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Regulation of Food Choice in *Drosophila melanogaster*

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ABSTRACT OF THE DISSERTATION

Regulation of Food Choice in *Drosophila melanogaster*

by

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The taste system enables animals to identify different water-soluble chemicals in their niches, enabling them to ingest nutritive food materials and reject toxic and noxious chemicals. The sense of taste is modulated both by internal factors like physiological state as well as external factors like presence of harmful contaminants in the food. Here we studied different factors that can modulate the taste system in flies. First, we studied yeast response in flies. Food preference for yeast is visible only in female flies and is dependent on mating state. Although earlier studies have explained mating realted physiological changes that are responsible for the dietary switch following mating, the cellular and molecular basis of yeast feeding was unknown. We found that a subset of free amino acids to be required for the feeding preference for yeast. We further identified tarsal taste neurons that are sensitive to amino acids.

We discovered that *Ir76b* an ionotropic receptor is necessary for amino acid taste. It combines with other Irs like *Ir20a* and other factors to confer amino acid sensitivity.

Nutritional imbalance changes dietary requirements in flies. They need to ingest more of the nutrient they are deprived of to maintain nutritional homeostasis. These changed requirements lead to changes in the taste preferences of flies. In order to study the compensatory changes following nutrient deprivation, we fed the flies with two different

types of food viz. sugar deprived/yeast enriched and yeast deprived/sugar enriched. Feeding with both sugar deprived/yeast enriched food increased preferences and sensitivity for sugar while decreasing preferences for amino acids. While feeding with sugar enriched/yeast deprived food decreased their preferences and sensitivity for sugar at the same time decreasing their preferences for amino acids. We identified a *Dop2R* dependent mechanism to be responsible for compensatory changes following feeding with sugar deprived/ yeast enriched diet. Compensatory changes upon feeding with sugar enriched/ yeast deprived diet however required *dilp5*, an insulin like peptide.

Furthermore, we found that both sweet taste neuron and low salt taste neurons are inhibited with increasing pH of the taste solution. Also, presence of high concentrations of different salts lead to decreased activity of the sweet taste neurons.

Taken together all the above discoveries will be helpful in understanding how food choice is regulated in flies.

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Chapter 1: Introduction

Chemosensation: Chemosensation is the property by virtue of which animals can sense chemicals in the surrounding environment. Identifying chemical moieties in one's niche allows for identification of food sources, mates as well as potential threats or noxious conditions. The ability to sense chemicals is an ancient one; organisms ranging from as simple as unicellular organisms to as complex as human beings can perceive chemicals in their surroundings. Bacteria are known to perceive different chemicals and exhibit chemotactic movement along concentration gradient of different chemicals (Berg, 1975; Spudich and Koshland, 1975). AMP as well as several other nucleotides act as chemoattractants in the single cellular amoeba of the slime mold- *Dictyostelium discoideum* (Konijn et al., 1969). In multicellular organisms chemosensation is broadly classified into smell, i.e. chemosenstaion of volatile objects and taste, i.e. contact chemosensation. Most multicellular organisms have a separate group of cells dedicated for this purpose. *Caenorhabditis elegans*, a nematode, bears 11 pairs of chemosensory neurons out of which AWA, AWB and AWC neurons are tuned for sensing volatile compounds i.e. for olfaction while ASE neurons are specialized for sensing water soluble compounds i.e. for gustation (Bargmann, 2006). Likewise, in the arthropod *Drosophila melanogaster*, there are separate groups of neurons for olfaction and gustation (Vosshall and Stocker, 2007). In mammals like rodents and humans, specialized taste receptor cells present in taste buds detect different tastants whereas odorants are detected by olfactory neurons present in nose or in vomeronasal organs (in rodents) (Buck, 2004; Chandrashekar et al., 2006).

The basic principles of chemosensation are identical among all the organisms. In all cases there are specialized proteins called receptors which bind to different chemical compounds and convey information about the chemical to the cell. The receptors vary widely in their ligand binding property and mode of downstream signaling.

Importance of taste: Taste is the term applied to refer to contact chemosensation in multicellular animals. The sense of taste allows animals to identify food substances rich in nutrients required for growth and reproduction. It also allows animals to avoid ingesting noxious or toxic substances. Since most naturally occurring food sources are mixtures of both aversive and attractive compounds, the sense of taste allows animals to evaluate whether the reward of choosing a particular food source surpasses its potential threats or vice versa. Also, nutritional requirements for an organism changes with its physiological state. The sense of taste can adapt to changing physiological needs to ensure intake for nutritional balance. The sense of taste also allows animals to sense pheromones (only for those animals that secrete pheromones), enabling them to choose mates, identify con-specifics and also efficiently executing various other complex social interactions.

Fly as a model system to study taste: *Drosophila melanogaster* has a simple neurophysiological organization with only about 100,000 neurons in their brain (van der Goes van Naters and Carlson, 2006). They are easy to grow, have short life cycles and are genetically tractable (Duffy, 2002; Hales et al., 2015). Flies use their sense of taste to make decisions on feeding, ovi-position as well as social interactions like mating (van der Goes van Naters and Carlson, 2006; Montell, 2009; Vosshall and Stocker, 2007). Inspite of being a much simpler organism compared to mammals, *Drosophila* exhibits a wide array of complex gustatory behaviors that is comparable to that observed in

mammals (Liman et al., 2014). Also, aspects of gustatory behaviors of flies are very similar to several closely related insect pest and disease vectors (Kessler et al., 2013).

For all these reasons *Drosophila melanogaster* has been a model system of choice to study different aspects of taste and gustatory behavior.

Taste modalities in *Drosophila*: The sense of taste has evolved to enable animals to identify and differentiate between palatable and unpalatable substances. The palatability of a substance is in many aspects a function of its nutrition content. As the most important purpose of feeding is to ensure a supply of nutrients required by the body, most nutritious substances are generally palatable.

Nutritional requirements of most animals are extremely identical, since all animals require carbohydrates, proteins, fats as well as vitamins and different minerals for proper growth and reproduction. Hence the taste modalities of animals from mammals to flies are generally identical.

Flies exhibit feeding preferences for sugars, certain amino acids, water, carbonation and low concentrations of salt and fatty acids (Cameron et al., 2010; Dahanukar et al., 2007; Fischler et al., 2007; Ganguly et al., 2017; Harris et al., 2015; Masek and Keene, 2013). Whereas bitter compounds, acids and high concentrations of salt are avoided by the flies(Alves et al., 2014; Charlu et al., 2013; Kim et al., 2017; Weiss et al., 2011; Zhang et al., 2013). Flies can also detect and avoid chemical nociceptive compounds using their taste system (Zhang et al., 2013; Kwon et al., 2010).

The fly uses its taste organs to detect these substances following which they decide whether or not to initiate feeding.

Taste organs of a fly: *Drosophila* bears specialized hair like structures known as taste bristles or taste sensilla in numerous parts of their body all of which could function at least to some extent as a chemosensor or a taste organ. Besides they also contain taste pegs and pharyngeal taste sensilla both of which are non-hair like sensory structures.

The main taste organs of the fly are the labellum, the pharyngeal organs and the tarsi. Other than these, they also have taste bristles on the anterior wing margins and in case of the females on their ovipositors.

Each sensillum generally houses multiple taste neurons along with a mechanosensory neuron and also has support cells at the base. At the tip of the sensillum there is a pore through which chemicals can diffuse into the sensillary lymph present within. The dendrites of taste neurons bear taste receptors on them, which are activated when they encounter chemical compounds.

The organization of taste sensilla on the different taste organs is highly stereotypical. Axons of taste neurons from the different taste organs project to different parts of the sub-esophageal zone and/or the ventral nerve chord where they relay the information to higher order neural circuits (Kain and Dahanukar, 2015; Yapici et al., 2016).

Labellum as taste organ: In flies, the tip of the proboscis is called the labellum and is a major taste organ analogous to the tongues in mammals. Because of its accessibility the structure and function of the labellum has been very well characterized. Labellum bears a total of approximately 60 sensilla which are stereotypically arranged. Based on their morphology, they have been categorized into three classes viz. large(L), intermediate(I) and small(S). Each of these sensilla bear upto 4 taste neurons, each of which are

specialized for the detection of specific taste modalities (Freeman and Dahanukar, 2015; Vosshall and Stocker, 2007).

The sensitivities of taste sensilla have been broadly characterized by extracellular tip recordings to various chemicals. Subsequently the role of different subclasses of taste neurons were validated by selectively silencing or ablating them and then investigating loss of cellular and behavioral responses to tastants. Taste neurons present in the labellar taste bristles exhibit response to wide array of sweet and bitter compounds, acids, water as well as salt (Charlu et al., 2013; Dahanukar et al., 2007; Freeman and Dahanukar, 2015; Hiroi et al., 2004; Weiss et al., 2011; Zhang et al., 2013).

Previous studies report that both L-type and S-type sensilla house four taste neurons. In L-type sensilla separate neurons are activated by sweet compounds, water, low concentrations of salt and high concentrations of salt (Freeman and Dahanukar, 2015). Interestingly, the low salt neuron is activated more at lower concentrations of salt than at higher concentrations and is required for appetitive responses to low salt concentrations while the high salt neuron is responsible for aversive responses to high salt concentrations (Zhang et al., 2013). The functional classes of S-type sensilla are identical to that of the L-type except that they have an aversive neuron which senses bitter compounds as well as acids.(Freeman and Dahanukar, 2015). Interestingly, two S-type sensilla are insensitive to bitter compounds and thus are functionally similar to the L-type (Weiss et al., 2011). The I-type sensilla consists of two neurons one for sweet taste and the other for aversive taste. The aversive taste neuron of the I-type sensilla can also respond to salt and low pH (Charlu et al., 2013; Zhang et al., 2013).

Sweet taste neurons are activated by multiple mono- and oligo- saccharides, alcohols, sugar acids and glucosides (Dahanukar et al., 2007). Sugars being appetitive, activation of these neurons is expected to lead to feeding. Indeed, artificially expressing a capsaicin receptor in these neurons leads to the fly feeding on capsaicin (Marella et al., 2006). Activation of these neurons by expressing a heat activated ion-channel and then applying heat also leads to proboscis extension in flies (Montell, 2009). Expectedly, silencing or killing these neurons leads to a decreased sugar ingestion by starved flies.

Based on calcium imaging and behavior analysis some studies report that labellar sweet neurons also detect low concentrations of fatty acids (Masek and Keene, 2013; Masek and Scott, 2010). According to these studies, Phospholipase C (PLC) pathway in the sugar neurons is instrumental in fatty acid detection. Fatty acid response is abolished when PLC pathway is disrupted (in *norpA* mutant flies) as well as when sugar neurons is silenced. Interestingly, flies with disrupted PLC pathway retained normal sugar responses suggesting two independent pathways of detection.

The water neuron essentially responds to low osmolarity (Cameron et al., 2010). The activity of this neuron in inversely related to the osmolarity of a solution. These neurons are however absent in the I-type sensilla.

The low salt neuron is activated by lower concentrations of salt while they are inhibited by higher salt concentrations (Zhang et al., 2013). Since low salt concentrations are attractive to flies (Liu et al., 2003; Zhang et al., 2013), activation of these neurons leads to feeding. However, detailed studies involving artificial activation of these neurons as described in case of sugar neurons have not been conducted.

These neurons are most activated at 100mM NaCl and beyond 500mM NaCl the activity reduces drastically (Zhang et al., 2013). Whether or not these neurons respond to low concentrations of other salts have not been studied in detail.

A different class of neurons detects high salts in flies. These neurons fire in a concentration dependent manner, exhibiting increasing firing frequency at higher concentrations of salt and relative activation of this neuron and the low salt neurons correlates with the fly's gustatory behavior to salt. In I type and S type sensilla the high salt neurons also respond to bitter compounds. Although earlier studies suggested presence of a high salt neuron in all L hairs (Vosshall and Stocker, 2007), based on a recent study by Zhang et al., 2013, presence of high salt neuron in the L-sensilla type could be debated.

Bitter neurons respond to bitter compounds including alkaloids, terpenoids and phenolic compounds. Thus, this class of neurons is the most broadly tuned of all classes of taste neurons. Based on the range of bitter compounds their bitter neurons respond to, S and I sensilla could be classified into various subtypes. S-a and S-b subtypes are the most broadly tuned while I-a and I-b are narrowly tuned to bitter compounds. Interestingly, S-c type, like the L-type sensilla does not have bitter-responsive neuron. Most bitter compounds are toxic and are harmful if ingested. Hence, they are perceived as aversive by flies and activation of the bitter neurons leads to cessation of feeding. Indeed, artificially activation of these neurons by expressing capsaicin receptors in them and presenting flies with sugar mixed with capsaicin decreases the fly's feeding of sugar (Marella et al., 2006).

Bitter neurons can also perceive organic and non-organic acids (Charlu et al., 2013; Weiss et al., 2011). According to Charlu et al., 2013 bitter neurons can sense low pH solutions. However, a pH sensor in flies is yet to be discovered. Bitter neurons of the S-b and I-b sensilla subtypes are highly sensitive to acids whereas the bitter neurons of the S-a and I-a subtypes have a much-reduced response (Charlu et al., 2013).

Interestingly, based on electrophysiological responses amino acid taste is completely absent among the labellar taste bristles (Dahanukar et al., 2007). This is somewhat surprising since amino acid is an important nutrient in flies.

Other than the taste bristles the labellum also contains around 30 peg sensilla, which are small sensory structures present on the inner surface of the labellum arranged in a line between the pseudotrachea. The contribution of the peg sensilla to taste in not yet fully understood. A single study reported that some peg neurons respond to carbonation but not to sweet or bitter compounds (Fischler et al., 2007).These neurons could be labelled by E-409 GAL4, an enhancer trap line, and silencing them reduced the consumption of carbonated water by flies. Carbonation is an appetitive stimulus in flies and expectedly, expressing capsaicin receptors in these cellsenhanced feeding of capsaicin mixed food. Since pegs contact the food during feeding, it is likely that the pegs have additional functions in taste. More studies are required to understand the role of the peg sensilla towards fly taste.

Role of the pharyngeal organs in taste: The pharynx comprises three different taste organs viz. the Lateral Sense Organ (LSO), the Ventral Cibarial Sense Organ (VCSO) and the Dorsal Cibarial Sense Organ (DCSO) (Stocker, 1994). The DCSO has two taste sensilla on each side of the midline, each sensillum containing three taste neurons.

On the other hand, both the LSO and VCSO have three taste sensilla on each side of the midline. Each sensilla of the LSO and VCSO has eight and ten taste neurons respectively (Gendre et al., 2004; Nayak and Singh, 1983).Previous studies have reported the presence of sweet neurons in pharyngeal taste organs (LeDue et al., 2015). Another study shows the presence of high salt neurons in the pharynx (Kim et al., 2017). Pharyngeal taste organs are located at the junction of the peripheral taste organs and the internal parts of the digestive tract. Because of this unique location, the pharynx is highly suited to function as a last check point to evaluate food prior to ingesting. As in pegs, pharyngeal taste organs have been understudied and more studies are required to elucidate its role in taste.

Tarsi as taste organ: The tarsi or the distal most segments of the fly leg plays an important role in the taste response of the fly. They are likely the first taste organs that come in contact with the food substrates and signal from the tarsi may inform the fly whether or not to probe further.

The tarsi are covered with a large number of taste bristles. In female flies there are 28,21 and 22 sensilla respectively in the foreleg, midleg and hindleg (Ling et al., 2014). In male flies, there are more sensilla in the foreleg but identical numbers in the midleg and hindleg (Nayak and Singh, 1983).Taste sensilla of the tarsi can be classified based on the morphology of the tip, which can be either straight or forked (Ling et al., 2014). Most tarsal sensilla are paired and are placed in a bilaterally symmetrical manner. The arrangement of the sensilla is highly stereotypical between different pairs of legs as well as between different flies.

The tarsi are covered by thick spines and the taste sensilla are interspersed among them. This makes electrophysiological recordings from the tarsal taste sensilla somewhat challenging. Nevertheless, several attempts have been made to characterize taste sensilla present on the tarsi using tip recordings (Ling et al., 2014; Meunier et al., 2003). An early study by (Hiroi et al., 2004) demonstrated the presence of four taste neurons in each sensillum a sweet neuron, a bitter neuron, a hypo-osmolarity or water neuron and a low salt neuron. A more recent study by Ling et al., 2014, analyzed the taste responses of the tarsal sensilla more systematically.

