired t test at each sucrose concentration. $*p < 0.05$; $**p < 0.005$. Error bars indicate SEM.

Supplementary Figure 2

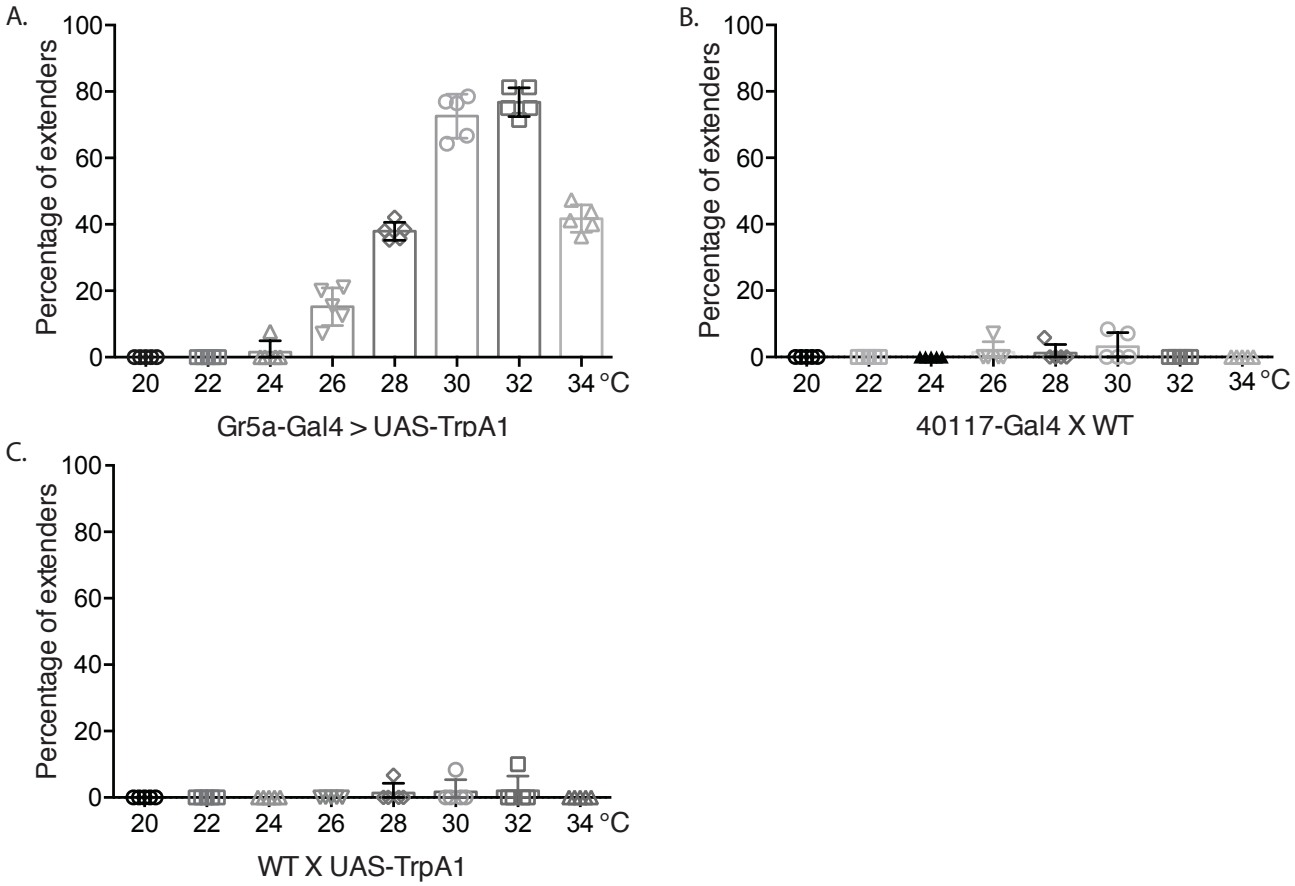


Figure 2S Temperature-course of Proboscis extension upon TrpA1-dependent inducible activation

A. Percentage of extenders in Gr5a-Gal4 > UAS-TrpA1 flies at a series of temperatures. Each bar represents 5 trials, n= 15 ~ 20 flies per trial. Error bars indicate SEM. The temperature course of Gr5a-Gal4 > UAS-TrpA1 flies is similar to it of R40117-Gal4 > UAS-TrpA1 flies.

B. Percentage of extenders in 40117-Gal4 X WT flies at a series of temperatures. Each bar represents 5 trials, n= 10 ~ 20 flies per trial. Error bars indicate SEM.

C. Percentage of extenders in UAS-TrpA1 X WT flies at a series of temperatures. Each bar represents 5 trials, n= 10 ~ 20 flies per trial. Error bars indicate SEM.

Supplementary Figure 3

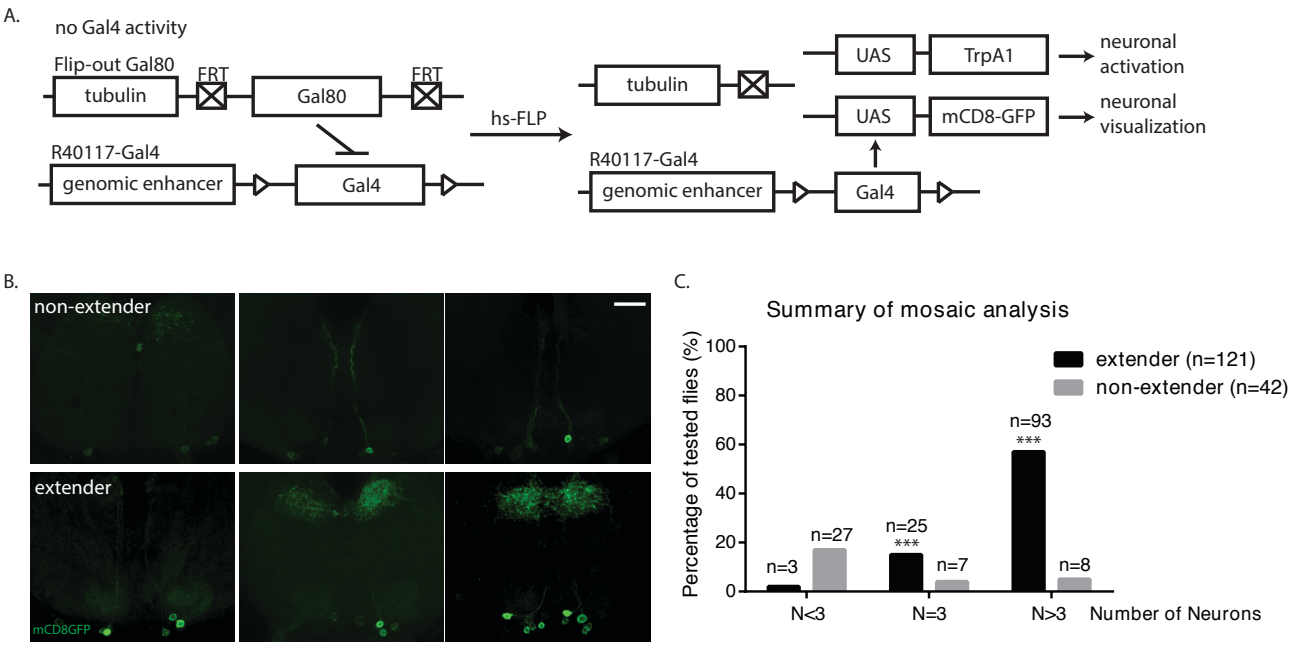


Figure 3S At least three VM neurons are required to elicit repetitive proboscis extension

A. Schematic illustration of the mosaic analysis strategy using hs-Flp; UAS-dTrpA1/tub > Gal80 >; R40117-Gal4/UAS-mCD8::GFP flies.

B. Representative brains from flies showing no extension (non-extenders) or extension at restrictive temperature (extenders). Scale bar represents 20 μm.

C. The summary plot of the frequency distribution of extender flies (black bars, n=121) and non-extender flies (grey bars, n=42) in three categories according to number of labeled neurons: N<3, N=3, N>3 (Fisher's exact test, p=1.7098238803442235e-17).

Supplementary Figure 4

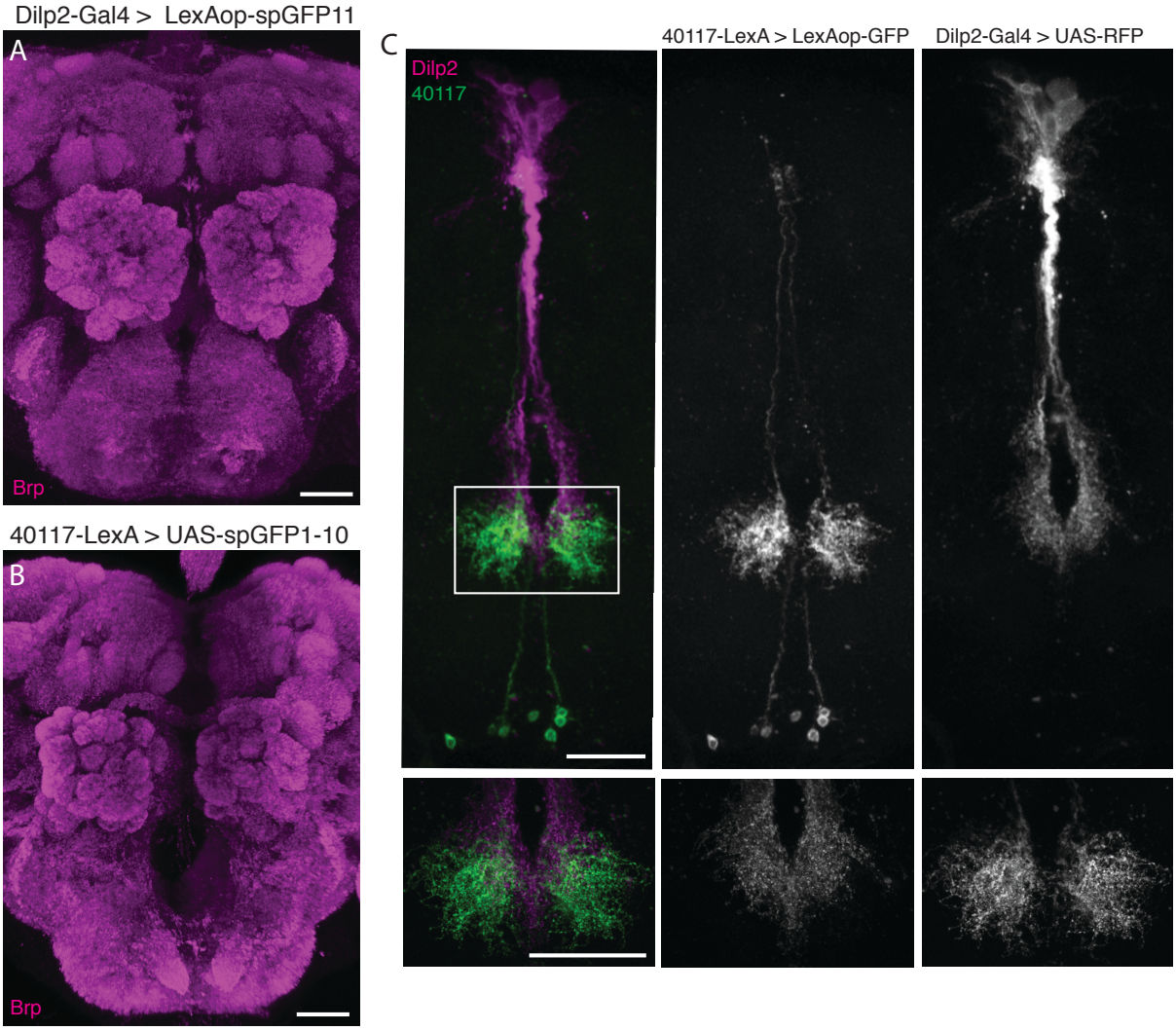


Figure 4S Overlay of insulin producing cells and VM neurons

A. Whole brain image of genotype (Dilp2-Gal4 > LexAop-spGFP11), no GRASP was detected.

B. Whole brain image of genotype (40117-LexA > UAS-spGFP1-10), no GRASP was detected.

C. An overlay of insulin producing cells (IPCs) and VM neurons in one brain by co-labeling IPCs with endogenous RFP signal and VM neurons with endogenous GFP signal. Scale bar represents 20 μm.