The results showed that tarsal sensilla are more diversely tuned than the labellar taste sensilla. Some sensilla, such as f5s are very broadly tuned,responding to sugar, bitter compounds and amino acids, whereas sensilla like f4b are more narrowly tuned. One particular sensillum, f5b, did not respond to any stimulus tested. Also, collectively the tarsal sensilla respond to a wider range of bitter compounds than the labellar taste bristles. The additional taste sensilla present in the male forelegs is speculated to contribute towards pheromone sensing.

Attempts have been made to characterize the tarsal taste neurons using calcium imaging. One such study (Ganguly et al., 2017) reported the presence of a few amino acid sensing neurons in the tarsi.

Wing as taste organ: The anterior wing margins bear taste sensilla although their functions are not well understood. It has been speculated that they aid the fly in tasting their surroundings while they are passing through very narrow spaces(Vosshall and Stocker, 2007).

Presence of sugar and bitter neurons have been demonstrated in wing sensilla using calcium imaging (Raad et al., 2016). Another study showed that stimulation of the taste sensilla on the wing margin by bacterial lipopolysaccharides can lead to grooming behavior (Yanagawa et al., 2014).

Molecular basis of taste: All taste neurons bear different kinds of taste receptors that are responsible for the detection of the taste compounds. So far four different kinds of taste receptors families have been identified viz. gustatory receptors (Gr) (Clyne et al., 2000; Scott et al., 2001), pickpocket (Ppk) receptors (Cameron et al., 2010; Chen et al., 2010; Pikielny, 2012),transient receptor potential (Trp) (Kang et al., 2010) and ionotropic receptors (Ir) (Croset et al., 2016; Ganguly et al., 2017; Hussain et al., 2016; Zhang et al., 2013).

Gustatory Receptors: Gustatory receptors (Gr) have been extensively studied in flies. Incidentally, receptors of the Gr family were first found in flies in an attempt to discover novel receptors having multiple transmembrane domains, using a BLAST algorithm (Clyne et al., 2000). Subsequently Grs have been reported in all arthropods and have homologs even in *Caenorhabditis elegans*, a nematode (Kent and Robertson, 2009). Although all Grs have a highly conserved C-terminus domain (Clyne et al., 2000), even within flies they vary widely with as little as 8% sequence identity (Robertson et al., 2003).

Unlike mammalian taste receptors, members of the Gr family have inverted topology resembling the Olfactory receptor (Or) proteins in flies (Zhang et al., 2011). Although mammalian taste receptors are G-protein coupled receptors the nature of the GRs has been highly debated. Since Grs are structurally similar to Ors, which are ligand gated

ion-channels, a similar mode of function has long been suggested for the Grs. This hypothesis was bolstered by the observation that both Gr43a in flies and BmGr9, its ortholog in *Bombyx mori* can function as cation channels in cell culture (Sato et al., 2011).

However, other groups have reported strong evidences in support of G protein function in taste neurons. Most of these studies targeted different components of the GPCR pathway, silencing them using genetic manipulation or pharmacologically and subsequently assayed for loss in taste behavior. Silencing Goα in the sweet neurons (with Grs acting as sweet receptors) using RNAi lead to decreased sucrose response as well as reduced feeding (Bredendiek et al., 2011). Same outcome was observed when a pharmacological approach was taken using PTX. Working independently, other groups reported reduced sugar responses when Gsα, Gγi or Gq were silenced (Kain et al., 2010; Ueno et al., 2006). Interestingly, although these studies bear evidence of G-proteins, residual responses still remained even after the components were silenced. Based on all the results published so far, it might be correct to assume that Grs function both as ligand gated ion channels and as GPCRs.

Grs are very widely expressed in the body of the fly. They are present in taste neurons, in the olfactory system, in the reproductive system, in neuroendocrine cells as well as in the central nervous system (Miyamoto et al., 2012; Delventhal and Carlson, 2016; Fujii et al., 2015; Park and Kwon, 2011; Sparks et al., 2013). Although called gustatory receptors, Grs are likely to have a much wider range of function.

The primary function of the Grs is in taste detection. Several Grs have been shown to be responsible for sweet, salt and bitter tastes as well as in pheromone detection

(Dahanukar et al., 2007; Kim et al., 2017a; Watanabe et al., 2011; Weiss et al., 2011). The sweet Grs are evolutionarily conserved with homologs in other arthropods as well as in *Daphnia* (Kent and Robertson, 2009; Peñalva-Arana et al., 2009).

Only 9 Grs required for sweet taste detection have so far been described namely *Gr5a*, *Gr61a*, *Gr64a-f* and *Gr43a* (Fernstrom et al., 2012; Freeman and Dahanukar, 2015; Yarmolinsky et al., 2009). Although all sweet neurons bear the sweet Grs, the exact combination of Grs expressed in one cell might vary from another. Since Grs are expressed in multiple combinations, the exact nature of the receptor complexes is difficult to understand. Studies involving mutant analysis have shown that Gr5a and Gr64a mediate responses to a non-overlapping group of sugars (Dahanukar et al., 2007). Studies have also shown the roles of certain Grs in mediating responses to particular sugars. For eg. *Gr64e* has been shown to mediate taste for glycerol while *Gr43a* is required for fructose (Miyamoto et al., 2012; Wisotsky et al., 2011). Out of all the Grs only *Gr5a* and *Gr43a* has been shown to be able to function on their own in cell culture (Chyb et al., 2003; Sato et al., 2011).

Interestingly, one sweet Gr, *Gr43a*, has been shown to be present in the CNS as well (Miyamoto et al., 2012). Being a fructose receptor, it serves as a sensor to measure fructose level in the hemolymph and helps in maintaining hemolymph sugar level (Miyamoto et al., 2012).

Bitter Grs are more numerous than sugar Grs. Like the sugar Grs they also occur in multiple combinations in bitter neurons, with some neurons expressing as many as 28 bitter taste Grs (LeDue et al., 2015). Of all the bitter Grs, *Gr32a*, *Gr33a*, *Gr66a*, *Gr39a* and *Gr89a* are the most common (Weiss et al., 2011).

Presence of either *Gr32a* or *Gr33a* and *Gr66a* has been demonstrated in most functional bitter neurons and mutant analysis have shown loss of bitter taste when any one of these is missing (Chen et al., 2009; Lee et al., 2009, 2010; Moon et al., 2006).The large number of bitter receptors gives rise to the possibility of an extremely large number of combinations, which is useful since the flies are expected to encounter more varieties of bitter compounds in the nature than any other tastant type. So far only for L-canavanine, the nature of the receptor complex required for detection has been discovered (Shim et al., 2015). Shim et al., 2015 reported that *Gr8a*, *Gr93b* and *Gr66a* together forms the receptor complex for detection of L-cannavanine. Interestingly ectopic expression of these three receptors in sweet neurons as well as in S2 cells have been sufficient to confer L-cannavanine taste response (Shim et al., 2015). Incidentally, this is till now the only known example of a complete functional Gr receptor complex.

Several of the bitter Grs have been shown to be important for detection of pheromone in the tarsi. *Gr32a* has been shown to be responsible for the detection of male inhibitory pheromone 7-tricosene and is responsible for suppressing male-male courtship (Wang et al., 2011).Mutating *Gr32a* increases male-male courtship in flies. Gr33a another bitter receptor is also involved in the suppression of male-male courtship (Hu et al., 2015). Another bitter Gr namely *Gr39a* is required for sustained male courtship (Watanabe et al., 2011).

A recent study has shown that *Gr2a* expressed in two neurons of the LSO in the pharynx is required for detection of high concentrations of sodium salts (Kim et al., 2017). When the *Gr2a* neuron is silenced or *Gr2a* mutated, the fly's ability to reject food with high salt concentration is impaired. Gene silencing revealed that *Gr23a*, another Gr expressed in the same neurons also plays the same role.

Since the other two Grs in these neurons namely *Gr57a* and *Gr93d* did not have an impact on high salt taste, it is likely that *Gr2a* and *Gr23a* might form a complete salt receptor by themselves. However, an ectopic expression system or expression in cell cultures is required to verify this. Importantly, *Gr2a* is absent in the high salt neurons in the labellum suggesting that there are probably other functional high salt receptors in the fly as well.

Other than taste, Grs have been implicated in olfaction as well. *Gr21a* and *Gr63a* present in the ab1c neurons of the fly antenna have been shown to detect carbon di oxide (Kwon et al., 2007). Recent studies have also discovered non-chemosensory roles of the Grs (Ni et al., 2013). Ni et al., 2013 demonstrated that *Gr28d* acts as a thermosensor, while an independent study by Xiang et al., found that *Gr28b* acts as a photoreceptor in larval body wall (Xiang et al., 2010).

Transient receptor potential (Trp) channels: Among all the taste receptors found in flies, Trp channels are the most conserved. Trp channels are involved in a multitude of function including mechanosensing, hearing, photo-transduction, thermosensing as well as in chemical nociception i.e. gustatory detection of noxious chemical compounds. *TrpA1* expressed in the s hairs in required for the detection of aristolochic acid. While *TrpL* another Trp receptor expressed in the s hair detects camphor. There are 9 other members of the Trp receptor family found in flies but no role in chemical senses have yet been ascribed to them.

pickpocket(Ppk) receptors: Ppk receptors are a class of degenesrin/epithelial sodium channels (Deg/ENac) i.e. they are cation channels which are not voltage-gated and are amiloride sensitive (Adams et al., 1998).

Role of Ppk receptors have been discovered in detection of tastes as well as pheromones (Cameron et al., 2010; Hill et al., 2017; Liu et al., 2003; Pikielny, 2012; Vijayan et al., 2014).

Cameron et al., 2010 discovered that *Ppk28* in involved in the detection of water and hypo-osmolarity in the labellum. They demonstrated that *Ppk28* knockout flies had impaired water neuron activity. Additionally, *Ppk28* could confer water sensitivity when expressed ectopically in the bitter neurons and in HEK cells.

Ppk receptos have been shown to play a role in salt taste detection in the larvae. While *Ppk19* (along with *sano*) was reported to be required for avoidance of high salt (Alves et al., 2014), other studies showed that *Ppk19* and *Ppk11* are required for attraction towards low salt (Liu et al., 2003). Surprisingly, these functions of *Ppk19* and Ppk11 could not be seen in adult flies (Zhang et al., 2013).

Three different Ppk receptors namely Ppk23, *Ppk25* and *Ppk29* are required for detection of female pheromones by males and is thus responsible for normal courtship behavior (Pikielny, 2012; Vijayan et al., 2014). Non-chemosensory roles of Ppk channels include liquid clearance from the tracheal system (Liu et al., 2003) as well as mechanical nociception in the larvae (Guo et al., 2014; Zhong et al., 2010).

Ionotropic Receptors: Ionotropic receptors(Irs) were first discovered from a bioinformatic analysis of protein coding genes expressed in the antenna of *Drosophila* (Benton et al., 2009).The Irs are derived from the ionotropic glutamate receptors (IGluRs), the chief difference being the ligand binding domain (Benton et al., 2009). While the ligand binding domain of IGluRs bind only to glutamate a very diverse ligand binding is observed in case of the Irs.

So far, 61 Irs have been founded in *Drosophila* (Benton et al., 2009). Out of them *Ir25a* and *Ir8a* are highly conserved not only across arthropods but protostomes in general (Croset et al., 2010). *Ir76b* is another highly conserved Ir with homologs in all insects as well as in *Daphnia* (Croset et al., 2010). The Irs are widely expressed in the chemosensory organs both olfactory and gustatory (Benton et al., 2009; Koh et al., 2014). Besides, some Irs are also expressed in different cells of the gut. Different groups of Irs are expressed in different groups of chemosensory neurons. Like the Grs, different combinations of Ir mediate responses to different stimulus.

A large body of work exists demonstrating the important role by played by different Irs in chemosensation of volatile molecules. They are required for olfactory response to acids, amines and aldehydes to name a few (Abuin et al., 2011; Ai et al., 2010). Investigations in the olfactory system shows that Irs mostly function as heteromeric complexes with Ir76b, Ir25a and Ir8a potentially acting as the co-receptors (Abuin et al., 2011).

In the recent years several groups have published their results on the role of Irs in gustation. Interestingly, all of these studies explained different roles played by the same Ir namely Ir76b (Croset et al., 2016; Ganguly et al., 2017; Hussain et al., 2016; Zhang et al., 2013). Zhang et al., 2013 reported that *Ir76b* is necessary and sufficient for low salt detection as well as preference in the flies. In *Ir76b* knockouts the salt neurons lost their salt sensitivity while expressing *Ir76b* ectopically in the sugar neuron or in cell culture could confer salt sensitivity. Patch clamp studies by the same group revealed that Ir76b acts as an ungated sodium channel.

A study by Hussain et al., 2016, revealed that *Ir76b* is required for gustatory detection of polyamines whereas independent studies by us (Ganguly et al., 2017) and Croset et al., 2016, have found Ir76b to be required for amino acid taste. Interestingly, the polyamine and the amino acid taste neurons do not respond to salts (Ganguly et al., 2017; Hussain et al., 2016).

Irs have non-chemosensory roles as well with all the evidences coming from three separate publications from Dr. Paul Garrity's lab (Knecht et al., 2016, 2017; Ni et al., 2016). They have described important roles of different Irs in hygrosensation i.e. sensing of humidity. *Ir25a* together with *Ir40a* and *Ir93a* confer sensitivity to dryness while Ir25a and Ir68a together are required for detection of high humidity. Together they mediate dry-seeking behavior in hydrated flies and moisture seeking behavior in dehydrated flies.

Modulation of taste in flies: The function of the gustatory system in flies goes beyond simple detection of chemical compounds. Taste preferences as well as activity of taste neurons are modulated by internal physiological states as well as external factors like contamination of food. This plasticity in the taste system is extremely important for the flies to survive and reproduce.

Internal physiological conditions like mating for female flies or nutritional imbalance changes the fly's needs to ingest particular kinds of nutrients in higher quantities. The fly's taste preferences are likewise modulated to enable them to feed on the required diet. In natural conditions, most available food sources are mixtures of both appetitive and aversive compounds. Since it is essential for the flies to avoid toxic substances, in presence of them, the neurons signaling for appetitive responses are often modulated to prevent feeding.

Modulation by mating status: Females of most multicellular animals invest a large amount of energy for reproduction, hence their nutritional requirements changes after mating. In flies, egg development necessitates larger supply of proteins and amino acids. Subsequently, preferences for yeast (natural source of proteins and amino acids for flies) and amino acids increase dramatically in female flies following mating (Ganguly et al., 2017; Ribeiro and Dickson, 2010; Vargas et al., 2010). Although sodium does not contribute towards reproductive success, yet interestingly female flies develop a higher preference for salt upon mating (Walker et al., 2015).

Sex peptide, a peptide deposited by the male on the female's reproductive tract during mating plays a significant role in the post mating dietary switches (Aigaki et al., 1991; Kubli, 2003; Liu and Kubli, 2003). Some early studies on post-mating dietary switch suggested that sex peptide works directly on sex peptide receptors present on sensory neurons detecting nutrients and pheromones thereby modulating responses at a peripheral level (Kubli, 2003). Many recent evidences however indicate the existence of a more sophisticated pathway. The sex peptides are detected by *Ppk*+, *fru*+,*dsx*+ sex peptide sensory neurons(SPSNs) present in the reproductive tract of the female flies (Häsemeyer et al., 2009; Ribeiro and Dickson, 2010; Yang et al., 2009). The activity of the SPSNs are inhibited as an outcome. This leads to the silencing of the sex peptide abdominal ganglion (SAG) neurons which connect the SPSNs to the central brain where egg-laying and mating related decisions are made (Feng et al., 2014). Octopamine secreted by a small group of sexually dimorphic *dsx*+ octopaminergic neurons on the abdominal ganglion has also been postulated to mediate increased yeast preference upon mating (Rezával et al., 2014). Interestingly, modulation of salt preference following mating has been shown to be independent of octopamine (Walker et al., 2015).

A role of serotonin has also been suggested since increased serotonin level leads to an increased preference for yeast (Vargas et al., 2010). But no evidence suggesting increase in serotonin level following mating has been found (Vargas et al., 2010).

TOR pathway has also been found to be important in the post mating dietary switch for yeast. Activation of *dS6K* a component of the fly TOR pathway increases yeast preference while silencing *dS6K* leads to decreased appetite for yeast (Ribeiro and Dickson, 2010; Vargas et al., 2010).

Most of these studies elaborate about the role of a more central pathway for the dietary switch but whether it correlates with shifts in the peripheral taste sensitivity is yet to be investigated.

Modulation by nutritional imbalance: Nutritional imbalance is caused by both starvation as well as deprivation of specific macronutrients, both of which are capable of altering preferences for different foods in flies.

When flies are wet-starved they have higher sensitivity towards sugar as well as lower sensitivity for bitter compounds (Inagaki et al., 2014). The enhanced sensitivity towards sugar is an outcome of increased secretion of dopamine (Inagaki et al., 2012; Marella et al., 2012). Flies fed on L-dopa a dopamine precursor have been shown to have increased sensitivity for sugar. Activation of the dopamine neurons by expressing the heat receptor *TrpA1* and then applying heat also has a similar effect on sugar sensitivity. It has been reported that dopamine can act directly on the sugar neurons via DopEcR, a class of dopamine receptors expressed in the sugar neurons, to elevate sugar evoked calcium influx, increasing presynaptic activity in response to sugar stimulus (Inagaki et al., 2012).

Another study reported that *Dop2R* function is required for increased sugar sensitivity following starvation (Marella et al., 2012). Interestingly, increased dopamine secretion is controlled by upstream *dNPF*+ neurons; however, whether dNPF itself is required for this process has not been studied.

A different group has reported that starvation leads to upregulation of Gr64a which could also be instrumental in increasing sensitivity to sugar (Nishimura et al., 2012). However, the role of differential regulation of Grs in the changing sugar sensitivity has not been explored. One reason might be that since their expression levels are very low, subtle differences in expression might be difficult to be understood.

The decreased bitter neuron response upon starvation is independent of the increased sugar sensitivity (Inagaki et al., 2014). Inagaki et al., 2014, reported that inhibition of bitter neurons infact is mediated by sNPF, a different neuropeptide. Starvation likely triggers the lateral neurosecretory cells(LNC) to secrete sNPF which acts on sNPFRs present on GABAergic interneurons. However how GABAergic interneurons inhibit bitter compound evoked activity of the bitter neurons is yet to be understood. Lateral neurosecretory cells secrete sNPF on the brain Insulin producing cells (IPCs) as well, however no role of the IPCs in decreasing bitter sensitivity could be found. Interestingly, sNPF secretion by the LNCs is under the control of upstream *AKH*+ cells. Artificially increasing the activity of the *AKH*+ cells using *TrpA1* decreases bitter neuron sensitivity.

Additionally, another study has suggested the role of the ventro-lateral cluster of octopaminergic/tyraminergic neurons (OA-VLs) in modulating bitter neurons upon starvation (LeDue et al., 2016). Starvation leads to a decrease in the neural activity in

OA-VL neurons which in turn leads to secretion of octopamine and tyramine. Both octopamine and tyramine can inhibit synaptic output of the bitter neurons via Oct-TyR receptors present on the bitter neurons.

Interestingly, increase of sugar sensitivity precedes decrease in bitter sensitivity upon increasing starvation (Inagaki et al., 2014).

Studies investigating the effect of macronutrient deprivation mostly focused on the outcome of deprivation of yeast which is a natural source of proteins and amino acids in flies (Liu et al., 2015; Ribeiro and Dickson, 2010; Vargas et al., 2010). Expectedly, flies deprived of yeast has an increased preference for yeast. One study suggests that it also leads to decreased preference for sugar (Liu et al., 2017). Earlier studies postulated that TOR pathway and serotonin plays significant roles in increasing yeast preference upon yeast deprivation (Ribeiro and Dickson, 2010; Vargas et al., 2010). Recently Liu et al., 2017, study suggested that increased yeast intake and decreased sugar intake following yeast deprivation to be the controlled by a group of dopaminergic neurons. Increased yeast preference is mediated by downstream neurons expressing *Dop2R* whereas *Dop1R* expressing downstream neurons mediate decreased sugar preference.

A recent study (Wang et al., 2016) found that when flies are fed with sucralose which is a non-nutritive sweet compound, they have higher preference, neuronal sensitivity and neuronal responses to sugar. *Gr64a+* sweet neurons were found to be modulated in these flies. Sequencing experiments revealed that insulin receptor *InR* was upregulated in these flies. This leads to increase in insulin signaling to octopaminergic and dopaminergic neurons which ultimately cause an increase in dNPF secretion.

dNPF directly acts on the NPF-receptors on the *Gr64a*+ sweet neurons thereby causing an increase in sugar sensitivity. However, another group refuted the claim stating that the sucralose fed flies were essentially starved and the phenotypes observed are indistinguishable from that of wet starved flies (Park et al., 2017).

Modulation by past experiences: A study by Zhang et al., 2013, demonstrated that components of fly taste can be modulated by prior experiences. They reported that when food is contaminated with camphor, a non-toxic bitter compound, for a long time, taste neurons are gradually desensitized to camphor. This is caused by a decline in E3 ubiquitin-ligase which downregulates *Trpl*, the camphor receptor in taste neurons. However, camphor sensitivity is returned to its normal level once flies are reintroduced to normal diet.

Modulation by presence of aversive compounds in food: Since most aversive bitter taste compounds are detrimental to the fly, their chances of survival as well as reproductive success depends on their ability to reject food sources contaminated with bitter compounds. Modulations of these type can occur both at the peripheral level as well as at the level of higher order neurons.

One study shows that sweet neurons are inhibited when sugars are contaminated by bitter compounds (French et al., 2015). They demonstrate that it was not the outcome of ephaptic interaction rather the bitter compounds directly inhibited the sweet neurons. Whether it was through the sweet taste receptors or by altering any other property of the sweet neurons have not yet been understood.

Another group has reported that an olfactory binding protein obp49a plays a significant role in inhibition of sugar neurons by bitter compounds (Zhang et al., 2013).

Obp49a is secreted by the support cells into the taste sensillary hemolymph. It apparently binds with the bitter compounds and carry them to the sweet neurons. However, whether the sweet neurons are inhibited by the bitter compound or by obp49a itself is not clear.

Besides bitter compounds, acids can also inhibit sweet neuron activity in flies (Charlu et al., 2013). One study using calcium imaging shows that in bitter silenced flies, inhibition of tarsal sweet neurons by bitter compounds can be inhibited by acids.

There is also evidence to suggest that GABAergic interneurons play an important role in inhibition of sweet neuron activity in presence of bitter compounds (Chu et al., 2014; Pool et al., 2014). These interneurons synapse with both sugar and bitter neurons. Bitter neuron activation leads to increased activation of the interneurons leading to increased secretion of GABA which decreases presynaptic activity of the sweet neurons via GABA-B receptors.

Another piece of evidence suggests that mechanosensory neurons are also capable of inhibiting sweet neuron activity (Jeong et al., 2016). Flies generally prefer softer food over harder food even with a higher concentration of sugar. Mechanosensory neurons which sense the hardness of the food can inhibit the sweet neuron activity via GABAergic interneurons. Expectedly, this process is also dependent on GABA-B receptor.

Conclusion: Although recent years have seen a remarkable progress in understanding different aspects of fly taste, more remains to be understood. The function of many members of the different taste receptor families expressed in the taste tissues are still unknown.

Among the receptors with known functions, little is understood about how they form functional receptor complexes. Although much remains unknown about the molecular basis of amino acid, acid and high salt tastes. Much remains ununderstood about how taste responses are modulated by nutrition imbalances.

Although there are some studies explaining how starvation affects taste responses, the entire picture is not yet clear. Even less is known about the effects of macronutrient deprivation. The molecular mechanism of sweet neuron inhibition by aversive compounds is not yet understood. Also, whether other aversive tastants beside bitter compounds and acid can inhibit sweet neuron activity is yet to be studied.

Here we attempt to unravel some yet undiscovered aspects of how fly taste is modulated by external and internal conditions. In Chapter 2 we study sexually dimorphic amino acid taste in flies. We discover that *Ir76b* is necessary for amino acid taste preference in flies. We find that other Irs like *Ir20a* combine with *Ir76b* to form functional amino acid receptors. Although we do not present any information about how amino acid responses could be modulated by mating, the study provides a thorough understanding of the molecular and cellular basis of amino acid taste. This is likely to act as a stepping stone in understanding mating and nutrient deprivation mediated changes in amino acid sensitivity at the peripheral level.

In Chapter 3 we study the effects of macronutrient deprivation on fly taste. We find that food preferences as well as behavioral and taste neuron sensitivity are altered in flies deprived of one macronutrient viz. either yeast (amino acids) or sugar. We find that the temporal pattern of the occurrence of the changes vary in these two types of nutrient deprivation and that they are mediated by two different pathways.

While *Dop2R* plays an important role in altered preferences and sensitivity in sugar deprived flies, *dilp5* an Drosophila insulin like peptide is required for compensatory changes following amino acid deprivation.

In chapter 4 we study the role of different classes of non-bitter aversive taste stimuli in sweet neuron inhibition. We discover that sweet neuron activity can be inhibited by high salt as well as high pH. Interestingly high pH also inhibits salt neuron firing suggesting a common strategy to inhibit feeding of any attractive taste stimuli when contaminated with potentially harmful substances.

These studies not only help filling up many gaps in the understanding of different aspects of fly taste but also has some translational properties. Our findings on amino acid test and sweet neuron inhibition can be used to prevent insect-borne diseases. Also, our results from the experiments studying macronutrient deprivation could potentially be extrapolated to better understand aspects of human metabolic disorders.

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Chapter 2: A molecular and cellular context-dependent role for Ir76b in detection of amino acid taste

ABSTRACT

Amino acid taste is expected to be a universal property among animals. Although sweet, bitter, salt, and water tastes have been well characterized in insects, the mechanisms underlying amino acid taste remain elusive. From a *Drosophila RNAi* screen, we identify an ionotropic receptor, Ir76b, as necessary for yeast preference. Using calcium imaging, we identify *Ir76b*$^{+}$ amino acid taste neurons in legs, overlapping partially with sweet neurons but not those that sense other tastants. *Ir76b* mutants have reduced responses to amino acids, which are rescued by transgenic expression of *Ir76b*, and a mosquito ortholog *AgIr76b*. Co-expression of Ir20a with Ir76b is sufficient for conferring amino acid responses in sweet taste neurons. Notably, Ir20a also serves to block salt response of Ir76b. Our study establishes the role of a highly conserved receptor in amino acid taste, and provides a mechanism for mutually exclusive roles of Ir76b in salt and amino acid-sensing neurons.

INTRODUCTION

The importance of dietary protein and amino acids has been investigated for several insects including *Drosophila*, and reveals that, like mammals, insects must acquire some essential amino acids via foods (Golberg and De Meillon, 1948; Hinton et al., 1951; House, 1962; Singh and Brown, 1957). Females, in particular, require large supplies of amino acids for synthesizing egg yolk (Dimond et al., 1956). Restriction of amino acids thus has a direct impact on female fecundity (Chang, 2004; Dimond et al., 1956; Fink et al., 2011).

Amino acid deprivation also significantly affects larval growth and development, as well as adult life span (Baltzer et al., 2009; Britton and Edgar, 1998; Chang, 2004; Grandison et al., 2009; Vrzal et al., 2010).

Given the importance of amino acids in food sources, it is perhaps not surprising that insects demonstrate taste sensitivity to amino acids. Behavioral analyses in various insects, including honeybees, ants, and the dengue fever vector, *Aedes aegypti*, show that mixtures of some amino acids and sugar are preferred over sugar alone (Alm et al., 1990; Ignell et al., 2010; Wada et al., 2001). Moreover, electrophysiological recordings show that selected amino acids evoke action potentials in taste hairs of some insects. For instance, in blowflies and fleshflies some individual amino acids were found to activate either sweet- or salt-sensing neurons; others were found to have inhibitory effects on these taste neurons (Shiraishi and Kuwabara, 1970). Studies in blood-feeding tsetse flies identified neurons in tarsal taste hairs that are exquisitely sensitive to several individual amino acids, as well as to a mixture of amino acids that are found in human sweat (van der Goes van Naters and den Otter, 1998). Amino acid-sensing neurons have also been described in cabbage butterflies (Van Loon and Van Eeuwijk, 1989) and *Helicoverpa* moths (Zhang et al., 2011; Zhang et al., 2010).

Drosophila exhibit strong feeding preference for yeasts and yeast extract, which serve as a major source of protein(Tatum, 1939). Mated females, as well as adult flies fed on a protein deficient diet, can identify and select yeast over sucrose in binary choice assays (Ribeiro and Dickson, 2010; Vargas et al., 2010). A recent study reports behavioral taste sensitivity to free amino acids, albeit only in flies raised on a diet lacking in protein (Toshima and Tanimura, 2012).

In these experiments, flies extended their proboscis upon stimulation of labellar taste hairs with amino acid solutions, indicating a role for taste hairs as amino acid sensors. However, little is known about the molecular and cellular basis of amino acid taste.

Many amino acids taste savory or sweet to humans. Mammals detect amino acids using a heteromeric receptor comprised of two subunits, T1R1 and T1R3, expressed in fungiform taste buds (Nelson et al., 2002). The T1R1/T1R3 receptor has broad specificity for L-amino acids and does not respond to the D isomers. T1Rs, which are G protein-coupled receptors related to metabotropic glutamate receptors, have no counterparts in insect genomes.

Here, we investigated behavioral and cellular responses in the fly to amino acids, identifying them as critical cues for feeding preference to yeast extract. We find that mated females exhibit feeding preference for individual amino acids, which are preferred to different extents in binary choice experiments with sucrose. From an *RNAi* screen, we identify a requirement for a highly conserved chemosensory ionotropic receptor, Ir76b, in mediating feeding preference for yeast extract. Using genetic silencing and calcium imaging experiments, we characterize the role of *Ir76b*$^{+}$ neurons in behavioral and cellular responses to amino acids in mated females. We find that responses to all tested amino acids are lost in *Ir76b* mutants, and rescued by transgenic expression of *Ir76b*. Moreover, Ir76b function is conserved across millions of years of evolution – expression of the *Ir76b* ortholog from *Anopheles gambiae* also rescues the behavioral deficits in *Ir76b* mutant flies. Ir76b has been recently described as a salt taste receptor (Zhang et al., 2013), however we find that amino acid-sensing neurons do not respond to salt. Analysis of additional candidates from our initial *RNAi* screen reveal additional Irs involved in amino acid taste.

Co-expression of one of these, Ir20a, with Ir76b, is sufficient to confer amino acid sensitivity to sweet taste neurons. Moreover, the presence of Ir20a blocks Ir76b-mediated salt response as measured in cellular and behavioral assays. Taken together, our results demonstrate a novel and highly conserved gustatory role for Ir76b in detection of amino acids, in addition to its function as a salt taste receptor. Our studies also identify a role for Ir20a in facilitating mutually exclusive functions of Ir76b in salt and amino acid taste neurons.

MATERIALS AND METHODS

Fly stocks and constructs: Flies were raised on standard cornmeal-dextrose media at 22–25°C. Unless otherwise indicated, wild type flies were w^{1118} (BL 5905). *Ir8a* (BL 41744), $Ir25a^{2}$ (BL 41737), $Ir76b^{05}$ (BL 9824), $Ir76b^{1}$ (BL 51309), $Ir76b^{2}$ (BL51310), *Df*(*Ir76b*) (BL 5126), *UAS-mCD8::GFP* (BL 5130), *UAS-Stinger* (BL 29648), *lexAop-mCherry::HA* (BL 52271), *UAS-GCaMP3* (BL 32236), *Gr64f-GAL4* (BL 57669), *Gr89a-GAL4* (BL 57676), P element transposase (BL 3664) and the DGRP lines were obtained from the *Drosophila* Bloomington Stock Center. *D. pseudoobscura* flies were obtained from the *Drosophila* Species Stock Center. *UAS-RNAi* flies for the *Ir* family, *SPR*, and *tra*, were obtained from the Vienna *Drosophila* RNAi Center. The following stocks were generously shared by others: $Ir76b\text{-}GAL4^{RB}$ and *UAS-Ir76b* (Richard Benton, University of Lausanne, Switzerland); $Ir76b\text{-}GAL4^{CM}$ (Craig Montell, University of California, Santa Barbara); *UAS-Kir2.1* (Kristin Scott, University of California, Berkeley); *fruP1-LexA* (Bruce Baker, Janelia Research Campus).