Supplementary Figure 5

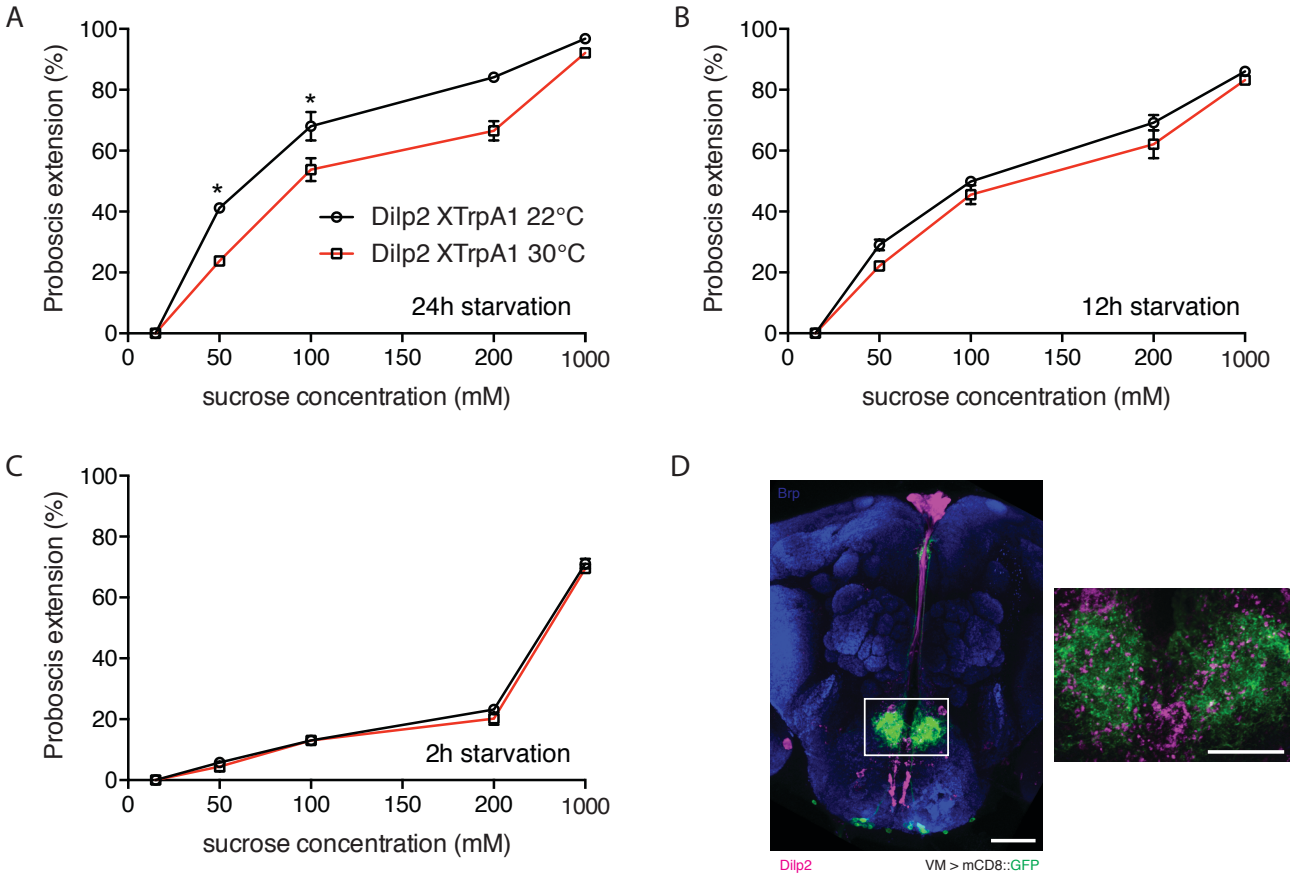


Figure 5S TrpA1-dependent inducible activation of IPCs reduces sweet sensitivity in highly starved animals.

A. PER response curve of UAS-TrpA1; R40117-Gal4 flies after 24h starvation. Flies were first tested at 22°C and then left at 30°C for 30minutes before testing with the same series of sucrose concentrations presented to flies at 22°C. For each data point, n = 2 trials of 11 flies each. Error bars indicate SEM. Students' paired t test at each sucrose concentration, $*p < 0.05$.

B. PER response curve of UAS-TrpA1; R40117-Gal4 flies after 12h starvation. Everything else remained the same as tests after 24h starvation.

C. PER response curve of UAS-TrpA1; R40117-Gal4 flies after 2h starvation. Everything else remained the same as tests after 24h starvation.

D) Staining of Dilp2 (magenta) in the brain of R40117-Gal4; UAS-mCD8::GFP flies. The neuropil is stained with Brp in blue. The inset clearly shows that Dilp2 peptide permeates the middle arborisations of VM neurons in puncta. Scale bars represent 20 μm.

Supplementary Figure 6

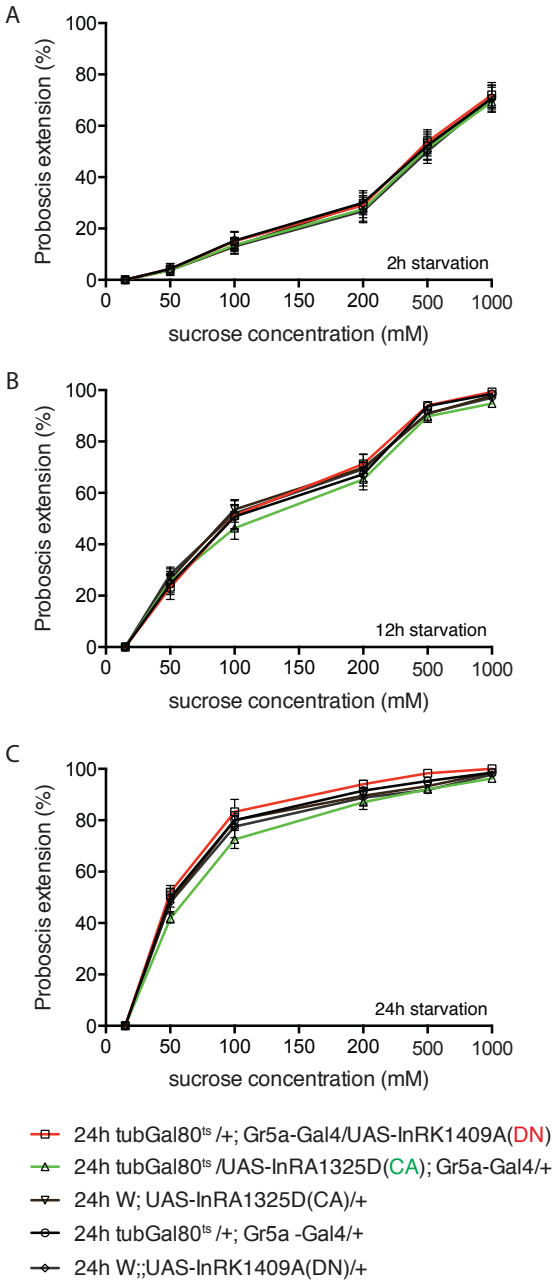


Figure 6S PER responses after manipulation of insulin signaling in Gr5a neurons.

A-C) PER response curves of tub- Gal80^{ts}; Gr5a-Gal4 flies expressing either UAS-InR.K1409A (DN) shown in red or UAS-InR.A1325D (CA) shown in green and indicated controls to a series of sucrose concentrations after 2h starvation, 12h starvation (B), and 24h starvation (C). Flies were raised at 18°C and shifted to 31°C after eclosion for 3 days prior to behavioural experiments. Experiments were performed at 31°C. Each each point represents n = 4 trials of 12 ~15 flies each. Error bars indicate SEM.

Supplementary Figure 7

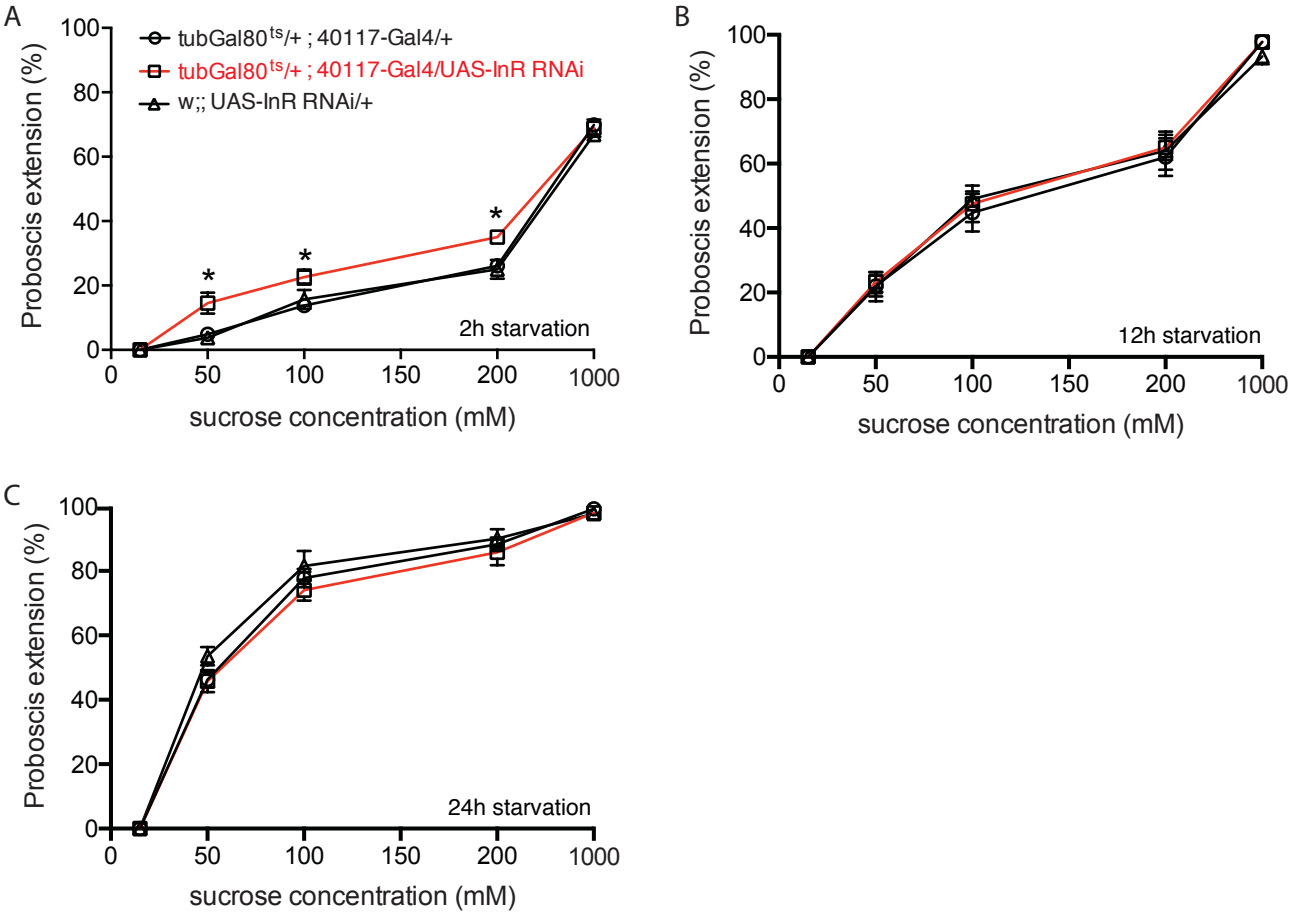


Figure 7S PER responses after knockdown of insulin signaling in VM neurons

A-C) PER response curves of tub-Gal80^{ts}; 40117-Gal4 flies expressing UAS-InR RNAi shown in red and indicated controls to a series of sucrose concentrations after 2h starvation, 12h starvation (B), and 24h starvation (C). Flies were raised at 18°C and shifted to 31°C after eclosion for 3 days prior to behavioural experiments. Experiments were performed at 31°C. Each each point represents n = 6 trials of 12 ~15 flies each. Two-way ANOVA followed by post hoc t-test with Bonferroni correction at each sugar concentration. *$p < 0.05$; **$p < 0.005$, ***$p < 0.001$. Error bars indicate SEM.

3.2 Identification and characterisation of other gustatory interneurons.

Our ultimate research goal is to understand the principles underlying the development and function of a simply circuit connecting sensory stimuli to motor behaviour. We are particularly interested in how the central circuit integrates different taste information and make an appropriate choice. In our TrpA1-inducible activation screen of over 200 Gal4 lines, we identified other interesting lines expressing Gal4 in gustatory interneurons. These interneurons were categorised into three groups: interneurons contributing to sweet taste behaviour, to bitter taste behaviour and to the integration of sweet and bitter information.

3.2.1 Sweet taste behaviour: Gal4 lines expressing interneurons similar to VM neurons

We identified two other Gal4 lines, R49904 and R49146 labelling a cluster of interneurons similar to VM neurons from the Rubin collection. Activation of these interneurons is sufficient to elicit repetitive complete extension of the proboscis while inhibition renders flies insensitive to attractive stimuli. Subpopulations of these interneurons are directly connected to sweet sensing GRNs. These interneurons likely represent first-order gustatory interneurons controlling sweet taste behaviour. The enhancer fragment for line R40117 lies upstream of gene B-H1, which encodes a homeobox binding domain. The enhancer fragments for line R49146 and line R49904 lie upstream of gene milt and gene Dll, which encodes the N-terminal domain of huntingtin-associated protein 1 (HAP1) and a homeobox DNA-binding domain respectively.

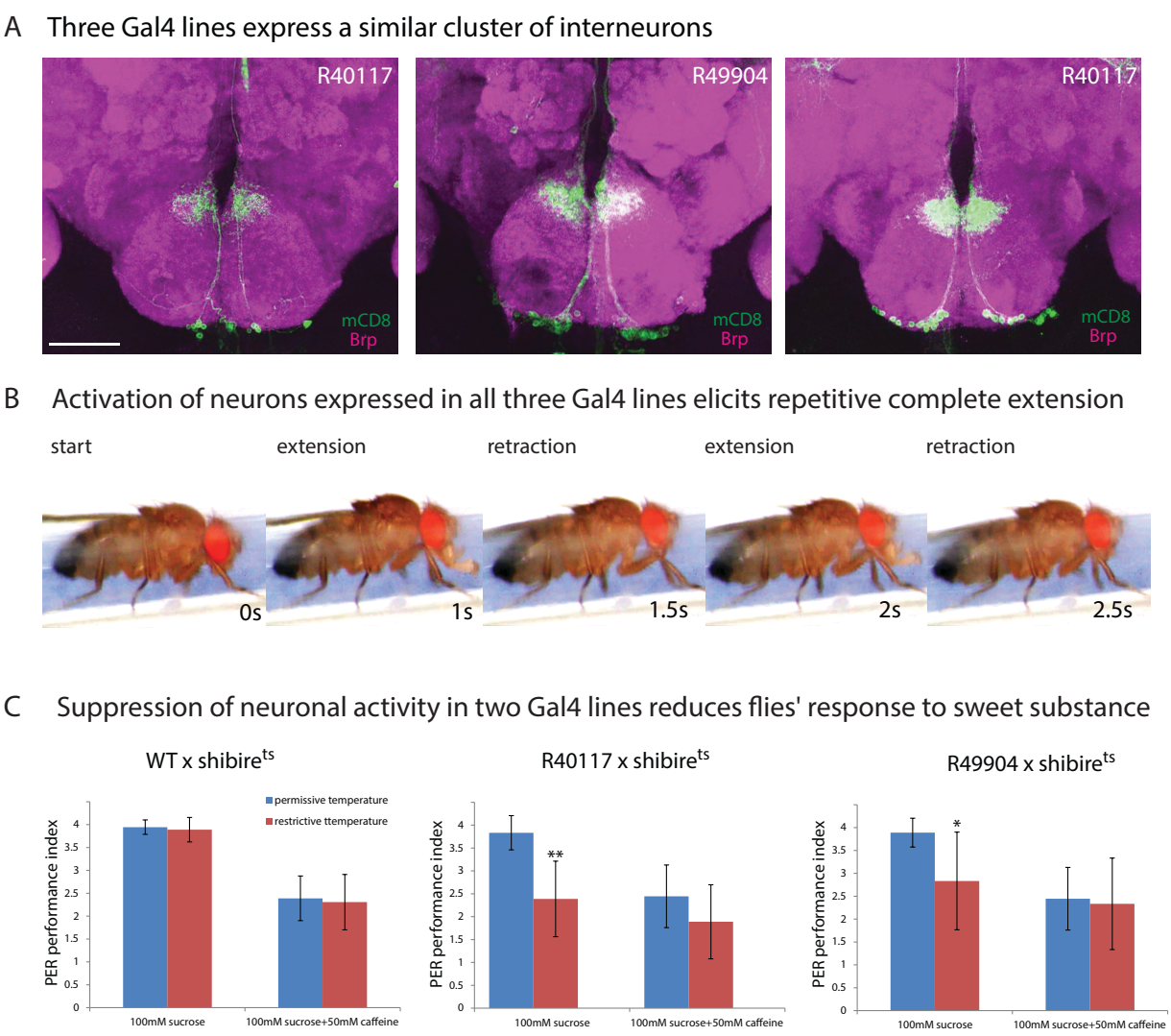


Figure 3.2.1 A) expression patterns of line R40117, R49904, R49146 visualised with 10XUAS-mCD8::GFP. Scale bar represents 50 µm. B) TrpA1-dependent activation of all three Gal4 lines was sufficient to elicit repetitive proboscis extension. Example snapshots show flies of genotype *UAS-TrpA1; 49146-Gal4* in a freely walking chamber at 30°C. C) Results of PER assays examining acute inhibition of these three lines impairs response to sucrose in the case of R40117 and R49904 but responses to bitter caffeine were not affected. A different scoring system was adopted in these assays in which 0 represents no extension, 1 represents a twitch, 2 represents one complete proboscis extension, 3 represents two complete proboscis extensions and 4 represents more than two complete proboscis extensions. This system results in large variance in comparison to the system counting extension probability. Nonetheless with both measuring schemes, the trend that acute inhibition impairs sweet sensitivity was recapitulated.

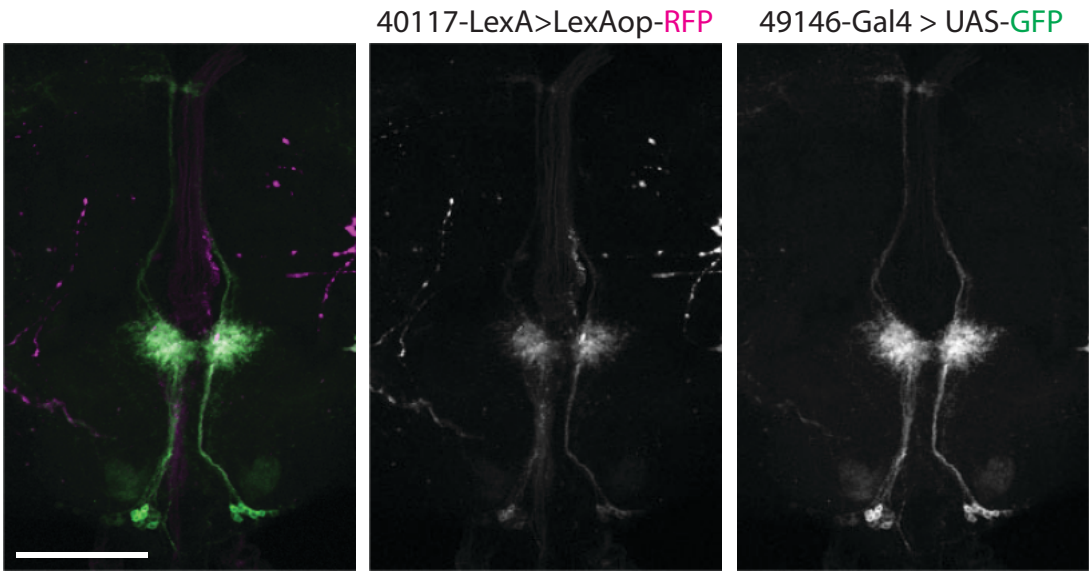


Figure 3.2.2 40117-Gal4 and 49146-Gal4 are expressed in the same neurons.

There are about 6 ~ 8 neurons, 12 ~ 16 neurons, 14 ~ 19 neurones expressing in line R49146, R40117 and R49904 respectively. To examine whether neurons labelled in line R49146 are the same neurons labelled in line R40117, we used 40117-LexA and 49146-Gal4 to drive LexAop-RFP and UAS-mCD8::GFP respectively in the same fly. We found that in all neurons expressing GFP there is magenta colour, suggesting that all neurons expressed in 49146-Gal4 are VM neurons. Scale bar represents 50 μm.

3.2.2 A second class of gustatory interneurons.

We identified three Gal4 lines labelling a different cluster of interneurons. Activation of all three lines via TrpA1 elicits prolonged complete extension of the proboscis. Interestingly in freely walking chambers, flies expressing TrpA1 in line R39523 could walk while extending proboscis at restrictive temperature. Previously a study reported that locomotion could suppress proboscis extension via activation of one pair of interneurons in the VNC (Mann, Gordon et al. 2013). It suggests that interneurons labelled in line R39523 process information after signal of location from VNC is integrated into the circuit. GRASP experiments demonstrated that interneurons expressed in line R39523 are in close proximity to both Gr5a and Gr66a gustatory receptor neurons. From the mosaic analysis, one flip-out clone expressing Gal4 in one cluster of unilateral neurons are sufficient to elicit complete extension after TrpA1-inducible activation. This unilateral cluster of interneurons are anatomically very similar to the secondary lineage SA1 in subesophageal ganglion (Kuert, Hartenstein et al. 2014). The enhancer fragment for line R49077 lies upstream of gene GRHRII, which encodes the corazonin receptor. The enhancer fragments for line R39523 and line R49158 lie upstream of gene mtt and gene abba, which encodes the metabotropic glutamate receptors and a ring finger domain protein respectively.