The promoter reported for $Ir76b\text{-}GAL4^{RB}$ is a 916bp fragment of the sequence immediately upstream of the predicted start codon (Silbering et al., 2011); Primers reported for amplifying the promoter fragment for $Ir76b\text{-}GAL4^{CM}$ are 5'-

GGTTGACCCAGTCTAATGTATGTAATTG and 5'- ACGAGTGCCTACTGTACTCTTTAG (Zhang et al., 2013), which yields a 922bp amplicon, also immediately upstream of the predicted start codon. Thus, the two constructs differ in 7 bp at the 5' end. Observed differences in the expression patterns of the two drivers are possibly due to differences in insertion sites. *Ir76b-LexA*^{RB} was created using a promoter fragment amplified using primer binding sites for 5'-CCAGTCTAATGTATGTAATTG and 5'-CGATACGAGTGCCTACTG. Several independent insertion lines were tested, which showed some variability in expression but the majority showed overlap in expression with *Ir76b-GAL4*^{RB}.

Generation of transgenic and mutant lines: A full-length *AgIr76b* cDNA sequence (VectorBase: AGAP011968) was synthesized by Genescript (Piscataway, NJ); a full-length *Ir20a* coding sequence was amplified from genomic DNA using primers 5'-ATGTTGGCAAGCTTGAA and 5'-TTACAAGCTATTGAAAAATACG. Both were cloned into pUASg-attB and integrated in the attP40 phiC31 landing site.

For generating the Ir20a mutants a region close to the 5' end of the only exon of the gene (**Figure 7B**) was targeted using the following oligos: GGATTGAAGTATACCAGTG and AAACCACTGGTATACTTCAATCC. They were ligated into pU6-BbsI-chiRNA (Addgene # 45946).

The resulting plasmid was directly injected into Vas-Cas9 (BDSC # 51324) embryos and the emerging adult flies were crossed with balancer lines to generate isogenic lines. Genotyping revealed that some of the resulting lines showed no change while several exhibited deletions resulting in frameshift alleles.

Tastants: Tastants were all obtained from Sigma: Caffeine (C8960), HCl 37% A.C.S. reagent (320331), D-phenylalanine (P17151), Glycine (320331), L-alanine (5129), L-

arginine (A8094), L-asparagine (11149), L-aspartic acid sodium salt monohydrate (92384), L-cysteine (30089), L-glutamic acid monosodium salt monohydrate (92834), L-glutamine (98540), L-histidine (53319), L-isoleucine (17403), L-leucine (61819), L-lysine (L5501), L-methionine (64319), L-phenylalanine (P5482), L-proline (81709), L-serine (84959), L-threonine (89179), L-valine (94619), sucrose (S7903), yeast extract (Y1625) and yeast nitrogen base without amino acids and ammonium sulfate (Y1251). Additional tastants were obtained from the following sources: HCl 37% (A.C.S. reagent, 320331); NaCl (Macron Fine Chemicals, 7647-14-5). Tastants were dissolved in water for behavior and calcium imaging experiments, and in 30mM tricholine citrate (Sigma, T0252) for electrophysiological recordings.

Behavior assays: 0-2 day old flies were transferred to fresh food vials (10 males and 10 females per vial), maintained at 25°C with >50% humidity under a 12:12 light:dark cycle, and tested at 5-7 days of age (except for the Ir76b>Ir20a experiments where 7-10 day old flies were tested). Prior to experiments, flies were starved for 24-26 hours in vials with water-saturated Kimwipe beds. Starvation time for *D. pseudoobscura* was 26 hours. This starvation regime was chosen to permit evaluation of innate or baseline preference for various tastants (including amino acids), as opposed to preferences modulated by specific dietary requirements. Tests were performed in tight-fit Petri dishes (Falcon 35-1006). Solutions of 0.75% agarose containing the stimuli and either 0.25 mg ml^{-1} indigo carmine (Sigma I8130) or 0.5 mg ml^{-1} sulforhodamine B (Sigma 230162) were prepared fresh and spotted in equal numbers in the Petri dishes. To account for any possible bias caused by the dyes, tests were typically performed with the same dye/stimulus combinations (as specified in accompanying figure legends). Flies were fed in the Petri dishes for 2 hours at 25°C in a Styrofoam dark humid chamber.

Feeding was performed between 2–6 PM, after which the flies were frozen and scored for color in the abdomen. Only trials in which >50% flies survived and >50% participated were included in the analysis. Preference indices were calculated using the following formula: $[N_{pink} + 0.5N_{purple}] / [N_{pink} + N_{blue} + N_{purple}]$.

Immunohistochemistry: Fly brains were dissected and fixed in paraformaldehyde and blocked using normal goat serum. Primary antibodies were mouse α-nc82 (1:20, DSHB AB2314866), rat α-CD8a (1:100, Invitrogen MCD0820), rabbit α-HA (1:100, Abcam ab9110) and chick α-GFP (1:500 or 1: 10,000, Abcam ab13970); secondary antibodies were Alexa-488 α-rat (1:150, Invitrogen A11004), Alexa-568 α-mouse (1:150, Invitrogen A11004), Alexa-488 α-chick (1:150, Invitrogen A11039), Alexa-568 α-rabbit (1:150, Invitrogen A11036), and Alexa-647 α-mouse (1:150, Invitrogen A21235). Confocal *z*-stack images were acquired using a Leica SP5 confocal microscope and analyzed using Image J.

Calcium imaging: Flies aged ≥7 days, maintained at 29 °C (to allow better expression of GCaMP) for ≥4 days were used for imaging. For single fly preparations for imaging, a fly was anesthetized briefly, decapitated, and glued to the base of a tight-fit Petri dish (Falcon 35-1006) using double-sided sticky tape. The sticky tape was also used to secure the forelegs such that the terminal 2-3 segments remain uncovered. A drop of water (100 µL) was used to cover the exposed part of the leg.

Tastants were applied by adding 100 µL drops at 2X concentrations to the water drop. Between stimuli, the foreleg was rinsed once with water before addition of a second water drop. GCaMP3 fluorescence was recorded using a Leica SP5 confocal microscope. A filter block with 488 nm excitation filter and 500–535 nm emission filter was used.

The focal plane was first adjusted to maximize the number of cell bodies that were visible in the fifth tarsal segment. The gain was reduced such that cell bodies were green in the spectrum log (mean intensity ≤10), after which images were acquired at ~2.5 frames per second using a 10X objective. Stimuli were added ~10–20 seconds after onset of recording, which was continued for ~2 minutes. Images were analyzed using the Leica SP5 LAS AF software (in quantify mode) to obtain heat maps and fluorescence intensity values. ΔF/F % values were calculated separately for each cell body using the mean intensity value of all frames in the 5-second period prior to addition of the stimulus ($F_{pre(cell)}$) and mean intensity value of all frames in the 5-second period around the peak response ($F_{post(cell)}$). Mean intensity values ($F_{pre(cell)}$ and $F_{post(cell)}$) were calculated similarly for one region of interest chosen in the vicinity of the labeled cell bodies. For wild type analysis, only cell bodies that showed ΔF/F% of ≥10 were included; all cells were included for the experiment in Figure 5A. In all cases, cells with ΔF/F% values that deviated >2 standard deviations from the mean were excluded from the analysis.

ΔF/F% was calculated with the following formula:

$$\frac{[F_{post(cell)} - F_{post(background)}] - [F_{pre(cell)} - F_{pre(background)}]}{[F_{pre(cell)} - F_{pre(background)}]} \times 100$$

Electrophysiological recordings: Extracellular tip recordings were obtained from L-type labellar sensilla as described previously (Benton and Dahanukar, 2011). Recordings from tarsal sensilla were modified from Ling et al., 2014. Flies were anesthetized with CO2 and decapitated. Using thin strips of double sided sticky tapes flies were attached to a glass slide with their forelegs protruding out of the slide. More strips of tape were used to keep the forelegs from moving. A reference electrode was inserted into their abdomen and using a reference electrode filled with tastant mixed with

electrolyte (30mM tricholine citrate) the recordings were conducted. For tarsal recording Female flies aged 8–10 days (labellar) or 5-7 days (tarsal) were used for recordings. All chemicals were dissolved in 30 mM tricholine citrate, which served as the electrolyte. Neuronal responses were quantified by counting the number of spikes in the first 500 ms upon contact with the stimulus.

Statistical analyses: Behavioral preference indices were compared using the Mann-Whitney *U* test or ANOVA with pairwise comparisons using Bonferroni adjustment for multiple comparisons or Tukey's *post hoc* analysis. Changes in calcium activity were compared using Mann-Whitney *U* tests.

Sample sizes and statistical tests were chosen based on previously published studies, and are cited in all figure legends. For all column and line graphs, error bars indicate s.e.m.; error bars in scatter plots indicate s.d.

RESULTS

Amino acids mediate sexually dimorphic feeding preference for yeast extract

To explore mechanisms underlying yeast taste detection, we first characterized feeding responses to yeast extract, which contains free amino acids, peptides, sugars, and salts as well as various B vitamins. We used binary choice feeding tests in which batches of cohabiting male and female flies were offered a choice between 5 mM sucrose and 1% yeast extract, after which their yeast preference indices (PI) were computed separately. By testing *D. melanogaster* and a distantly related species, *D. pseudoobscura*, we found that female flies of both species preferred yeast extract to a greater extent than male flies (**Figure 2.1A**). Control experiments with sucrose alone revealed little if any sex-specific variation in sugar feeding (**Figure 2.1B**).

Relative preference for yeast extract was concentration-dependent in both sexes, but nevertheless lower in males than observed in females (**Figure 2.1C**). In addition, flies without antennae (wild type, antennae-less) or without any functional olfactory neurons (Benton et al., 2009; Larsson et al., 2004) (Δ*orco*, antennae-less) showed the same preference for yeast extract as intact wild type flies (**Figure 2.1D**), suggesting the capability to evaluate it as a food source even in the absence of olfactory input. Surprisingly, flies failed to display the same behavioral preference for yeast extract depleted of amino acids (**Figure 2.1D**). Together, the results suggest that amino acids mediate behavioral responses to yeast extract.

Individual amino acids are preferred to different extents

We next characterized behavioral responses to individual amino acids. We elected to test individual L-amino acids in binary choice tests with 5 mM sucrose, which evokes robust feeding responses by itself. Amino acids were tested at 25 mM, which is within the range reported in a number of commercially available yeast extracts, and feeding choice was monitored along with overall feeding participation of both males and females. The test conditions were so chosen to reveal variations in preferences for individual amino acids, which could be more easily observed relative to sucrose. We found that different amino acids were preferred to greatly different extents relative to sucrose (**Figure 2.2A**), as compared to the results of a previous study in which mean preferences of individual amino acids were found to be more similar to each other (Toshima and Tanimura, 2012). As observed for yeast extract, male flies tested in parallel showed little or no preference for any of the amino acids when sucrose was offered as an alternative (**Figure 2.2A**). The strongest behavioral responses were elicited by serine, phenylalanine, alanine, threonine, and glycine.

We performed additional control experiments to examine the validity of these responses: First, we tested L- and D-phenylalanine in parallel and found that, by contrast to L-phenylalanine, flies exhibit no preference for the D isomer over sucrose (**Figure 2.S1**). We then tested the L and D isomers of phenylalanine against each other, and as predicted, found that flies preferred L-phenylalanine over D-phenylalanine (**Figure 2.S1**). Simultaneous tests in which L-phenylalanine was added to both dyes yielded an iso-preference index (PI = 0.4563±0.0353, mean±s.e.m., *n*=6).

We then carried out a series of experiments in which we tested serine against one of three other amino acids. The results from these experiments were consistent with the preferences derived from binary choice assays with sucrose, and showed that serine was preferred to the same extent as phenylalanine, slightly preferred over glycine, and strongly preferred over proline (**Figure 2.S2**). Flies showed equal preference (PI = 0.5083±0.0083, mean±s.e.m., *n*=6) in control experiments in which serine was added to both dyes (**Figure 2.S2**).

The five amino acids that elicited the strongest preference were together sufficient to restore behavioral activity to amino acid-deprived yeast extract (**Figure 2.2B**). Among these, phenylalanine and threonine are essential dietary amino acids (Sang and King, 1961). Glycine, although not essential, is required for normal growth and development (Sang and King, 1961). The activity of the five amino acids was mimicked by a subset of three, which included two essential amino acids, phenylalanine and threonine, along with serine (**Figure 2.2B**). In fact, males exhibited a higher preference for this mixture than to yeast extract alone, possibly due to differences in relative amounts of attractive amino acids in yeast extract.

Using the same paradigm, we tested a mixture of the five amino acids that elicited the weakest preference and found that this mixture did not confer any change in palatability (**Figure 2.2B**).

Feeding preference elicited by each of the top five amino acids was roughly concentration dependent across a range up to 100 mM (50 mM for phenylalanine, due to limitations of solubility) (**Figure 2.2C**). Moreover, experiments to compare behavioral preferences of mated and virgin females revealed that the choice to feed on amino acids was significantly elevated upon mating (**Figure 2.2D**), consistent with previous studies that showed increased yeast preference in mated females (Ribeiro and Dickson, 2010; Vargas et al., 2010).

A Screen of DGRP lines did not yield any meaningful result

To identify the genes necessary in amino acid taste we conducted a screen of 101 DGRP (Drosophila Genetics Reference Panel) fly lines. The DGRP collection consisting of more than 200 lines stems from fly population occurring in the wild at Raleigh, North Carolina, USA. The wild caught lines were inbred to isogenize them and sequenced to identify the SNPs present. The lines were observed to include most common polymorphisms. We tested the flies in a binary choice assay by giving them a choice between 5mM sucrose and 25 mM phenylalanine. The lines exhibited a wide range of preferences for phenylalanine starting from no preference (non-responder) to very high preferences (hyper responders) **(Figure 2.3)**. We expected to uncover the genes necessary for amino acid taste by identifying the common SNPs in the non-responders as well as the hyper-responders. A preliminary analysis using the DGRP website did not reveal a SNP in a receptor gene or genes involved in metabolic regulation. Hence this approach to detect the genes involved in amino acid taste was discarded.

More meaningful results could have been obtained by testing more amino acids or a mixture of amino acids, but since we took a more direct approach of testing RNAi lines for receptors, the line of investigation using DGRP flies were no longer pursued.

An *RNAi* screen identifies a requirement for *Ir76b* in mediating feeding preference for yeast extract

To identify receptors involved in mediating feeding preference for yeast extract, we used *RNAi* to knock down expression of candidate genes using the pan-neuronal *elav-GAL4* driver and tested adult flies in binary choice assays with sucrose and yeast extract. We focused on *Ionotropic receptor* (*Ir*) genes (Benton et al., 2009), which have been associated with amine and acid sensing in the fly olfactory system (Ai et al., 2010; Min et al., 2013; Silbering et al., 2011), and more recently have been found to be expressed in taste neurons as well (Koh et al., 2014). We found that preference for yeast extract was weakest in *Ir76b-RNAi* females (**Figure 2.4A**).

Notably *Ir76b*, whose expression was previously reported in both olfactory and gustatory tissues (Benton et al., 2009), was among chemoreceptor genes expressed at high levels in taste tissue transcriptomes (rpkm=16.49 in female proboscis, rpkm=5.92 in female legs, *n*=2). Mean preference for yeast extract was reduced for a number of other *Ir-RNAi* lines, including for some reported to be expressed in taste neurons, such as *Ir20a*, *Ir47a*, *Ir52a*, *Ir52d*, *Ir56a*, and *Ir56d* (Koh et al., 2014). Although a significant reduction was observed only for *Ir20a-RNAi*, a few other candidates yielded PI values for most independent trials that were lower than the value of mean PI–standard deviation of the *GAL4* control (**Figure 2.4B**).

We confirmed the absence of a role for Ir8a and Ir25a, the other broadly expressed receptors, by testing available null mutants, neither of which showed significant

reduction in preference for yeast extract (**Figure 2.S3**). A few instances of *Ir-RNAi* lines as well showed significant increases in their preference for yeast extract as compared to *elav-GAL4* controls. These results raise the possibility that few Irs may be involved in detecting components of yeast extract, either volatile or non-volatile, that are repulsive to some degree. Alternatively, since flies were always given a choice between sucrose and yeast extract, the *Ir* genes associated with this phenotype may be involved in sucrose response.

Tarsal *Ir76b*$^{+}$ neurons respond to amino acids

We found no evidence for amino acid sensitivity in a previous electrophysiological analysis of L-type taste hairs (Dahanukar et al., 2007), which are among the extensively characterized taste sensilla of the labellum. A previous study has however reported alanine responses from the tarsal taste bristle f5s present on foreleg (Ling et al., 2014). We therefore elected to record from tarsal taste hairs with 100mM of serine and alanine the two amino acids eliciting greatest preference in taste assays. Other than f5s we also recorded from f4s and f4c, both from foreleg, since these two were easily accessible from the same preparations. We recorded with 100mM sucrose and 10mM denatonium as well to verify the identity of the hairs being tested. Consistent with Ling et al., 2014, both f4s and f5s were activated by both sucrose and denatonium (**Figures 2.5A and 2.5B**) and f4c was strongly activated by denatonium but showed little or no activity to sucrose (**Figures 2.5A and 2.5B**). Interestingly, both serine and alanine elicited responses significantly higher than that elicited by the solvent alone from f5s and f4c (**Figure 2.5A and 2.5B**). No amino acid response was however observed from f4s. This indicated that at least a subset of tarsal bristles harbor amino acid sensitive neurons.

We therefore focused on characterizing the role of tarsal *Ir76b*$^{+}$ neurons in detection of amino acids. *Ir76b-GAL4* is broadly expressed in the tarsi, and reporter expression could be visualized in ~10-25 neurons in each of the four distal tarsal segments in both sexes (**Figure 2.6A**), suggesting that *Ir76b* may label multiple cells in each sensillum.

We expressed GCaMP3 under the control of *Ir76b-GAL4* and measured tastant-evoked changes in calcium activity in neurons of the fourth and fifth tarsal segments. We first measured responses to serine, the amino acid that evoked the strongest behavioral response. Tarsi of mated female flies were stimulated with 100 mM serine and changes in GCaMP3 fluorescence were monitored in *Ir76b-GAL4* cells in the fourth and fifth segments of the tarsi. Application of 100 mM serine resulted in a significant increase in fluorescence, which was not observed with water alone (**Figures 2.6B and 2.6C**), and mean change in fluorescence increased with serine concentrations between 15 and 100 mM (**Figure 2.6D**). The threshold concentration of serine for visualizing a single cell response was higher than that observed for a behavioral response, as has been previously reported for sugars. The number of serine-activated neurons ranged from 1–9 in the different samples; differences in the number and intensity of labeled cells, along with differences in alignment of individual preparations, likely contribute to some of the observed variability. A larger fraction of serine-responsive cells was obtained from the 5th tarsal segment (18 of 21) as compared to the 4th segment (3 of 21), but we could not map the identity of hairs innervated by the activated cells.

We next measured responses to each of the top five amino acids that evoked strong behavioral responses, and observed stimulus-evoked increases in fluorescence in each case (**Figure 2.6E**). Each amino acid was tested at a concentration of 100 mM (except phenylalanine at 50 mM). Interestingly, *Ir76b-GAL4* neurons in male tarsi did not show

strong responses to the five amino acids (**Figure 2.S4**), suggesting that sex-specific differences in peripheral sensitivities may account for differences in taste preference, at least in part.

Taste neurons are typically divided into sub-populations that are selective for a single taste category (Freeman and Dahanukar, 2015). To determine the specificity of *Ir76b-GAL4* cells we measured activity of serine-activated cells to other categories of tastants. First, we identified neurons activated by 100 mM serine. For these experiments, the focal plane was selected to visualize such cells that could be easily identified by their position and relative arrangement among labeled cells for sequential application and imaging using other stimuli. We then applied other tastants and measured calcium activity in these identified cells.

We found that serine-responsive cells were not activated by 50 mM NaCl, 10 mM caffeine, or pH2 HCl (**Figure 2.6F**). However, we observed an overlap between serine- and sucrose-sensing neurons in the tarsi. It is possible that this may have some functional significance, as interactions between sweet and amino acid taste have been reported previously (Alm et al., 1990; Wada et al., 2001); however, by contrast, the detection of other categories of tastants appears to occur independently.

***Ir76b* is necessary for cellular responses to amino acids**

To investigate the role of *Ir76b* in cellular responses to amino acids, we measured responses in *Ir76b* mutant flies. We obtained a null allele, $Ir76b^{2}$, which was generated by imprecise excision of a P-element inserted in the third intron of *Ir76b* (Zhang et al., 2013). We compared responses of $Ir76b^{2}$ mutants with those of flies in which *Ir76b* expression was rescued using the *Ir76b-GAL4* driver. Imaging analysis revealed that

response to 100 mM sucrose was not significantly different between the mutant and rescue flies (**Figure 2.7A**).

However, expression of *UAS-Ir76b* resulted in significantly increased responses to serine, phenylalanine and threonine (**Figures 2.7B and 2.7C**). Although not significant, mean fluorescence changes in response to alanine and glycine were also higher in the rescue flies than those observed in the mutant (P=0.0881, n=11–16 and P=0.0554, n=25–30 respectively, Mann-Whitney U tests versus $Ir76b^{2}$ mutant).

We also tested a second mutant $Ir76b^{MB00216}$ which has a Minos allele in the third intron of *Ir76b* gene. Responses to three tested amino acids (serine, phenylalanine and alanine) were greatly diminished in $Ir76b^{MB00216}$ flies (**Figure 2.S5**), consistent with a role for *Ir76b* in amino acid detection. Expression of *UAS-Ir76b* via *Ir76b-GAL4* in these flies resulted in elevated responses to serine and alanine, and to a lesser extent to phenylalanine as well (**Figure 2.S5**). These results demonstrate that *Ir76b* is necessary for taste neuron responses to amino acids.

***Ir76b* is necessary for behavioral responses to amino acids**

To begin to examine the contribution of *Ir76b* in driving behavioral responses to yeast extract and amino acids we investigated the effect of varying the gene dosage of *Ir76b*. Females with one copy of *Ir76b* (*Df(Ir76b)/+*) showed a reduced mean preference for yeast extract as compared to control flies bearing two copies of the gene ($Ir76b^{+}$) (**Figure 2.8A**). Similarly, overproduction of *Ir76b* via the *GAL4*/*UAS* system (*Ir76b>Ir76b*) caused an increase in mean response to yeast extract in both sexes, and significantly so in male flies (**Figure 2.8A**).

Together, these results support a role for *Ir76b* in the behavioral switch from sucrose to yeast preference. We next tested *Ir76b* mutant females and found that they showed a

drastic reduction in mean preference for yeast extract as compared to *Ir76b*$^{+}$ control flies (**Figures 2.8B and 2.S6A**). We confirmed that the deficit in yeast extract preference was due to the loss of *Ir76b* by three independent rescue experiments.

First, precise excision of the *Ir76b*^{05} P element showed that preference for yeast extract was restored in the revertant (*Ir76b*^{rev}) flies (**Figure 2.5B**).

Second, expression of *UAS-Ir76b* using *Ir76b-GAL4* restored behavioral preference for yeast extract in *Ir76b*^{2} mutants (**Figure 2.5B**).

We also rescued Ir76b expression in Ir76b ^{MB00216} flies using *Ir76b-GAL4* and found that behavioral preference to yeast extract was restored (**Figure 2.S6B**). There were only minor differences in sucrose feeding behavior across all the genotypes tested (**Figures 2.5B and 2.S6A**).

Given that the selection of yeast extract in feeding choice experiments is dependent on the presence of amino acids, we tested the role of *Ir76b* in behavioral responses to amino acids. In a series of feeding choice experiments with each of the top five amino acids we found that behavioral responses in *Ir76b*^{05} and *Ir76b*^{2} mutants were greatly reduced (**Figures 2.5C and 2.5D**). Moreover, those of *Ir76b*^{rev} flies were significantly higher than those in *Ir76b*^{05} flies (**Figure 2.5C**). Similarly, transgenic rescue in *Ir76b*^{2} flies by expressing *UAS-Ir76b* via *Ir76b-GAL4* rescued behavioral responses to each of the five amino acids (**Figure 2.5D**). Behavioral responses to amino acids were somewhat reduced in the *Ir76b* ^{MB00216} as well (**Figure 2.S6B**). Transgenic rescue in *Ir76b* ^{MB00216} flies by expressing *UAS-Ir76b* via *Ir76b-GAL4*, resulted in elevated behavioral responses to four of the five amino acids tested (**Figure 2.S6B**). Based on these results, the simplest interpretation is that *Ir76b* is necessary for taste acceptance of amino acids.

Ir76b function is evolutionarily conserved

All insect genomes sequenced to date reveal one or more orthologs of Ir76b, which belongs to a group designated as “antennal Irs” whose expression in the antenna is potentially conserved in all insects (Croset et al., 2010). Although gustatory expression of *Ir76b* has not been investigated in other insects, we were curious whether a distantly related Ir76b ortholog could substitute for fly Ir76b function. We elected to test *Ir76b* from the malaria vector *Anopheles gambiae*, which is separated from *D. melanogaster* by ~260 million years of evolution (Moreno et al., 2010).

We constructed *UAS-AgamIr76b* and tested behavioral responses to yeast extract in animals in which the *UAS-AgamIr76b* transgene was the only source of *Ir76b*. Results of feeding experiments showed that AgamIr76b restored preference for yeast extract in $Ir76b^{2}$ mutants (**Figure 2.8E**). Additional experiments showed that behavioral responses to each of the top five amino acids were also rescued by *AgamIr76b* (**Figure 2.8F**). Thus, Ir76b function in mediating taste responses to amino acids appears to be evolutionarily conserved in flies and mosquitoes.

Ir76b marks functionally distinct subsets of taste neurons

Two lines of evidence suggest that *Ir76b* is associated with multiple functional categories of taste neurons.

First, we found that *Ir76b-GAL4* labeled multiple neurons per sensillum in tarsi, and these are known to be functionally distinct (Ling et al., 2014). Second, *Ir76b* is involved in taste responses to salt (Zhang et al., 2013), polyamines (Hussain et al., 2016), as well as to amino acids (**Figures 2.7 and 2.8**). We were therefore prompted to further characterize expression of *Ir76b-GAL4*. In addition to the *Ir76b-GAL4* driver used above

(Silbering et al., 2011), hereafter referred to as *Ir76b-GAL4*^{RB}, we obtained a second *Ir76b-GAL4* line (Zhang et al., 2013), named *Ir76b-GAL4*^{CM} for comparison. We observed that both drivers were broadly expressed in external and internal taste organs, and there was little difference in expression in tarsi (61±0.7 and 62±1.73 cells respectively, mean±s.e.m., *n*=3) and pharyngeal taste organs (not shown) between them. Both drivers also labeled taste pegs that line the oral surface of the labial palps (**Figure 2.9A**).

However, *Ir76b-GAL4*^{RB} appeared to be excluded from labellar taste hairs that house salt-sensing neurons, which are labeled by *Ir76b-GAL4*^{CM}. We confirmed this difference by creating an *Ir76b-LexA*^{RB} transgene and performing double labeling experiments with *Ir76b-GAL4*^{CM}, which revealed the presence of numerous cells in the labellum, and axonal projections in the subesophageal zone (SEZ) labeled exclusively by *Ir76b-GAL4*^{CM} (**Figure 2.9A and 2.9B**).

We next determined the overlap of *Ir76b-LexA*^{RB} with markers for sweet (*Gr64f-GAL4*) and bitter (*Gr89a-GAL4*) taste neurons. Consistent with the results of our calcium imaging experiments (**Figure 2.6F**), we found overlap between *Gr64f-GAL4* and *Ir76b-LexA* (**Figure 2.9C**). The *Ir76b*$^{+}$/*Gr64f*$^{+}$ cells are likely those activated by both serine and sucrose. Interestingly, *Ir76b-LexA* expression also partially overlapped with that of *Gr89a-GAL4*. Although serine-activated neurons did not respond to a bitter tastant (**Figure 2.6F**), the expression analysis predicts that one or more bitter compounds would activate a distinct sub-population of *Ir76b*$^{+}$ cells. Visualization of axonal projection patterns in the subesophageal zone revealed patterns of overlap between *Ir76b*$^{+}$ termini and those of sweet and bitter neurons (**Figure 2.9D**), as observed in the periphery.

Given our observation that serine-responsive *Ir76b*$^{+}$ tarsal neurons were not activated by salt (**Figure 2.6F**), we wanted to explore the idea that different subsets of *Ir76b*$^{+}$ neurons may be involved in appetitive responses to salt and amino acids. While *Ir76b-GAL4*^{CM} and *Ir76b-GAL4*^{RB} were co-expressed in tarsal neurons, the main difference between them appears to be that *Ir76b-GAL4*^{CM} labeled salt-sensing neurons in the labellum, whereas *Ir76b-GAL4*^{RB} did so weakly, if at all. We therefore took advantage of the two drivers to perform two sets of experiments.

First, we rescued *Ir76b* function in an *Ir76b*^{2} mutant background using either of the two drivers, which showed that both are sufficient to drive expression in a pattern that rescues salt and amino acid responses (**Figure 2.S7**).

Second, we silenced the two populations of *Ir76b*$^{+}$ neurons using the inwardly rectifying potassium channel Kir2.1 and compared consequences on behavioral responses to various tastants. As expected, *Kir2.1* expression under the control of either driver resulted in a significant loss of preference for yeast extract as compared to the level observed in *GAL4* or *UAS* control flies (**Figure 2.9E**).

By contrast, only silencing of *Ir76b-GAL4*^{CM} neurons caused significant defects in behavioral preference for salt (**Figure 2.9E**). In both cases, minor but significant reductions were observed for behavioral responses to sucrose as compared to control flies (**Figure 2.9E**), likely stemming from sucrose sensitivity of tarsal *Ir76b*$^{+}$ neurons (**Figure 2.6F**). There was no effect on rejection of caffeine (**Figure 2.6E**), suggesting that Ir76b may not be associated with caffeine-sensing class of bitter taste neurons. Alternatively, changes in caffeine-sensitivity may need to be evaluated across a range of concentrations. Overall, these results support the idea that Ir76b is expressed in multiple

functional categories of taste neurons, and largely distinct sets of *Ir76b*$^{+}$ taste neurons mediate appetitive responses to low salt and amino acids.

Ir20a alters Ir76b response from salt to amino acids

We next wished to investigate molecular mechanisms that underlie the difference in Ir76b function in salt and amino acid neurons. Based on previous findings that implicate Ir76b as an independently functioning Na^{+} channel in salt taste neurons (Zhang et al., 2013), and as a co-receptor in olfactory neurons (Abuin et al., 2011), we hypothesized that other members of the Ir family expressed in amino acid sensing neurons may serve to gate the conductance of Ir76b.To identify candidate co-receptors, we returned to the results of our initial *RNAi* screen and re-tested several candidate *Ir-RNAi* lines (**Figure 2.3B**), including *Ir76b-RNAi* as a positive control.

Binary feeding choice experiments were performed using sucrose and amino acid-depleted yeast extract with a mixture of serine, threonine and phenylalanine. As expected, *Ir76b-RNAi* yielded a dramatic loss of preference for the amino acid mixture (**Figure 2.10A**).

Four other *RNAi* lines, for *Ir20a*, *Ir47a*, *Ir56d*, and *Ir64a* respectively, showed significant reductions in amino acid preference as compared to wild type flies (**Figure 2.10A**), suggesting that one or more of these receptors may function with Ir76b to mediate amino acid taste. As would be expected, the expression of at least three of these candidates (*Ir20a*, *Ir47a*, *Ir56d*) has been reported in tarsal taste neurons (Koh et al., 2014). *RNAi* knock-down of *Ir7f*, *Ir8a*, *Ir41a*, *Ir48c*, and *Ir85a* had no effect in this assay, suggesting that these receptors may be involved in sensing other amino acids or other cues in yeast extract.

We next wished to test the possibility that Ir76b functions along with one of the other Irs to mediate amino acid response by expression in sweet taste neurons. We selected *Ir20a* for this analysis, because *Ir47a* and *Ir56d* are likely to already be present in *Gr5a*$^{+}$ sweet neurons based on reporter analysis (Koh et al., 2014). We first confirmed the role of *Ir20a* in detecting amino acids by generating CRISPR/Cas9-mediated mutants (**Figure 2.10B**). Of the alleles recovered, we selected two for further analysis: *Ir20a*^{1} had a 1-nt deletion, which predicted a truncated protein product of 53 amino acids; *Ir20a*^{2} had a 2-nt deletion, which predicted a protein product of 74 amino acids. We also tested a control in which the *Ir20a* sequence had not been altered. *Ir20a*^{1} mutants showed a significant reduction in feeding preference for the amino acid mixture as compared to control flies (P<0.001 versus control, n=11). Although not statistically significant, mean feeding preference was also reduced in *Ir20a*^{2} mutants (P=0.136 versus control, n=13). Consistent with the *Ir20a-RNAi* phenotype, the magnitude of the defect is small, which may account for the discrepancy between *Ir20a*^{1} and *Ir20a*^{2} mutants. As expected, *Ir20a-GAL4* expression overlaps with *Ir76b-LexA* in tarsi and axonal projections from tarsal and pharyngeal neurons (**Figure 2.10C**). Together, these results are consistent with the model that amino acid detection is at least partially dependent on Ir20a. The observation that Ir20a is expressed only in a small subset of Ir76b neurons, and that loss of *Ir20a* has a weaker consequence than that of *Ir76b*, also suggests that Ir20a may have some functional redundancies with other Irs.