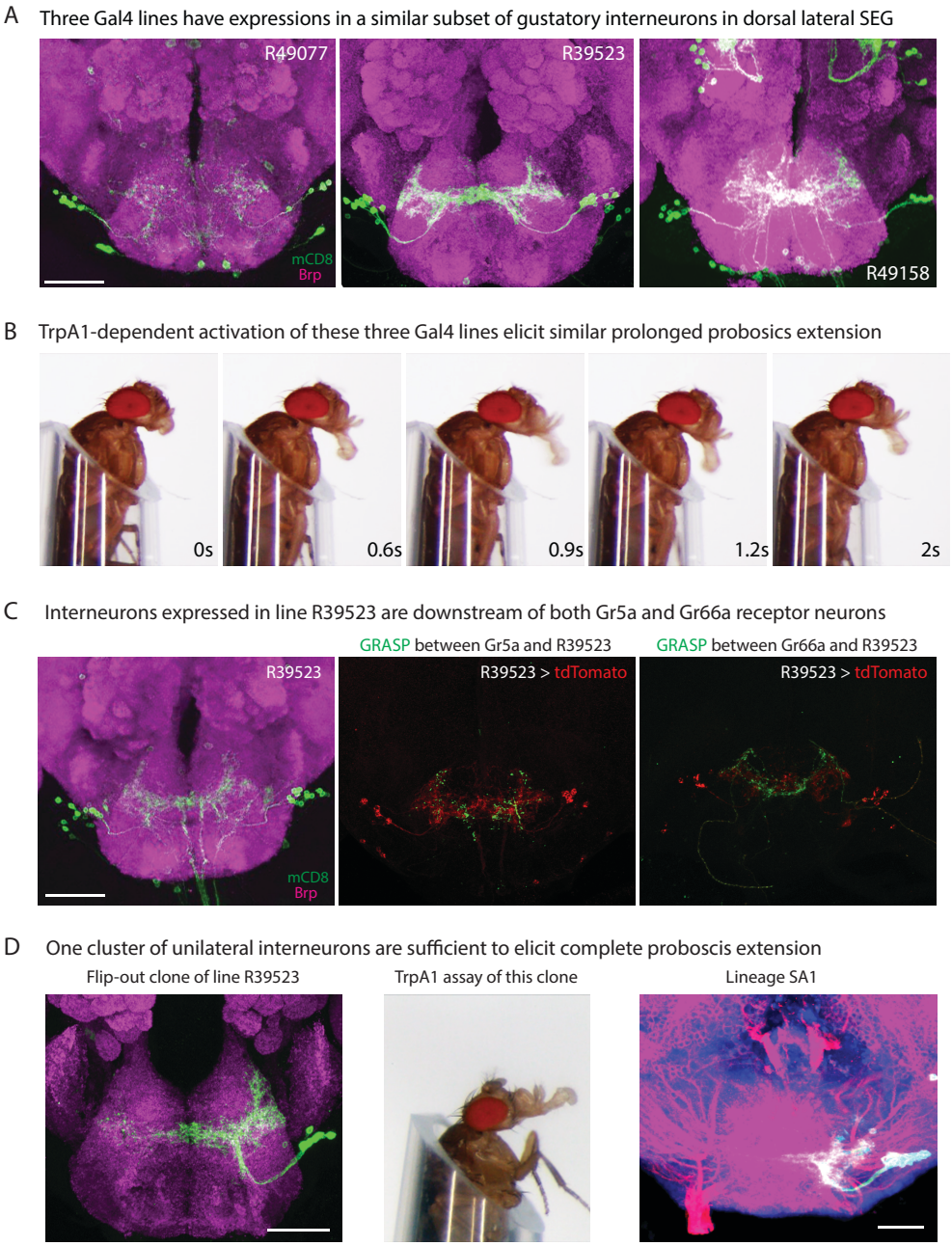


Figure 3.2.3 Identification of a second class of interneurons in the dorsal lateral SEZ.

A) expression patterns of line R40117, R49904, R49146 visualised with 10XUAS-mCD8::GFP. B) TrpA1 activation assay in which flies are fixed in a pipette tip. C) GRASP signals are endogenous from the reconstituted GFP. Tdtomato is stained against RFP antibody. D) Flip-out clone and the analysis was performed similarly as it with line R40117. Lineage SA1 is a MARCM clone stained with BP104 (photo courtesy of Volker Hartenstein). All scale bars represent 50 μm.

3.2.3 Potential interneurons that integrate sweet and bitter taste information

We identified a single Gal4 line which contains potential neurons contributing to the integration of sweet and bitter taste information, line R48613. The enhancer fragment for line R48613 lies upstream of gene trpm, which encodes a transient receptor potential channel. TrpA1-dependent activation of this line elicits repetitive complete proboscis extension. Acute silencing renders flies insensitive to both sweet and bitter stimuli. We postulate that the interneurons in this line either embody or lie downstream of where sweet and bitter taste information converge. An alternative explanation is that these interneurons impair general taste sensation, regardless of taste modality.

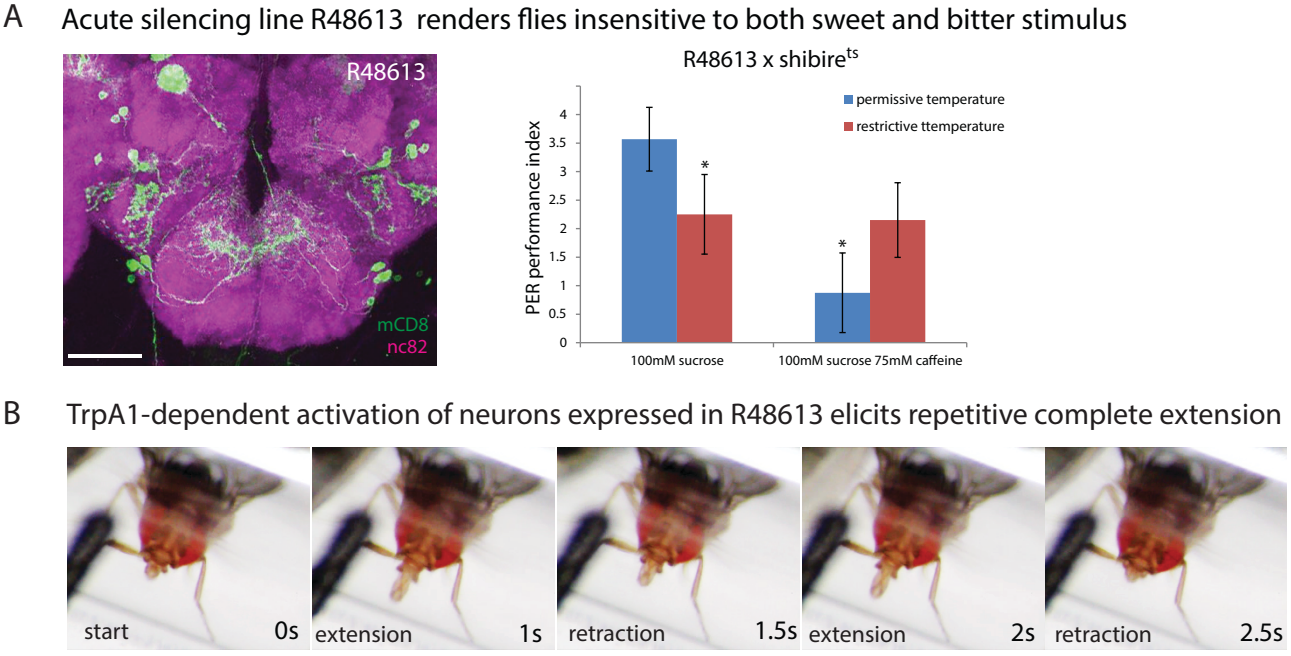


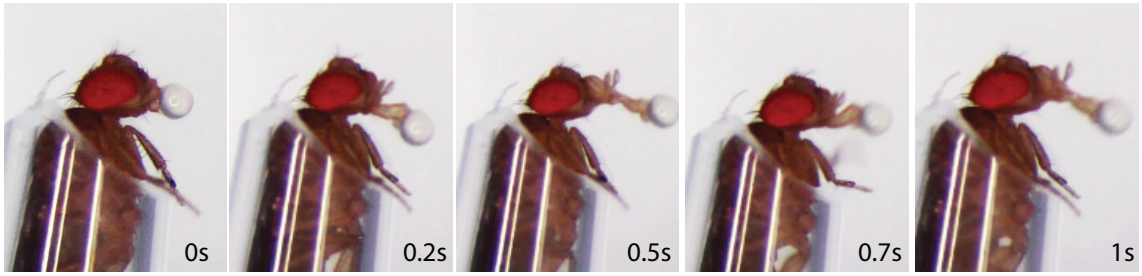
Figure 3.2.4 Identification of potential integrative interneurons.

A) expression patterns of line R48613 visualised with 10XUAS-mCD8::GFP. PER assay was analysed using the 0-4 scoring scheme. B) TrpA1 activation assay in which flies display repetitive extension in a freely walking chamber. Scale bar represents 50 µm.

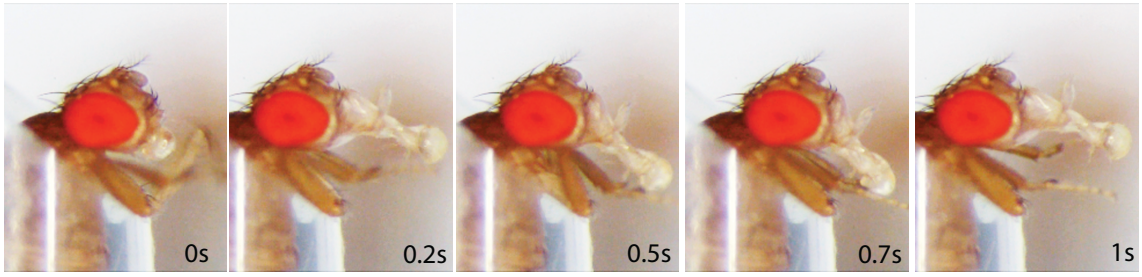
3.2.4 Potential interneurons mediating bitter taste behaviour

We identified a Gal4 line that exhibited prolonged extension but also developed a drop at the tip of labellum after TrpA1-dependent activation (which is an indicator that flies feel nauseous). The enhancer fragment for line R49128 lies upstream of gene lozenge, which encodes a runt domain that has DNA binding and transcriptional factor activity. This pattern resembles the behavioural pattern when activating Gr66a bitter GRNs. Inhibition of these neurons impaired aversive responses. Interestingly, activation of Gr66a bitter GRNs results in a decrease of proboscis extensions responses to sweet stimuli. These two assays, silencing Gal4 lines in search for increased bitter responses and activating Gal4 lines in search for a drop at the tip of labellum, can be used in combination to identify additional interneurons specifically mediating aversive gustatory responses.

A TrpA1-dependent activation of Gr66a sensory neurons elicits prolonged extension and a drop.



B TrpA1-dependent activation of line R49128 elicits prolonged extension and a drop at the labellum



C Screen paradigm for potential interneurons mediating aversive taste behaviors

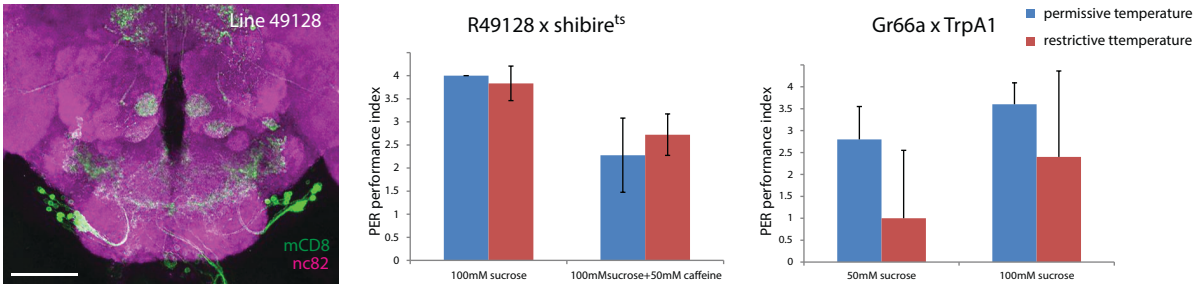


Figure 3.2.5 Identification of potential interneurons mediating bitter taste behaviour. TrpA1 assay, PER assay, and immunostaining are similarly perform as before. Scale bar represents 50 μm.

3.2.5 Summary

In our initial behavioural screen, we identified several interneuron Gal4 lines that are sufficient to elicit the complete proboscis extension responses. We identified two classes of first-order interneuron (VM from R40117 and DL from R39523) which relay information to high-order interneurons or motor output. Line R39523 contains a second set of potential first-order DL (dorsal-lateral) neurons which are sufficient to elicit prolonged complete proboscis extension after TrpA1-dependent activation. DL neurons are connected to both sweet and bitter GRNs.