We used *Gr5a-GAL4* to ectopically express *Ir76b*, either by itself or in combination with *Ir20a*, in sweet taste neurons. Ectopic experiments were conducted in an *Ir76b*^{2} mutant background to eliminate the activity of the salt taste neuron (Zhang et al., 2013), which is housed along with the *Gr5a*$^{+}$ sweet taste neuron in L-type sensilla.

We used single sensillum recordings to measure responses of sweet taste neurons in L-type sensilla to a mixture of serine, threonine and phenylalanine. While Ir76b alone did not confer sensitivity to the amino acid mixture, we found that co-expression of Ir20a and Ir76b together was sufficient to do so in the milieu of the sweet taste neuron (**Figure 2.10D**).

Given our observations that responses to salt and amino acids appear to be mutually exclusive, we next tested whether co-expression of Ir20a affected the Ir76b-mediated response to salt. As reported before (Zhang et al., 2013), we found that expression of Ir76b in *Gr5a*$^{+}$ neurons was sufficient to confer a salt response, although the strength of the response was somewhat lower than that observed in the endogenous context (**Figure 2.10E**). However, sweet taste neurons in which both Ir20a and Ir76b were expressed showed a reduced mean response to NaCl (**Figure 2.10E**).

To further test the idea that the presence of Ir20a can block salt response of Ir76b, we expressed Ir20a using *Ir76b-GAL4* and measured responses to salt. As predicted, expression of Ir20a caused a significant reduction in cellular and behavioral responses to salt as compared to that observed in *Ir76b-GAL4* or *UAS-Ir20a* control flies (**Figure 2.10F**). Although we cannot rule out the possibility that Ir20a interferes with Ir76b in a non-physiological manner, the simplest interpretation of our results is that Ir76b activity is gated by Ir20a to mediate amino acid taste.

DISCUSSION

Here we report the identification of the cellular and molecular basis of amino acid detection in *Drosophila* taste neurons. Genetic analyses, combined with behavior assays and calcium imaging studies reveal that Ir76b, an ionotropic receptor previously found to

mediate salt taste (Zhang et al., 2013), is necessary for amino acid detection by tarsal taste neurons. Analysis of Ir76b expression and function is consistent with a model (**Figure 2.10G**) in which this receptor marks two functionally exclusive populations of cells, one that responds to salt and another that responds to amino acids. In the latter, Ir76b combines with Ir20a, and possibly other Irs, which gate its activity to amino acid ligands.

Ir genes encode proteins related to ionotropic glutamate receptors and represent an ancient family of chemoreceptors, based on their occurrence in genomes of all protostomes (Croset et al., 2010). Their expression and function has been extensively characterized in the fly olfactory system, in which they are expressed in combinations of up to four receptors in olfactory receptors neurons (Abuin et al., 2011). In keeping with their ancient origin, Irs have been associated with detection of broadly appealing or noxious stimuli, including acids, amines, and ammonia (Abuin et al., 2011; Ai et al., 2010; Grosjean et al., 2011; Min et al., 2013).

More recently, *Ir* gene expression has been analyzed in gustatory neurons of both adult and larval stages, and accords possible roles in taste recognition to several members of the family (Koh et al., 2014; Stewart et al., 2015). However, with the exception of Ir76b, taste functions of Ir proteins remain to be characterized. Given that many *Ir* genes are co-expressed with either *Gr5a* or *Gr66a* in sweet or bitter taste neurons (Koh et al., 2014), another open question is whether, and if so how, Ir proteins coordinate with other classes of receptors.

Ir76b has been proposed to function as a Na^+ leak channel that is fixed in a permeable state (Zhang et al., 2013). In this model, Ir76b-mediated sodium conductance

remains low until contact with salt-laced foods, because the sensillar lymph is rich in potassium but contains low sodium. Ectopic expression of Ir76b yields the predicted outcome – sensitivity to sodium chloride in a concentration dependent manner (Zhang et al., 2013). This was surprising because Ir76b is expressed in a variety of neurons that do not respond to salt, including amino acid-sensing neurons in tarsi.

The identification of Ir20a as one co-receptor that promotes amino acid response and blocks salt response is consistent with the idea that Ir76b conductance is regulated differently in salt and amino acid taste neurons by other members of the Ir family (**Figure 2.10F**). Notably, although expression of Ir20a blocked salt response of *Ir76b*$^{+}$ neurons in L-type sensilla, it was not sufficient to confer sensitivity to amino acids (not shown). Moreover, *Ir* candidates may have been missed within the limited scope of the initial *RNAi* screen using yeast extract, which could have several redundant attractive cues. Thus, in all likelihood, additional Irs operate in combination with Ir76b and Ir20a to form amino acid receptors. The presence of Ir47a and Ir56d in tarsal neurons as well as labellar sweet taste neurons makes them appealing candidates for such roles.

It is also possible that different Irs fulfill the role of Ir20a in other amino acid-sensing neurons. A few observations support this idea. First, *Ir20a* mutants do not phenocopy *Ir76b* mutants (**Figure 2.10B**). Second, Ir20a displays a restricted pattern of expression in 2-3 neurons in the fifth segment (**Figure 2.10C**), representing only a small fraction of *Ir76b*$^{+}$ neurons. Third, there appears to be some diversity in amino acid responses across taste neurons, invoking differences in receptor repertoires. Notably, there is precedent for participation of Ir76b in functional heteromeric receptors with two other Irs in olfactory neurons (Abuin et al., 2011; Benton et al., 2009; Silbering et al., 2011).

An appealing hypothesis is that Ir76b might operate likewise in taste neurons, in complexes with combinations of Irs that may have distinct amino acid recognition properties.

The occurrence of receptor combinations may also explain why different amino acids evoke responses of different strengths.

Sex-dependent variations in food choice have been described previously (Ribeiro and Dickson, 2010), but the extent to which they depend on variation in sensitivity of taste neurons remains to be examined. The results of our calcium imaging experiments suggest that differences in tarsal sensitivity to amino acids may underlie sexual dimorphism in yeast and amino acid preference (**Figure 2.S4**). Moreover, the observation that overexpression of *Ir76b* caused an increase in the preference for yeast extract implies that levels of Ir76b are limiting, particularly in male flies. We therefore expected sexual dimorphism in expression levels of Ir76b. However, transcriptome analysis revealed otherwise. Moreover, neither *Ir76b-GAL4* nor *Ir20a-GAL4* showed any sexual dimorphism in expression in tarsal or pharyngeal neurons, where both are expressed.

Thus, the mechanisms by which amino acid taste and yeast preference are enhanced in females as compared to males are likely to be dependent on as yet unknown sex-specific factors in *Ir76b*$^{+}$ neurons. Interestingly, *Ir76b-GAL4* is not expressed in *fru*$^{+}$ neurons (not shown), suggesting that *fru* circuitry may not underlie the sex-specificity of peripheral amino acid responses.

Amino acid and yeast preferences are also upregulated in females upon mating. We and others have found that virgin females behave much like males in binary choice assays.

Previous studies have shown that the post-mating shift in food preference depends on sex peptide (Ribeiro and Dickson, 2010), which is synthesized by male accessory glands and transferred to the female reproductive tract during copulation, although the manner in which sex peptide receptor (SPR) circuitry impinges on taste circuitry is not known. A recent study found that SPR function in *Ir76b*$^{+}$ neurons plays a role in sexually dimorphic responses to polyamines (Hussain et al., 2016).

However, we found that *RNAi*-mediated knockdown of SPR in *Ir76b*$^{+}$ neurons did not affect the behavioral shift to yeast extract in mated females (**Figure 2.S8**). Thus, the functional overlap between SPR^{+} and amino acid-sensing circuitry is likely to occur downstream of the sensory neuron. Consistent with this model, a role has been identified for fru+/dsx+/ppk+/SPR+ neurons in the reproductive tract that convey information either directly or indirectly to the subesophageal zone (Rezaval et al., 2012).

In mammals, amino acids are detected by a dedicated population of taste receptor cells (Nelson et al., 2002). By contrast, we found that amino acid-sensing neurons overlap with sucrose-sensing neurons in fly tarsi. However, behavioral experiments show that the fly can differentiate between sucrose and amino acids, supporting the idea that the two have distinct percepts in the brain.

The lack of amino acid sensitivity in labellar sweet taste neurons might provide one avenue with which to distinguish the two categories of tastants. Furthermore, previous studies in other insects suggest possible synergistic interactions between sugars and amino acids when presented in mixtures (Alm et al., 1990; Wada et al., 2001). Such interactions may be achieved, at least in part, via the co-expression of amino acid and sweet taste receptors in a subset of neurons. Indeed, this appears to be the case in

fleshflies and blowflies that detect some amino acids via sweet-sensing neurons (Shiraishi and Kuwabara, 1970).

Ir76b is highly conserved in insect genomes (Croset et al., 2010), and the functional substitution of DmIr76b with AgIr76b suggests that its role in taste detection is conserved as well. Although our study highlights the importance of Ir76b and amino acid detection for selection of proteinaceous food sources by phytophagous insects like *Drosophila*, free amino acids are also found in human sweat (Hier et al., 1946) and may serve as critical cues for blood-feeding disease vectors such as mosquitoes and tsetse flies. The identification of Ir76b as a receptor for amino acid taste invites further exploration of molecular mechanisms of amino acid taste in human disease vectors and may lead to targets for control of insect feeding behaviors.

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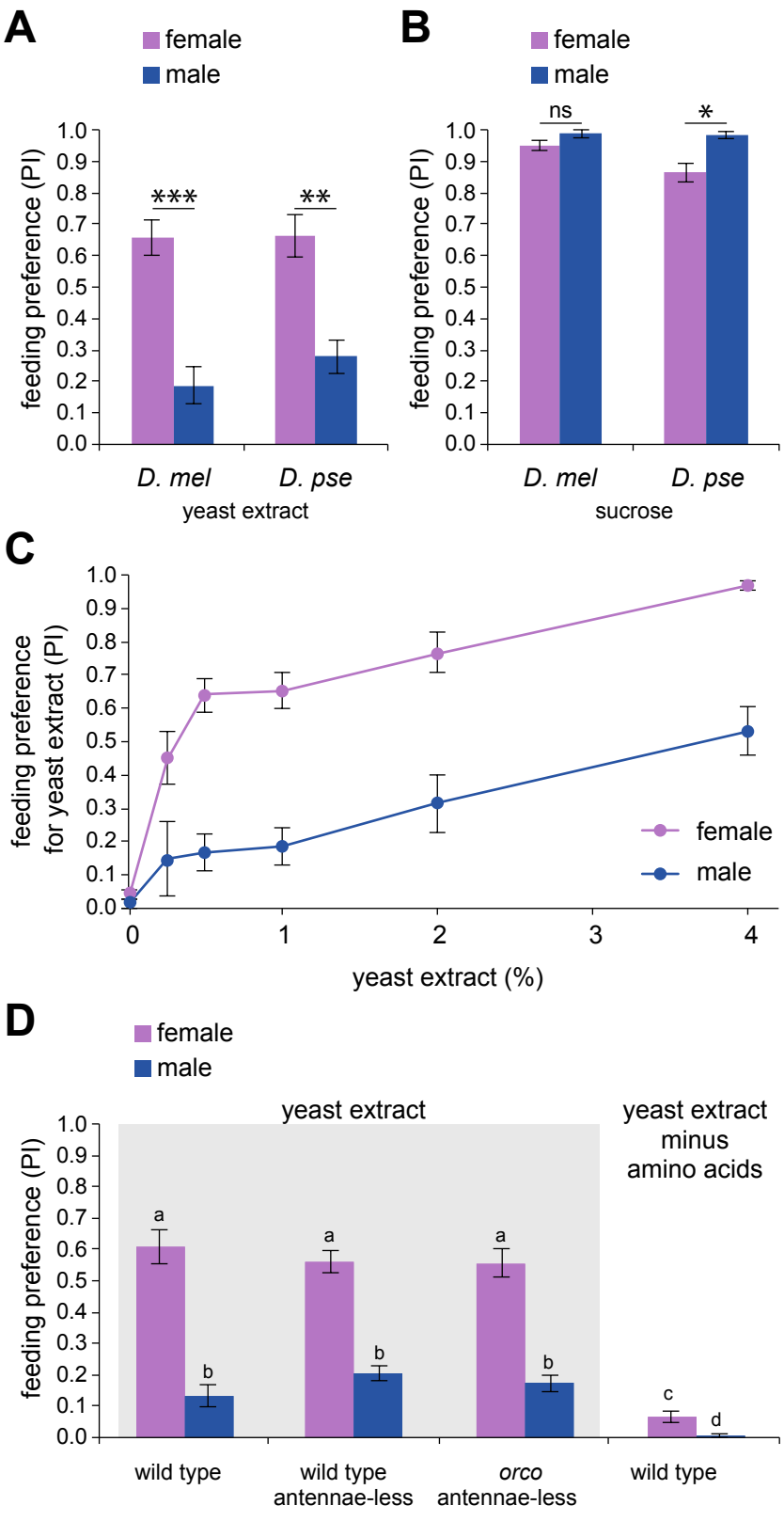

A
female
male
feeding preference (PI)
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
***
**
D. mel
D. pse
yeast extract
B
female
male
ns
*
feeding preference (PI)
D. mel
D. pse
sucrose
C
feeding preference for yeast extract (PI)
female
male
0
1
2
3
4
yeast extract (%)
D
female
male
yeast extract
yeast extract minus amino acids
feeding preference (PI)
a
b
a
b
a
b
c
d
wild type
wild type antennae-less
orco antennae-less
wild type

Figure 2.1. Yeast preference of *Drosophila* females is driven by amino acids

(A and **B)** Mean preference indices (PI) of *Drosophila melanogaster* (*D. mel*) and *Drosophila pseudoobscura* (*D. pse*) to 1% yeast extract (pink dye; tested against 5 mM sucrose, blue) and 5 mM sucrose (blue dye; tested against water, pink) obtained from binary feeding assays. n=6–7 (*D. mel*), n=6–12 (*D. pse*). *P<0.05, **P<0.01, ***P<0.001, Mann-Whitney U test.

(C) Behavioral responses to indicated concentrations of yeast extract (pink dye) tested against 5 mM sucrose (blue dye) in binary choice feeding tests. Genotype was w^{1118}. n=8–13.

(D) Mean PI of intact w^{1118} (wild type), and w^{1118} (wild type, antennae-less) or Δ$Or83b^{2}$ mutants (Δ*orco*, antennae-less) with surgically ablated antennae, obtained from binary feeding tests with choices between 1% yeast extract with or without amino acids (pink dye) as indicated and 5 mM sucrose (blue dye). n=10–18. Different letters indicate significantly different groups, P<0.05, two-way ANOVA with pairwise comparison. Error bars indicate S.E.M.

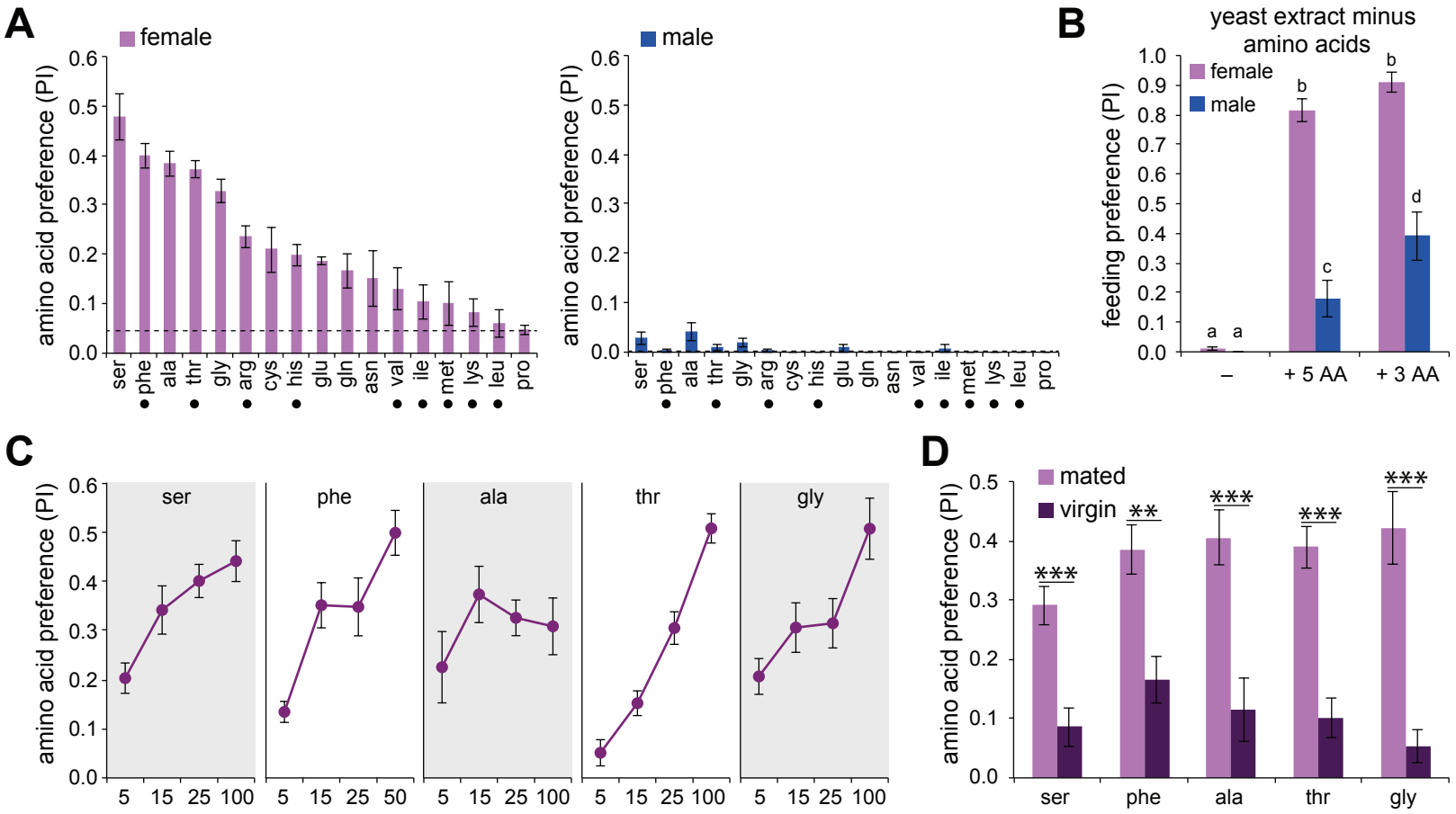

A
female
male
amino acid preference (PI)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
ser
phe
ala
thr
gly
arg
cys
his
glu
gln
asn
val
ile
met
lys
leu
pro
B
yeast extract minus amino acids
female
male
feeding preference (PI)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
a
a
b
c
b
d
–
+ 5 AA
+ 3 AA
C
ser
phe
ala
thr
gly
amino acid preference (PI)
5
15
25
50
100
D
mated
virgin
amino acid preference (PI)
0.0
0.1
0.2
0.3
0.4
0.5
**
***
***
***
***
ser
phe
ala
thr
gly

Figure 2.2. Mated females show increased preference for some amino acids

(A) Mean PI for 25 mM of indicated amino acid (pink dye; tested against 5 mM sucrose, blue dye). Dashed lines indicate preference for water solvent control (pink dye) tested against 5 mM sucrose (blue dye). Black dots indicate the essential amino acids. *n*=6–24.

(B) Mean PI for 1% amino acid-deprived yeast extract alone (–) or supplemented with 25 mM of each of the top five amino acids (+5AA^{top}) from (A), or 25 mM each of serine, threonine, and phenylalanine (+3AA), or 25 mM of each of the bottom five amino acids (+5AA^{bot}) from (A). Each of these combinations (pink dye) were tested against 5 mM sucrose (blue dye). *n*=7–12. For each stimulus, different letters indicate significantly different groups, $P<0.05$, two-way ANOVA with pairwise comparison.

(C) Mean PI of mated females for indicated concentrations of each of the top five amino acids (pink dye) tested against 5 mM sucrose (blue dye). For each concentration, *n*=5–11.

(D) Mean PI of mated or virgin females for 25 mM of named amino acid (pink dye) tested against 5 mM sucrose (blue dye). *n*=6. $^{**}P<0.01$, $^{***}P<0.001$, two-way ANOVA with pairwise comparison. For all experiments, genotype was w^{1118}. Error bars indicate S.E.M.

Preference (P.I.) for 25mM phenylalanine
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
BL 25175
BL 25176
BL 25177
BL 25185
BL 28127
BL 28129
BL 28137
BL 28141
BL 18161
BL 28166
BL 28172
BL 28177
BL 28178
BL 28194
BL 28211
BL 28181
BL 28192
BL 28139
BL 28138
BL 28183
BL 28136
BL 28182
BL 28157
BL 25201
BL 28164
BL 25199
BL 25197
BL 28201
BL 25210
BL 28167
BL 25190
BL 28122
BL 28149
BL 28171
BL 28190
BL 28191
BL 28204
BL 25192
BI 28160
BL 25200
BL 28151
BL 28175
BL 28135
BL 28186
BL 28148
BL 28159
BL 28173
BL 28208
BL 28197
BL 25203
BL 28132
BL 28156
BL 25184
BL 28212
BL 28193
BL 28180
BL 25204
BL 28153
BL 25174
BL 28155
BL 28134
BL 28179
BL 28187
BL 28176
BL 25209
BL 25191
BL 25206
BL 28189
BL 25193
BL 28147
BL 28150
BL 28165
BL 28152
BL 28202
BL 28142
BL 28170
BL 25181
BL 25195
BL 25194
BL 28126
BL 25744
BL 28124
BL 28203
BL 28213
BI 25187
BL 25179
BL 28144
BL 28168
BL 28140
BL 28143
BL 28184
BL 25189
BL 28185
BL 28163
BL 28131
BL 28199
BL 25745
BL 25186
BL 25188
BL 28125
BL 25207

Figure 2.3: Preference for 25mM phenylalanine in DGRP flies.

Mean PI of mated females for 25mM phenylalanine (pink dye) tested against 5mM sucrose (blue dye). All the fly lines tested are from the DGRP collection, the Bloomington stock number of the fly lines are as indicated. n=1-5. Error bars indicate S.E.M.

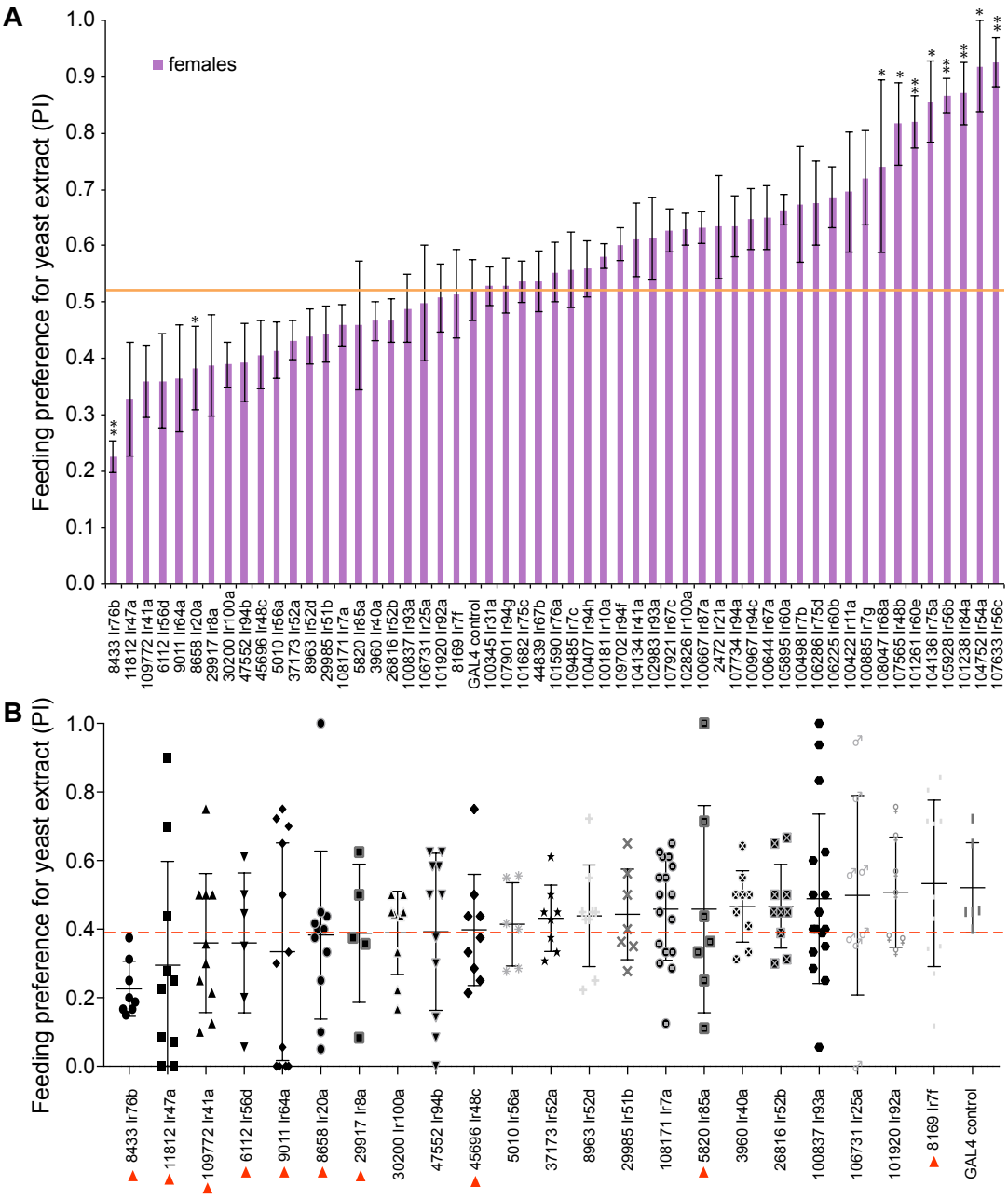

A
females
Feeding preference for yeast extract (PI)
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
8433 Ir76b
11812 Ir47a
109772 Ir41a
6112 Ir56d
9011 Ir64a
8658 Ir20a
29917 Ir8a
30200 Ir100a
47552 Ir94b
45696 Ir48c
5010 Ir56a
37173 Ir52a
8963 Ir52d
29985 Ir51b
108171 Ir7a
5820 Ir85a
3960 Ir40a
26816 Ir52b
100837 Ir93a
106731 Ir25a
101920 Ir92a
8169 Ir7f
GAL4 control
100345 Ir31a
107901 Ir94g
101682 Ir75c
44839 Ir67b
101590 Ir76a
109485 Ir7c
100407 Ir94h
100181 Ir10a
109702 Ir94f
104134 Ir41a
102983 Ir93a
107921 Ir67c
102826 Ir100a
100667 Ir87a
2472 Ir21a
107734 Ir94a
100967 Ir94c
100644 Ir67a
105895 Ir60a
100498 Ir7b
106286 Ir75d
106225 Ir60b
100422 Ir11a
100885 Ir7g
108047 Ir68a
107565 Ir48b
101261 Ir60e
104136 Ir75a
105928 Ir56b
101238 Ir84a
104752 Ir54a
107633 Ir56c
B
Feeding preference for yeast extract (PI)
1.0
0.8
0.6
0.4
0.2
0.0
8433 Ir76b
11812 Ir47a
109772 Ir41a
6112 Ir56d
9011 Ir64a
8658 Ir20a
29917 Ir8a
30200 Ir100a
47552 Ir94b
45696 Ir48c
5010 Ir56a
37173 Ir52a
8963 Ir52d
29985 Ir51b
108171 Ir7a
5820 Ir85a
3960 Ir40a
26816 Ir52b
100837 Ir93a
106731 Ir25a
101920 Ir92a
8169 Ir7f
GAL4 control

Figure 2.4: An RNAi screen to identify Irs involved in feeding preference for yeast extract.

(A) Mean PI values of mated females for 1% yeast extract (pink dye) in binary choice tests with 5 mM sucrose (blue dye). Genotypes were *elav-GAL4/UAS-Ir-RNAi; UAS-Dcr2* or *elav-GAL4; UAS-Dcr2/UAS-Ir-RNAi*. Ir gene name and Vienna Drosophila RNAi Canter stock number is listed for each bar. Control flies are *elav-Gal4/+; UAS-Dcr2/+* (GAL4 control). The orange line indicates mean PI of wild type (w^{1118}) females. n= 5-19. *P<0.05, **P<0.01, ***P<0.001, Mann-Whitney U tests versus GAL4 control.

(B) Scatter plot depicting PI values for individual trials for all lines that yielded mean PI values less than that of the GAL4 control. Red dashed line indicates value of Mean-S.D. for the GAL4 control. Red arrowheads indicate lines that were chosen for further analysis.

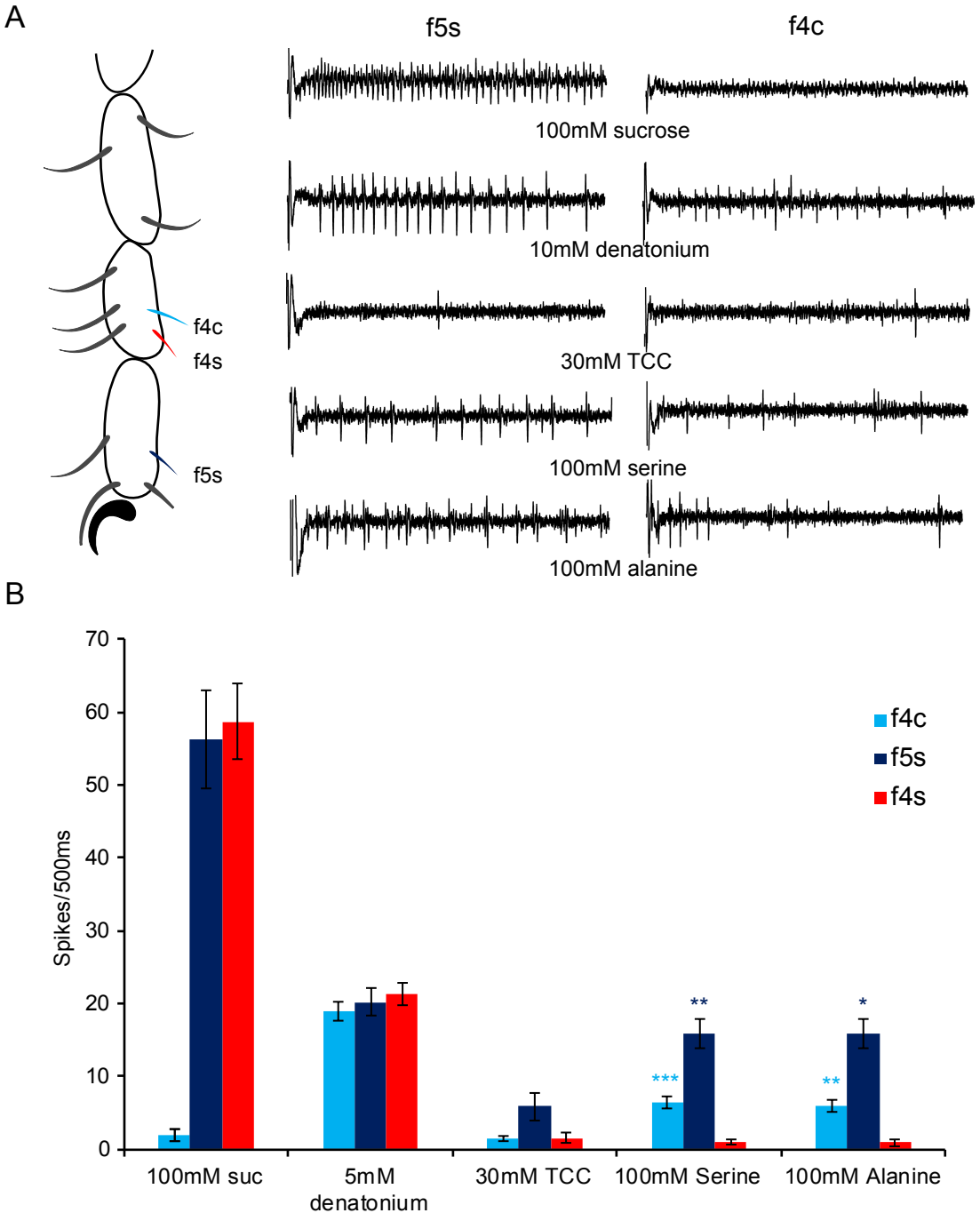

A
f5s
f4c
100mM sucrose
10mM denatonium
30mM TCC
100mM serine
100mM alanine
f4c
f4s
f5s
B
Spikes/500ms
70
60
50
40
30
20
10
0
f4c
f5s
f4s
***
**
**
*
100mM suc
5mM denatonium
30mM TCC
100mM Serine
100mM Alanine

Figure 2.5: Amino acids elicit responses from certain tarsal taste sensilla

(A) Left: Schematics of the arrangement of the taste bristles on the tarsal segment of the foreleg of female flies. Right: Examples of traces obtained from the tarsal bristles f5s and f4c of the foreleg (of mated females) for the first 500ms upon stimulation with the indicated tastants.