In the future, we will examine whether VM and DL interneurons can be directly activated by sweet or bitter stimuli using calcium imaging. We will further map the connectivity between VM/DL interneurons and downstream interneurons (potential premotor interneurons).

Further we identified line R48613 containing interneurons which are potentially required for integration of attractive and aversive stimuli and line R49128 expressing interneurons which might be involved in bitter-taste circuit. With the two established behavioural assays, we could identify more interneuron lines specifically required for the aversive pathway.

With availability of Gal4 lines which express interneurons mediating aversive behaviour, we will be able to answer questions such as whether attractive and aversive taste information converge on certain integrating interneurons or they remain separated throughout the circuit. Mapping connectivity of these interneurons will offer novel insights into how information is relayed and processed within a central gustatory circuit.

In addition split-Gal4 and flip-out methods could be used in the future to identify the essential interneurons mediating the different taste behaviours in these Gal4 lines. GRASP assays will enable us to identify potential connectivity between these interneurons and premotor interneurons. Finally we will combine these studies with functional imaging (Ca^{2+}). Together, these approaches will allow us to identify the principles underlying the development and function of a simple circuit connecting sensory stimuli to motor behaviour.

3.3 Measuring food intake after manipulating gustatory interneuron activity.

3.3.1 Potential mechanisms regulating feeding behaviour.

A key aspect for metabolic homeostasis is the balance between food intake and energy expenditure. There are many parameters about feeding behaviour from taste sensitivity (feeding threshold), food preference, feeding frequency, meal size to feeding termination. The interaction between physiological state and other motivational processes influence feeding decisions. We found that silencing VM neurons impairs sweet sensitivity in starved animals, silencing IPCs increases sweet sensitivity in satiated animals and blocking insulin signaling in VM neurons increases sweet sensitivity in satiated animals. We also wants to examine whether these manipulates lead to changes in food intake and whether there is a relationship between food intake and taste sensitivity.

To sense and satisfy physiological signals of basic survival needs, it is expected that organisms evolve functional components called interoceptive neuron populations, which monitor circulating levels of the nutrients and hormones that reflect energy status of an animal. In addition, interoceptive neuron populations could communicate with the feeding circuit at different levels such as receptor neurons or interneurons to determine when to eat, what to eat and how much to eat.

Many peptide hormones and their receptors have been shown to be important in regulating feeding behaviour and maintaining energy homeostasis. Some of these peptides may serve as messengers between the interoceptive neurons and the feeding circuit. In drosophila, neuropeptides implicated in regulating feeding behavior include the NPY homolog NPF which promotes food intake (Wu, Zhao et al. 2005), hugin which regulates feeding initiation (Melcher and Pankratz 2005), leucokinin (Al-Anzi, Armand et al. 2010) which promotes feeding termination and allatostatinA (Hergarden, Tayler et al. 2012) which inhibits feeding without changing metabolism. NPF as a signal of the hunger state could relieve the tonic dopamine (DA) inhibition of the mushroom body from six identified MB-MP neurons and promote appetitive memory (Krashes, DasGupta et al. 2009). High insulin level in a satiety state leads to reduced sensitivity to food odour by diminishing sNPF peptide signalling in olfactory sensory neurons (Root, Ko et al. 2011).

Thus we propose that a potential mechanism for regulating feeding behaviour and maintaining energy homeostasis functions as follows: when animals are hungry, interoceptive neurons sense the low internal energy levels and transmit the information to the neuromodulatory system which then acts only the feeding circuit to initiate food foraging and feeding. After feeding, high levels of nu-

trients in the circulatory system as well as distension of the digestive system signal that animals are satiated. The related neuropeptides then modulate the circuit to eventually terminate feeding.

3.3.2 Methods to measure food consumption in drosophila

Despite the existence of powerful genetic toolbox and a rich repertoire of behaviour assays, precise measurements of food intake remain challenging in drosophila. So far there are several common methods: the Capillary Feeder (CAFE) (Ja, Carvalho et al. 2007), food labelling with a radioactive tracer or colorimetric dye, and observations of proboscis extension (Deshpande, Carvalho et al. 2014) . Most recently a high-resolution food ingestion assay, called Expresso, is reported. This Expresso system could measure ingestion of individual flies in real time at nanoliter resolution (Yapici, Cohn et al. 2016).

1) Food-labelling with colorimetric dye

In this method, soluble and non-absorbable dyes are mixed into food. Animals are homogenised after feeding on the labelled food and absorbance of the dye in solution is used to quantify the volume of consumed food. The rationale behind this method is the Beer-lambert law, which relates the absorption of light to the properties and concentration of the material through which the light is travelling.

We used the dye Brilliant Blue FCF which has been shown not to influence feeding and measured absorption at 630 nanometers. We further made a calibration curve proving that absorption of light is linearly correlation with concentration of the dye (Figure 3.3.1). However we noticed this dyes can be quickly excreted and coloured fecal spots appeared in less than 30minutes after exposure to coloured food. Therefore dye-labelling method could only reflect the volume of consumed food in the initial phase of accumulation. It was reported that dye labelling can only detect large feeding differences (about 100% or larger) and thus has limited resolving power (Deshpande, Carvalho et al. 2014).

Food consumption was calculated using the Beer-lambert law. In general, summed absorption intensity for 20 flies in 30minutes-feeding is between 0.4 and 0.8, which equals to between 0.125µl and 0.2528µl volume ingested and between 0.43µg and 0.865µg sucrose consumed per fly. In comparison, previous study using CAFE assay reported that each fly consumes 1.4µg sucrose on average (Ja, Carvalho et al. 2007).

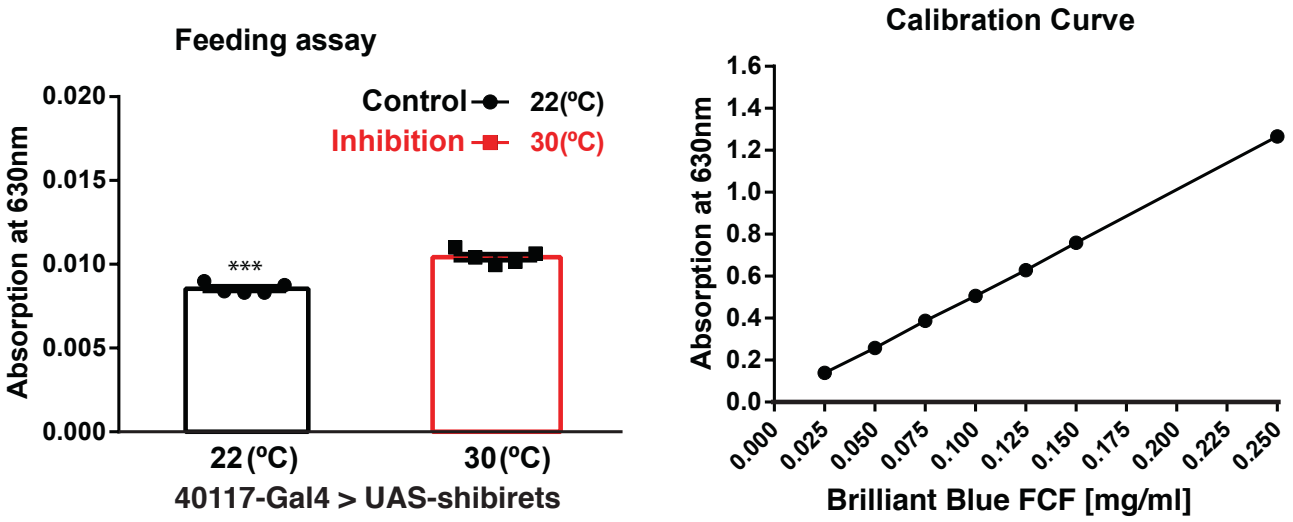


Figure 3.3.1 Measurement of food intake with dye-labelling method.

Flies were tested at 7 days old. After eclosion, adult flies were raised on experimental food mixed with dye to habituate for four days prior to testing. They were transferred to fresh food every day. Feeding assay was performed from 10am in the morning on food (1% agrose, 10mM sucrose, 1.25mg/ml blue dye). After feeding for 30minutes, feeding was interrupted and flies were immediately dissected for the crop and gut. We put the crop and gut from 20 flies to 20µl 50% ethanol (ethanol made in 1× PBS with 0.01% Triton X-100) in a 1.7ml Eppendorf tube and homogenised with a disposable pestle and mortar. After centrifugation to remove the debris, the supernatant was measured for absorption at 630nm using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific). Flies fed on non-labelled food were used as controls and were subtracted from experimental readings. Homogenisation of the crop and gut instead of the whole fly significantly reduces the variance and increases the resolving power of dye-labelling method.

A) Feeding assays with flies of genotype 40117-Gal4 > UAS-shibire^{ts} simultaneously at restrictive and permissive temperatures for 30minutes. Five independent trials with 20 flies in each trial were tested. Unpaired two-tailed t-test was used to assess statistical significance. ***$p < 0.001$. Error bars indicate SEM.

B) Calibration curve ($R^2 = 0.9999$) was obtained by measuring absorption of a serial dilutions of FCF blue dyes in 20µl 50% ethanol.

2) Food-labelling with radioisotope tracers

Radioisotope-labelling is has been used for a long time to monitor fly feeding (Geer, Olander et al. 1970, Thompson and Reeder 1987, Thompson, Reeder et al. 1991) . It has several advantages: 1) no need for homogenisation as radioisotopes can be detected through the cuticle; 2) radioisotope when used at low concentration does not affect ingestion volume; 3) variability with this method has been shown to be very low; 4) when the radioisotope 32P was used, its high sensitivity allows measuring consumption of individual flies.

Most importantly in contrast to dyes which are not absorbed by the gut and excreted quickly after feeding, radioactive labels may be absorbed in the digestive system and thus accumulates as feeding time increases. Among various labels, 32P-dCTP shows very high retention rate over several days without plateauing (Deshpande, Carvalho et al. 2014).

We analysed both the absolute volume of food consumed and relative amount between groups (normalised against control). The results show that chronic silencing with UAS-Kir2.1 significantly increase food consumption. Intriguingly both inactivation and constitutive activation of insulin signaling in VM neurons significantly reduces food consumption, suggesting a different modulatory system from solely inhibition of VM neurons or other indirect consequence after manipulating insulin signaling in VM neurons (Figure 3.3.2).

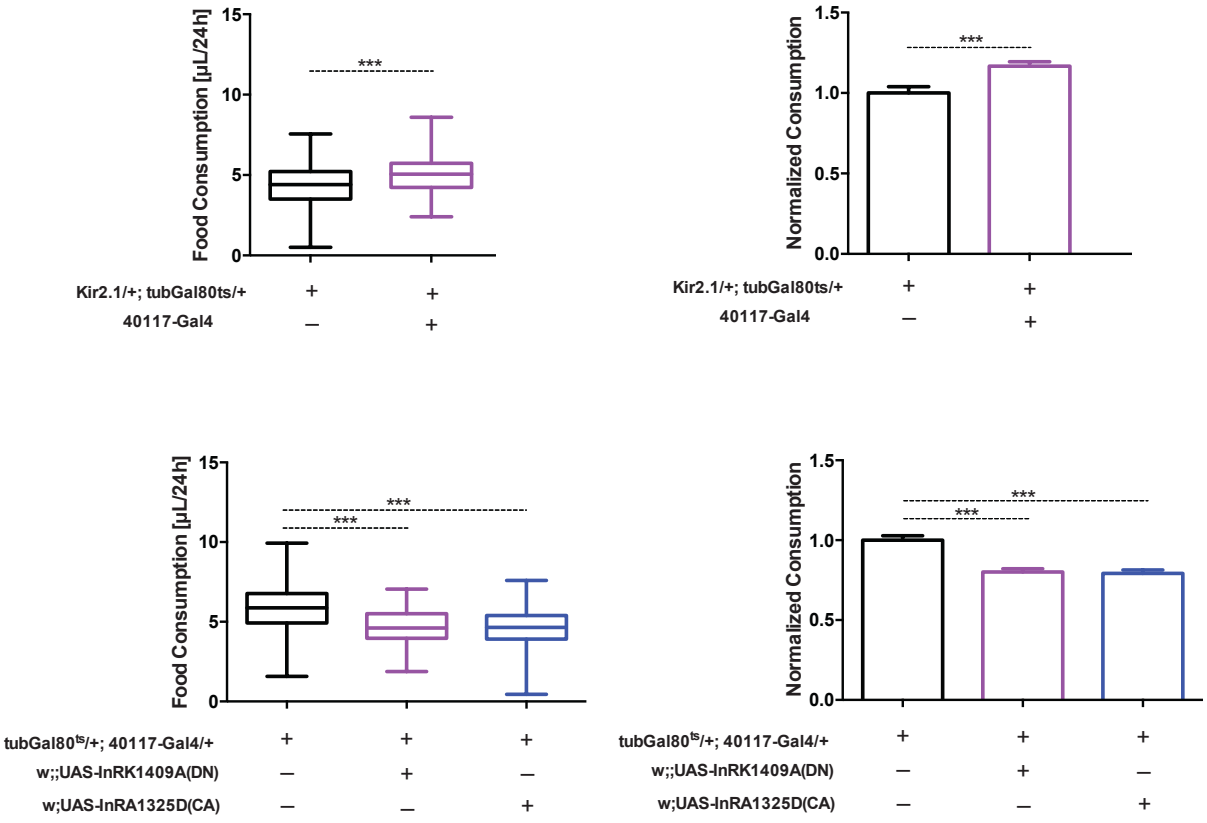


Figure 3.3.2 Measurement of food intake with radioactive labels.

We used 32P-dCTP as labels to test ad libitum feeding in animals with VM neurons silenced and insulin signaling manipulated in VM neurons. In general flies were habituated on the experimental food for one day prior to testing. To examine steady-state feeding, flies were not starved and transferred to empty vials after feeding for 24h. Then flies were first allowed to groom for 15 minutes to clean the radioactive remnant from themselves and then flies were killed by freezing, counted, and then assayed in the scintillation fluid in a Multi-Purpose Scintillation Counter. For each condition, 3 independent trials with 18–20 flies each were tested. Unpaired two-tailed t-test and one ANOVA with post hoc Tukey test was used to assess statistical significance. ***$p < 0.001$. Error bars indicate SEM.

3) CAFE assay

The Capillary Feeder assay, the CAFE assay, could measure precise, real-time measurement of ingestion by individual or grouped flies on the scale of minutes to days. In this assay, flies were fed from a glass micro-capillary, enabling direct measurement of consumption (Ja, Carvalho et al. 2007) . Food is presented in liquid form. One potential caveat is that animals were forced to feed from upside-down as opposed to normal feeding on solid food on the bottom. However overall results obtained with the CAFE assay are very reproducible and CAFE assay has therefore facilitated many studies of feeding behaviour.

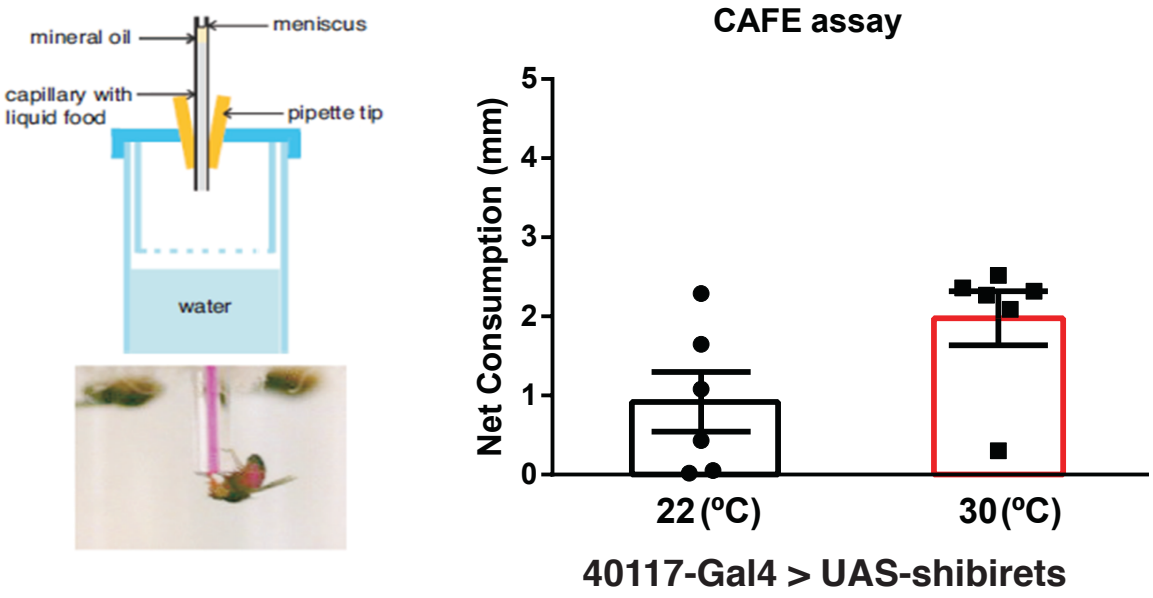


Figure 3.3.3 Measurement of food intake with CAFE assay.

The original CAFE assay setup is as shown in the schematic diagram. In our adapted setup, 4 flies were put in one 15ml vial with a cotton plug on top which allows air exchange. Two 200 µl pipette tips were inserted through the cotton plug and used to hold the glass capillary filled with 50mM sucrose solution. Both capillaries were topped with mineral oil to minimise evaporation. Kimpwipe tissues soaked up with water placed on the bottom was used to provide humid environment. All the experimental vials were placed in a large airtight Tupperware rubber storage container. The container has a thick layer of water-soaked tissue paper to create a highly humid environment.

Flies were habituated in this setup for two days prior to testing and were water-starved on the second day. The test was performed for 30minutes with capillary filled with sucrose and 1.25mg/ml blue dye in order to better visualise solution in the capillary and to compare with dye-labeled feeding assay. The position of the top meniscus of the solution was marked. In each condition, at least six vials were tested simultaneously with two vials empty of flies as controls for evaporation. The average decrease in distance seen in six capillaries subtracted from the average decrease in distance seen in the two controls, is used to estimate the consumed volume of food. Five independent trials with 6 flies in each trial were performed under two temperatures. Unpaired two-tailed t-test was used to assess statistical significance. ***$p < 0.001$. Error bars indicate SEM.

4) Expresso assay

Recently an automated version of the CAFE assay, the Expresso assay, which captures nanoliter-volume meal-bouts of single flies simultaneously with high temporal resolution. Expresso consists of multiple single-fly feeding chambers, each connected to a sensor bank. When a fly drinks from the capillary, Expresso analysis software automatically detects individual meal-bouts in real time by measuring the rapid decrease in liquid level (Yapici, Cohn et al. 2016).

5) Comparison of dye-labelling, radioactive tracer labelling and CAFE assays.

The CAFE and radioisotope-labelling provide the best sensitivity (the smallest change in feeding that could be detected) and reproducibility over a broad range of conditions. Dye-labelling is relatively inferior. Further radioisotope-labelling requires the fewest samples to distinguish a feeding difference, followed by CAFE and dye-labelling. The main limitation for radioisotope-labelling method is that it underestimates consumption by 5–25%. CAFE assay could be used to complement radioisotope-labelling and further validates the results as it measures direct food intake instead of relying on food marker.

In addition, currently there are only methods for measuring food consumption or ingestion in drosophila. To better understand how organisms control energy homeostasis, techniques that assess nutrient absorption and assimilation need to be developed in the future.

6) Summary

We used three different methods, dye-labelling, radioisotope-labelling and CAFE assay, to measure total food consumption after genetic manipulation in VM neurons. When silencing VM neurons,

results from three methods consistently showed increase in food intake, from starved flies feeding for a short term and from ad libitum fed flies feeding for a long term. A possible explanation is that flies compensate the decrease in perceived food palatability by over-consuming food. Concordant with the possibility, it has been show that flies consume a larger volume of food when the food is diluted than not (Deshpande, Carvalho et al. 2014). It suggests VM neurons convey taste information of sweetness (reflected in sucrose concentration in our experiments).

4. Discussion

4.1 VM neurons provide a non-linear gain to the gustatory circuit

In drosophila, attractive taste stimuli (sweet, low-salt) elicit proboscis extension and aversive taste stimuli (bitter, high-salt) lead to flies' avoidance in general. These responses are regulated by internal nutritional state and can be modified by learning and experience. The primary gustatory circuit, which is hypothesized to process and integrate taste information with other information such as neuromodulation, remains largely unknown. This circuit receives taste input with limited attributes (meaning concentration of stimuli and taste modality) and generate distinct behavioral response (meaning proboscis extension or rejection) on individual level.

A functional circuit should generate outputs within certain bounds as the inputs from environment change drastically. At the integration point of synaptic and modulatory inputs, neurons must perform mathematical operations to transform inputs into output firing rate. Usually the mathematical operation is an additive operation which represents the sum of synaptic inputs. But in many cases multiplication is essential. For example, multiplication by a constant or the gain factor could produce a change in the slope of the input–output relationship by scaling up or down the sensitivity of the neurons. Within the circuit, if the gain factor at the integration points can be adjusted, it allows large space for modulation and increases the scope of responses that the circuit could yield. These integration points with non-linear gain factors contribute to the mechanism of adaptive gain control. Adaptive gain control is seen very often at the sensory periphery to adjust the gain of sensory neurons and compensate for external variations. It is also likely to be employed at higher levels of information processing, such as in the example of VM neurons, where gain changes is modulated by internal nutritional state.

Our results showed that VM neurons could modulate behavioural sensitivity to sucrose depending on the internal nutritional state of animals. VM neurons mediate sweet taste behaviour, not in a 'on-and-off' manner but rather play a fine-tuning role. VM neurons could elevate sweet sensitivity in starved animals, presumably through adding weight onto synapses with downstream neurons, and enable flies to respond to sugar within naturally available concentration range with behavioural sensitivity that meet internal physical needs.

4.2 Gustatory circuitry in maintaining metabolic homeostasis.

Animals use gustatory information to assess the safety and palatability of potential food, and then integrate the gustatory information with both short-term and long-term signals regarding nutritional states. In times of nutrients sufficiency and ample fat stores, some signals of energy surfeit such as insulin, leptin and long-chain free fatty acids, send input into key brain areas, leading to inhibition of energy intake and glucose production as well as simultaneous increase in energy expenditure. Conversely in times of nutrients unavailability and deficient energy store, brain activates responses that promote food intake, reduce energy expenditure, and raise circulating nutrient levels. In this way, through diverse afferent signals, information regarding nutrient status and energy stores is communicated to the brain and integrated with cognitive, visual, olfactory and taste cues to maintain energy homeostasis. There are mainly two neuronal systems governing these processes, the systems responsible for food-seeking (meal initiation) and satiety perception (meal termination) respectively. Under the coordination of these two systems, both the motivation to find food and the size of individual meals tend to increase in response to weight loss until energy stores restore.

Among the circulation signals that inform the brain of changes in body fat stores, leptin and insulin, which circulate at levels proportionate to body fat content, could promote weight loss by acting on neuronal systems implicated in energy homeostasis and block neuronal actions that increase food intake. In drosophila, insulin functions as a key signal of energy surfeit and thus a negative-feedback regulator of fuel stores whereas leptin has not been detected so far. Reduced leptin and insulin signaling in neurons induce hyperphagia and weight gain in rodent models (Bruning, Gautam et al. 2000, Munzberg, Flier et al. 2004)

The system that govern satiety perception includes gastrointestinal factors released upon gastric distension such as cholecyskinin (CCK), other satiety signals transmitted through the vagus nerve and the nucleus tractus solitarius (NTS) which promotes the termination of a meal. Leptin, as the long-term homeostatic adiposity signal, could on one side inhibits orexigenic neurons and activates anorexigenic neurons in the ARC which yields an overall negative signal to the downstream node NTS, and on the other side directly enhances the response of NTS to satiety signals. The combinatorial effect of long-term homeostatic signals and short-term satiety signals is decreased reward in feeding, satiated feeling and eventual meal termination.

In drosophila, similarly the system governing satiety perception constitutes the hormonal system and input from gastrointestinal system (or the crop and gut). The median abdominal nerve (MAN) transmit signals of crop distention, which regulates meal cessation. The recurrent nerve (RN) transmit signals of foregut contraction, which regulates meal initiation. The presence of food in the midgut, the high level of hemolymph sugar, and foregut contractions together produce a satiety effect. Hyperphagia happens when either MAN or the RN was severed. Insulin is the principal hormone that signal nutrient availability and orchestrate metabolic homeostasis. Cholecystokinin (CCK) signaling appears well conserved through evolution. In drosophila, the CCK-like sulfakinins regulate gut function, satiety, and food ingestion. It is released from the median neurosecretory cells that produce insulin-like peptides and have axon terminations in neurohemal regions of the corpora cardiaca and anterior aorta, crop duct and anterior midgut. In insets, a region in the dorsal midline of the brain, designated pars intercerebralis, contains a group of median neurosecretory cells (MNCs) that produce a number of peptide hormones. MNCs is considered functionally analogous to the hypothalamus. The DSK-expressing MNCs are a subpopulation of the 14 IPCs (Soderberg, Carlsson et al. 2012). Another neuropeptide that regulates food intake is leucokinin, which innervates the foregut. Mutant flies of leucokinin neuropeptide and leucoknin receptor genes take larger but fewer meals comparing to wild-type flies, suggesting a defect in meal termination that arises from impaired communication of gut distension signals to the brain (Al-Anzi, Armand et al. 2010). Leucokinin is homologous to vertebrate tachykinin and injection of tachykinin reduces food consumption. Leucokinin neurons express the insulin receptors (dInR) and the serotonin receptor 5-HT1B. Both over expression InR signaling and serotoninergic signaling reduce leucokinin peptide expression and lead to a decrease in feeding (Liu, Luo et al. 2015).

Circulating insulin-like peptide level is high in state of satiation and ample nutrients storage and low when starved and nutrients store is mobilised. Insulin secretion is precisely tuned to the nutritional state of the animal. Increasing circulating glucose stimulates beta cell depolarisation and insulin secretion. In concert with glucose, gut-derived incretin hormones amplify glucose-stimulated insulin secretion after ingestion of carbohydrates and tune insulin output to the feeding status of the animal. Conversely after nutrient restriction or fasting, decretin hormones signal through G protein-coupled receptors to attenuate glucose-stimulated insulin secretion (Alfa, Park et al. 2015). In addition a peptide signal, Upd2, released from fat body which is a homologue of liver and adipose tissue after the levels of fat acids and amino acids increase, could release the inhibitory control over in-

sulin producing cells and stimulate insulin secretion (Rajan and Perrimon 2012). Interestingly human leptin can rescue the Upd2 mutant phenotypes.

The perception of reward after feeding begins from gustatory receptor cells to NTS, and then to multiple sites that receive taste information including ventral tegmental area (VTA) in the midbrain and nucleus accumbens (NAc) in the forebrain. Taste information from NTS neurons is forwarded by the thalamus to the primary taste neurons in the insular cortex and the secondary taste neurons in the orbitofrontal cortex to integrate with olfactory, visual and other cognitive inputs (Rolls 2005). One hypothesis is that the hunger-dependent firing rates of secondary taste neurons are correlated with the reward values of foods. When the activity in secondary taste neurons reduces as food is eaten, reward value of food diminishes, contributing to meal termination via projections to the striatum and amygdala (Kringelbach, O'Doherty et al. 2003). How does the VTA-NAc dopaminergic reward system communicate with the gustatory circuitry? One mechanism is through projections from the VTA- NAc pathway to the lateral hypothalamic area (LHA). Activation of the reward pathway could relieve the tonic inhibition on the food-intake-stimulatory neurons in LHA, which then engage the feeding motor programs (Kelley, Baldo et al. 2005). LHA area is connected in addition to NTS, ARC, striatum and orbitofrontal cortex, serving as an integrative node for homeostatic, satiety and reward-related inputs.

Reward perception is subject to homeostatic regulation in a manner that fasting augments reward value. One mechanism is that leptin and insulin tonically inhibit brain reward circuitry. Consistently energy restriction, which could lower circulating levels of insulin and leptin, increases the sensitivity of reward circuits and motivates animals to seek and obtain palatable foods. Conversely central administration of insulin or leptin diminishes sucrose preference (Figlewicz, Bennett et al. 2004). The negative effect of insulin on sucrose preference has been corroborated in drosophila by our study.

Next we will discuss systems that promote food intake. Neurons co-expressing neuropeptide Y (NPY) and agouti-related protein (AgRP) in the arcuate nucleus of hypothalamus are well studied to exert a stimulatory effect on food intake. These NPY/AgRP neurons are inhibited by leptin, insulin and are stimulated by ghrelin (Flier 2004). NPY and AgRP peptides could potently stimulate food intake and reduce energy expenditure. Adjacent to NPY/AgRP neurons are neurons that express pro-opiomelanocortin (POMC), the polypeptide precursor from which melanocortins are derived. AgRP could antagonise the catabolic effects of melanocortin signaling. Many POMC neurons in the

arcuate nucleus express anorexic neuropeptides α-melanocyte stimulating hormone and the CART peptide which reduce food intake. POMC neurons are stimulated by leptin and insulin but inhibited by neighbouring NPY/AgRP neurons (Cowley, Smart et al. 2001). When weight is lost, insulin and leptin levels in the plasm drop while ghrelin levels increase. The anabolic NPY/AgRP neurons are released from inhibition and catabolic POMC neurons are inhibited instead. Working in concert, the responses increase the rewarding perception of food and hence the motivation to eat through direct effects in the striatum or indirect effects in the lateral hypothalamic area, to promote the recovery of lost weight until it returns to its biologically defended level (Porte, Baskin et al. 2005).

Our understanding of the neural circuit of hunger and satiety is being updated and continued, with advent of new technologies. Despite a large body of knowledge on the important orexigenic functions of AgRP neurons, the resource of excitatory input onto AgRP neurons remains obscure until recently. Researchers used modified rabies virus to trace presynaptic neurons providing input to AgRP neurons and shockingly they found strong excitatory input from the hypothalamic paraventricular nucleus (PVH), which was a long-though satiety center (Krashes, Shah et al. 2014). Neurons in the arcuate nucleus could not only drive food intake but also stimulate energy expenditure. A subset of "non-POMC, non-AgRP" neurons are responsible for leptin-mediated thermogenesis, partly through the direct inhibition on the PVH neurons projecting to the NTS (Kong, Tong et al. 2012). POMC-expressing neurons located in the arcuate nucleus (ARC) and the nucleus tractus solitarius (NTS) regulate feeding at different time scale. Acute activation of NTS POMC produced an immediate inhibition of feeding behaviour whereas chronic activation of ARC POMC neurons are required to suppress food intake. Ablation of ARC POMC neurons, but not NTS POMC neurons, increase food intake and reduce energy expenditure, ultimately resulting in obesity and metabolic disorder (Zhan, Zhou et al. 2013).

The cellular mechanisms for reward processing in fly brain has been studied intensively in the context of behavioral reinforcement. Octopamine has historically been considered as the reward signal in flies. Dopamine neurons could posit both negative and positive reinforcement. Discrete dopaminergic neurons expressing the OAMB octopamine receptor convey the short-term reinforcing effects of sweet taste (Burke, Huetteroth et al. 2012). The rewarding dopaminergic neuron populations for sweet taste and nutrient properties of sugar are separate and have distinct projection patterns (Huetteroth, Perisse et al. 2015). Additionally a group of dopamine neurons in the PAM cluster, are activated by sugar ingestion and the activation is increased on starvation, endows a positive predictive value to the odour in the MBs (Liu, Placais et al. 2012).

There are several anorexigenic pathways elucidated in drosophila. One is the MIP peptide and its related signaling pathway which could reduce food intake and promote satiety. Activation of MIP neurons greatly decreased food intake and body weight, as well as to markedly blunt the sensitivity of starved flies toward food. However after terminating the activation of MIP neurons, body weight reverts rapidly to the normal level through a strong feeding rebound (Min, Chae et al. 2016). Another pathway is set by the allatostatin A expressing neurons that mimic the state of satiety (decreased food intake and behavioural responsiveness to sugar) without causing any change in energy reserves and exert an inhibitory influence on the motivation to feed (Hergarden, Tayler et al. 2012). Activation of AstA neurons inhibits starvation-induced feeding and starvation-enhancement on sucrose sensitivity, which can be antagonised by simultaneous activation of NPF neurons. AstA-exressing nerve fibers ramify within the SOG and exhibit varicosities in close proximity to projections of Gr5a sweet receptor neurons. It is possible that AstA neurons act on the primary gustatory circuit in the SOG. Additionally AstA regulates AKH and DILP signalling, partly via the AstA receptor that is expressed in both the insulin and AKH producing cells (Hentze, Carlsson et al. 2015). Obesity-blocked neurons have been reported that when they are silenced, fat store levels increase through increase in food intake and altered metabolism whereas hyper activation of these neurons leads to depletion of fat stores by increasing metabolic rate and decreasing fatty act synthesis, suggesting that these neurons could regulate fat utilisation and exert inhibitory effect in food intake. Interestingly most of these neurons express insulin-like peptides and tyrosine hydroxylase, necessary for dopamine synthesis (Al-Anzi, Sapin et al. 2009).

There are many distributed and potentially overlapping neural circuitry that evolve to signal hunger and promote feeding. Recently a small set of serotonergic neurons were identified which upon acute activation induce feeding in sated flies that resemble starved flies however synaptic silencing of these neurons in starved flies didn't decrease starvation-driven behaviours (Albin, Kaun et al. 2015). These subset of serotoninergic neurons are unlikely to contribute directly to taste, nutrients sensing or the feeding motor program because PER and nutritive preference in starved flies are normal, instead they might lie between these elements and convey the hunger signal and increase the motivation to feed because they could provide the positive value for appetitive memory formation. Neuropeptides such as serotonin have different and even contradictory effects on feeding (activation of entire serotonergic population decreases feeding, even in starved animals) depending on the wiring diagram and spatial organization of neuropeptidergic neurons. These serotoninergic neurons don't signal hunger through modulation of sensory gain but more likely lowering the activation

thresholds for command neurons so that a feeding motor program is more readily evoked in presence of appropriate taste and nutritional cues. VM neurons are special because it directly receives sweet taste information. It provides gain in starved animals. Without the stimulatory effect that VM neurons impart animals are less motivated to eat and behave like unstarved animals. Moreover VM neurons are modulated by satiety signal hormone insulin, making it different from the solely information-relaying neurons. A group of 12 cholinergic local interneurons (IN1, for ingestion neurons) recently reported belong to the orexigenic pathway (promote eating) (Yapici, Cohn et al. 2016). Inhibition of IN neurons led to reduced total food intake. Photo activation of IN neurons, like activation of Gr5a sweet taste receptor neurons that elicit robust light-controlled proboscis extension, can only evoke proboscis extension followed by ingestion in presence of food. IN neurons are connected to the afferent sensory axons of the sweet pharyngeal taste receptors neurons labeled by Gr43a and Gr64e but not to Gr5a sweet taste neurons on the labellum. Concomitantly proboscis stimulation with high concentration sucrose can't activated IN neurons without ingestion. In addition high concentration sucrose (1M) elicits a persistently active state in fasted but not sated flies and this persistent activity drops to baseline as flies consume food. Even in fasted flies, relatively low concentration sucrose (100mM) can not evoke responses in IN neurons. These neurons are functionally and anatomically distinct from VM neurons. It is very likely that IN neurons promote sustained ingestion whereas VM neurones amply the sweet taste information and encode a positive motivational force to feed.

5. Materials and Methods

Fly Stock

Fly stocks and crosses were maintained on standard fly food at 25°C and 75% relative humidity if not stated differently. Enhancer-Gal4 lines were obtained from the Bloomington Stock Center (Jenett, Rubin et al. 2012) and the Vienna Drosophila Resource Center (VDRC, Dickson and Stark). In addition, the following stocks were used in this study: w^{1118}, *Gr5a-Gal4* (Wang et al., 2004), *UAS-Kir2.1* (Baines et al., 2001), *tub- Gal80^{ts}* (McGuire et al., 2004), *UAS-dTRPA1* (Hamada et al., 2008), *UAS-shibire^{ts}* (Kitamoto, 2001), *UAS-InRDN* (*UAS-InR.K1409A*; Bloomington 8253), *UAS-InRCA* (*UAS-InR.A1325D*; Bloomington 8263), *UAS-InR RNAi* (V992), *UAS-Chrimson* (Klapoetke et al., 2014), *cha-Gal80* (Kitamoto, 2002), *Gr5a-LexA; UAS-tdTomato::LexAop-CD4::spGFP11; UAS-CD4::spGFP1-10* (Gordon et al., 2009), *UAS-tdTomato::LexAop-CD4::spGFP11; UAS-CD4::spGFP1-10* (made by Dr. Yunpo Zhao), *UAS-DenMark::syt.eGFP* (Bloomington 33065), *10XUAS-IVS-mCD8::GFP, UAS-RFP* (Bloomington 30556), 13XLexAop2-mCD8::GFP (Bloomington 32203). *40117-LexA* driver was generated as described below. *Dilp2-Gal4* was a generous gift from Dr. Hugo Stocker at ETH Zurich.

PER assay

PER assay was performed largely as described in the published protocol (Shiraiwa and Carlson 2007), except that flies were glued to glass slides using nail polish and stimulated on the tarsi via pipette tips instead of paper wicks. For PER experiments with *tub-Gal80^{ts}*, flies were raised at 18°C on standard food and shifted to 31°C after eclosion for 3 days to inactivate *tub-Gal80^{ts}*. Starvation was performed with wet tissues. After being glued to coverslips, flies were allowed for recovery in a humid chamber (75% humidity) for 1h before testing. In PER assays, flies were first water-satiated and then selected for responsiveness to high-concentration sucrose and non-responsiveness to water. For concentration curves, tarsi were stimulated with increasing concentrations of sucrose (50mM–1M sucrose) for three times at each concentration and water was presented in between stimulations. The percentages of extending flies were calculated and averaged over 3 repetitions as the final value of one trial. In general, at least 4 independent trials with 11-15 flies in each trial were performed for every genotype in every condition. We compared PER responses between controls and treatment groups at 31°C instead of comparing PER responses of a single genotype between 18°C and 31°C because we observed very different PER responses between 18°C and

31°C in control flies starved for same amount of time. For PER experiments with *UAS-shibire^{ts}*, flies were raised at 22°C on standard food and tested at 3-5 days old. The same PER assay procedure was performed. Flies were first tested at 22°C and then left for 30 minutes at 30°C before the same round of sucrose stimulation. The percentages of extending flies at 22°C and 30°C respectively were compared and the significance was assessed using paired student's t-test at each sucrose concentration. For PER experiments with flies expressing *UAS-TrpA1* in *Dlip2* cells, these flies didn't exhibit spontaneous extensions so that we could perform standard PER assay. Flies were first tested at 22°C and then left for 30 minutes at 30°C before the same round of sucrose stimulation, similar to the procedure with experiments using *UAS-shibire^{ts}*. All flies used for behavioural experiments were raised under a 12h light/12h dark cycle from 8am to 8pm and tested between 10am and 2pm.

TrpA1-dependent activation

Enhancer-Gal4 lines were crossed to *UAS-TrpA1* at 22°C. Flies were tested at 3-7 days old. They were first glued to glass coverslips and then left to recover in a humid chamber for 30 minutes. The behaviour was observed in a custom-made heating chamber and recorded (Canon EOS 60D) at control (22°C) and activation (30°C) temperature. In all our tested lines, male and female flies exhibit no obvious difference in PER response at 30°C. Proboscis extension frequency was counted as the number of total extensions in 30 seconds following the first extension divided by 30 seconds.

Chrimson-dependent activation

Enhancer-Gal4 lines were crossed to *UAS-Chrimson*. These flies were raised at 22°C in darkness on standard food mixed with 200uM all trans retinal. Male flies of 3-7 days old were glued to glass coverslip and left for recovery in total darkness 30 minutes prior to testing. We found that cyan light couldn't elicit proboscis extension in *Gr5a-Gal4 > UAS-Chrimson* flies. Therefore cyan light (490 nm) was used as a background light to see the fly during observation and recording with camera (Canon EOS 60D). Red light (625 nm) was used to activate Chrimson expressed in flies.

Mosaic analysis of *R40117-Gal4*

To generate mosaic clones labelling very few VM neurons, flies of the genotype *hs-FLP; UAS-TrpA1/UAS-mCD8::GFP; tub84B(FRT.Gal80)/40117-Gal4* were raised at 22°C and subjected to a 30 minutes heat shock at 37°C during pupal stages. Flies were collected at 3–5 days old and tested for

proboscis extension at 30°C. Extenders were defined as flies that extend proboscis in the first 2 minutes and those showing no detectable extension were seen as non-extenders. All flies were dissected and assessed for *mCD8::GFP* expression by immunohistochemistry.

Immunohistochemistry and Imaging

Adult flies at age 3-5 days old were pre-fixed with 4% PFA (prepared in 1× PBS, with 0.2% Triton X-100) for 3 hours at 4°C. The fixative was removed by washing three times with 0.2% PTX (0.2% Triton X-100 in 1× PBS) for 15 minutes each at room temperature. Flies were then dissected in the same solution on ice. Blocking was performed for 30 minutes at room temperature in 5% goat serum (prepared with 0.2% Triton X-100 in 1× PBS). Both primary and secondary antibodies were diluted in 5% goat serum. Samples were incubated with primary antibody for 3 days and with secondary antibody for 1 day at 4°C on a shaker. Washing of antibodies was performed for three times (30 minutes each time) with 0.2% PTX (0.2% Triton X-100 in 1× PBS) at room temperature. Vectashield media (Vector Laboratories) were used for mounting purpose.

Primary antibodies were used at the following dilutions: guinea pig anti-Discs large (1:1000), mouse anti-mCherry (1:1000, Clontech, to visualise Denmark), rat anti-CD8 (1: 500; Invitrogen), rabbit anti-GFP (1:1000; Invitrogen, to visualise syt-eGFP), rabbit anti-RFP (1:250; abcam, to visualise tdtomato), mouse nc82 (1: 400), rabbit anti-Dilp2 (1:2000, gift from Dr. Hugo Stocker). Secondary antibodies (1:1000; Invitrogen) conjugated with Alexa fluor-488, 568 and 647 were used in all immunostaining procedures. Imaging was carried out with a Zeiss LSM 700/710 laser scanning confocal microscope with a 10x (NA 0.3) objective, a 20x (NA 0.7) oil immersion objective, or a 40x (NA 1.25-0.75) oil immersion objective. Z-stacks were collected with optical sections at 1 μm intervals and the maximal projection was shown unless otherwise noted. Images were processed using Imaris (Bitplane) and Adobe Photoshop software.

To acquire endogenous GRASP, RFP, and GFP signals, a different preparation procedure was performed as described in the GRASP section.

GFP Reconstitution Across Synaptic Partners (GRASP)

For GRASP between *R40117-Gal4* and *Gr5a-lexA,* flies of the genotype *Gr5a-LexA/+; UAS-td-Tomato::LexAop-CD4::spGFP11/+; UAS-CD4::spGFP1-10/R40117-Gal4* were dissected. For GRASP between *R40117-lexA* and *Dilp2-Gal4,* flies of the genotype *Dilp2-Gal4/LexAop-*

CD4::spGFP11; 40117-LexA/UAS-CD4::spGFP1-10 and *Dilp2-Gal4/UAS-tdTomato::LexAop-CD4::spGFP11; 40117-LexA/UAS-CD4::spGFP1-10* were used. Flies were first dissected in ice cold PBST. Brains were incubated in PFA fixative for 20 minutes on ice and washed with PBST for 3x10 minutes. Then brains were directly mounted and imaged for endogenous GRASP, GFP (*40117-lexA > lexAop-GFP*) , and RFP (*Dilp2-Gal4 > UAS-RFP*) signals. Alternatively brains were incubated with primary anti-nc82 and anti-RFP antibodies (to visualise tdTomato) overnight at 4°C and with secondary antibodies for 2 hours at 4°C, which well preserved the endogenous GRASP signals.

Generation of transgenic line *40117-LexA*

We cloned the enhancer fragment of line R40117 from genomic DNA by PCR using the pENTR TOPO cloning kits (ThermoFisher). Primers were used as reported previously (Jenett, Rubin et al. 2012): left primer: ttctccatccccaacactctttcct, right primer: ggtgacgaagcgcactaaaaggcta. The enhancer fragment in entry vector was sequenced and transferred to the destination vector: pBPnls-LexA::p65Uw. The final constructs were inserted into the attP2 integration site using the phiC31 site-specific integration system to generate transgenic fly strain. Embryo injections and screening for transgenic flies were performed by BestGene (Chino Hills, US).

Statistical Analyses

In analysing mosaic clones, all flies were categorised to be extenders or non-extenders to construct a 2 X 2 contingency table. In this case, Fisher's exact test was used to assess the statistical significance. In PER assay experiments using *tub-Gal80^{ts},* there are two factors influencing the outcome, i.e. sucrose concentration and genotypes (more than 2). Instead we transform the two categorical variables (extension or not) into relatively continuous variables (extension probability) on the scale from 0 to 100%. We already expect that sucrose concentration has a significant effect on the outcome. Moreover we don't care about whether differences among genotype groups are identical at every concentration point, or we don't expect interaction between genotypes and concentration. We want to know which genotype makes a statistically significant difference in extension probability at each concentration point. Considering the logic of experimental design and our scientific goals, we used Graphpad Prism to perform two-way ANOVA with pairwise comparisons using a Bonferroni correction. For post tests, Prism can only compare within the same row. Thus when we make sucrose concentration as the row factor, Prism perform post hoc comparisons between control geno-

types and treatment genotypes at each concentration point with an adjusted cutoff point for P value, which serves our purposes well. In PER assay experiments using *UAS-TrpA1 and UAS-shibire^{ts}*, student's paired t test were used for statical tests as the same subjects were used before and after temperature-dependent activation. In all graphs, mean and SEM were shown.

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6.2 Abbreviations

GRASP: GFP Reconstitution Across Synaptic Partners

PER: Proboscis Extension Response

SEZ: Subesophageal Zone

IPCs: Insulin-Producing Cells

ILP: Insulin-Like peptides

MNCs: Median Neurosecretory Cells

CAFE: Capillary Feeder Assay

NTS: Nucleus Tractus Solitarius

ARC: Arcuate nucleus

PVH: hypothalamic Paraventricular nucleus

LHA: Lateral Hypothalamic Area

POMC: Pro-OpioMelanocortin

VTA: Ventral Tegmental Area

NAc: nucleus Accumbens

CCK: Cholecystokinin

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If you can't explain it simply, you don't understand it well enough.

Albert Einstein