(B) Mean responses in the first 500ms obtained from the indicated tarsal sensilla from the foreleg of mated females elicited by the indicated tastants. n=5-9 for f5s and f4, 7-13 for f4s. *P<0.05, **P<0.01, ***P<0.001, Student's T-test. Error bars indicate S.E.M.

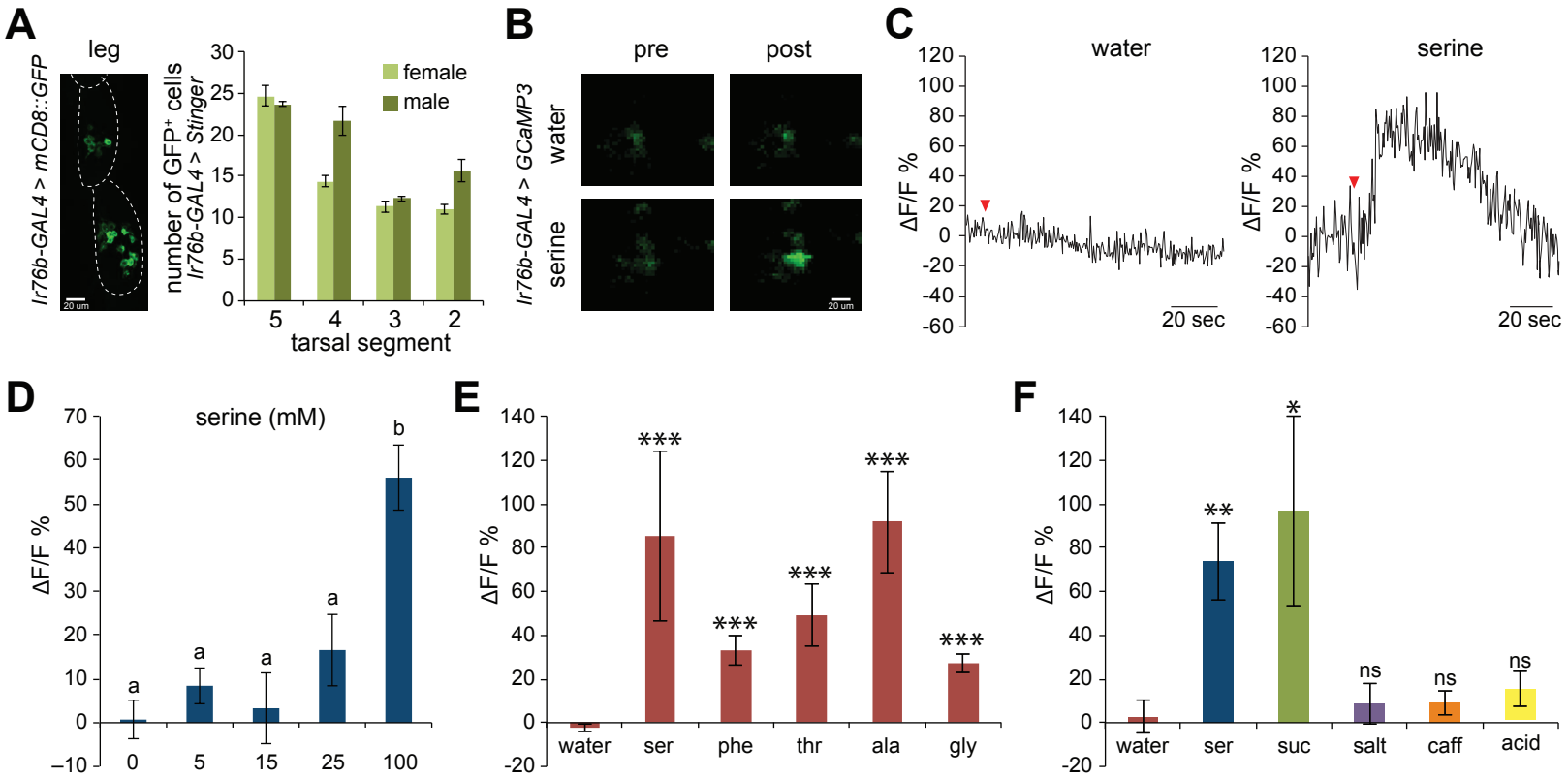

A
leg
Ir76b-GAL4 > mCD8::GFP
20 um
number of GFP+ cells
Ir76b-GAL4 > Stinger
female
male
tarsal segment
B
pre
post
Ir76b-GAL4 > GCaMP3
water
serine
20 um
C
water
serine
ΔF/F %
20 sec
D
serine (mM)
ΔF/F %
E
ΔF/F %
water
ser
phe
thr
ala
gly
F
ΔF/F %
water
ser
suc
salt
caff
acid
ns

Figure 2.6. *Ir76b-GAL4* neurons in female tarsi are activated by amino acids

(A) Representative image of GFP^+ cells in distal segments of female tarsi (left) and mean numbers of GFP^+ cells in indicated tarsal segments in female and male flies (right). *n*=3; error bars indicate s.e.m. Genotypes were *Ir76b-GAL4*^{RB}; *UAS-mCD8::GFP* (left) and *Ir76b-GAL4*^{RB}; *UAS-Stinger* (right). Scale bar, 20 μM. Error bars indicate S.E.M.

(B) Images of GCaMP3 fluorescence in a representative cell before (pre) and after (post) application of water control or 100 mM serine. Genotype was *Ir76b-GAL4*^{RB}; *UAS-GCaMP3*. Scale bar, 10 μM.

(C) Mean change in fluorescence (ΔF/F %) in representative *Ir76b-GAL4*^{RB}; *UAS-GCaMP3* cells in forelegs of female flies. Red arrowheads denote application of water control or 100 mM serine as indicated.

(D) Mean percent changes in GCaMP3 fluorescence after application of indicated concentration of serine. *n*=10–21 cells from 2–5 flies tested per concentration. Measurements were taken from cells that responded to 100 mM serine. For each stimulus, different letters indicate significantly different groups, $P<0.05$, one-way ANOVA with Tukey's *post hoc* test. Error bars indicate S.E.M.

(E) Mean percent changes in GCaMP3 fluorescence after application of indicated stimuli. Amino acids were tested at 100 mM, except phenylalanine, which was tested at 50 mM. ***$P<0.001$, Mann-Whitney *U* tests versus water. *n*=8–41 cells from 3–10 flies per stimulus. Error bars indicate S.E.M.

(F) Mean percent changes in GCaMP3 fluorescence after application of indicated stimuli. Measurements were taken from 100 mM serine-responsive cells. *P<0.05, **P<0.01, Mann-Whitney U tests versus water. n=10–13 cells from 4–5 flies. Error bars indicate S.E.M.

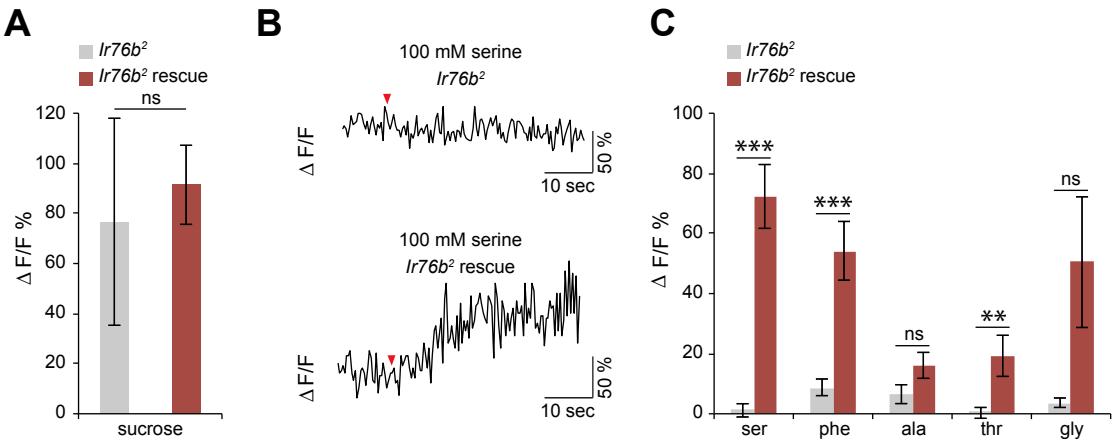

A
Ir76b²
Ir76b² rescue
ns
120
100
80
60
40
20
0
ΔF/F %
sucrose
B
100 mM serine
Ir76b²
ΔF/F
50 %
10 sec
100 mM serine
Ir76b² rescue
ΔF/F
50 %
10 sec
C
Ir76b²
Ir76b² rescue
100
80
60
40
20
0
ΔF/F %
***
***
ns
**
ns
ser
phe
ala
thr
gly

Figure 2.7. *Ir76b* is necessary for cellular responses to amino acids

(A) Mean percent changes in GCaMP3 fluorescence in *Ir76b*^{2} (*Ir76b-GAL4*^{RB}; *Ir76b*^{2}, *UAS-GCaMP3*) and *Ir76b*^{2} rescue (*Ir76b-GAL4*^{RB}; *Ir76b*^{2}, *UAS-Ir76b/Ir76b*^{2}, *UAS-GCaMP3*) flies upon application of 100 mM sucrose. *n*=18–32 cells from 3–5 flies, Mann-Whitney *U* tests. Error bars indicate S.E.M.

(B) Representative ΔF/F traces showing changes in GCaMP3 fluorescence in *Ir76b*^{2} (*Ir76b-GAL4*^{RB}; *Ir76b*^{2}, *UAS-GCaMP3*) and *Ir76b*^{2} rescue (*Ir76b-GAL4*^{RB}; *Ir76b*^{2}, *UAS-Ir76b/Ir76b*^{2}, *UAS-GCaMP3*) flies upon application of 100 mM serine.

(C) Mean percent changes in GCaMP3 fluorescence in *Ir76b*^{2} (*Ir76b-GAL4*^{RB}; *Ir76b*^{2}, *UAS-GCaMP3*) and *Ir76b*^{2} rescue (*Ir76b-GAL4*^{RB}; *Ir76b*^{2}, *UAS-Ir76b/Ir76b*^{2}, *UAS-UGCaMP3*) flies upon application of indicated stimuli. Amino acids were tested at 100 mM, except phenylalanine at 50 mM. *n*=11–32 cells from 2–7 flies (amino acids). ***P*<0.01, ****P*<0.001, Mann-Whitney *U* tests. Error bars indicate S.E.M.

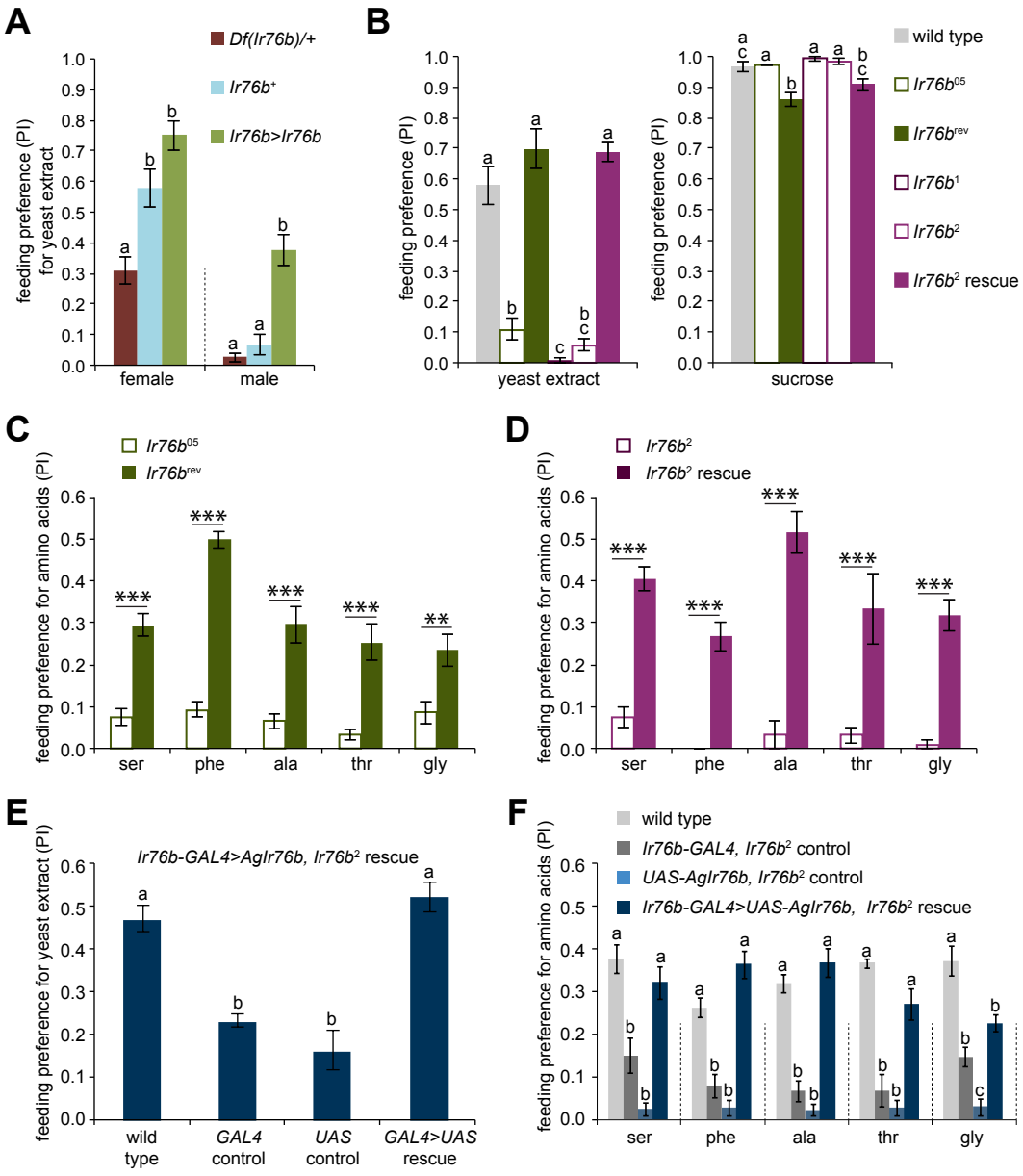

A
Df(Ir76b)/+
Ir76b+
Ir76b>Ir76b
feeding preference (PI) for yeast extract
female
male
B
wild type
Ir76b05
Ir76brev
Ir76b1
Ir76b2
Ir76b2 rescue
feeding preference (PI)
yeast extract
sucrose
C
Ir76b05
Ir76brev
feeding preference for amino acids (PI)
ser
phe
ala
thr
gly
D
Ir76b2
Ir76b2 rescue
E
Ir76b-GAL4>AgIr76b, Ir76b2 rescue
feeding preference for yeast extract (PI)
wild type
GAL4 control
UAS control
GAL4>UAS rescue
F
wild type
Ir76b-GAL4, Ir76b2 control
UAS-AgIr76b, Ir76b2 control
Ir76b-GAL4>UAS-AgIr76b, Ir76b2 rescue

Figure 2.8. *Ir76b* is necessary for behavioral responses to amino acids

(A) Mean PI for 1% yeast extract (pink) tested against 5 mM sucrose (blue) of *Df*(3L)XS533/+ (*Df*(*Ir76b*)/+), w^{1118} ($Ir76b^{+}$) and *Ir76b-GAL4*^{RB}; *UAS-Ir76b* (*Ir76b>Ir76b*) flies. *n*=5–9. For each sex, different letters indicate significantly different groups two-way ANOVA with pairwise comparison.

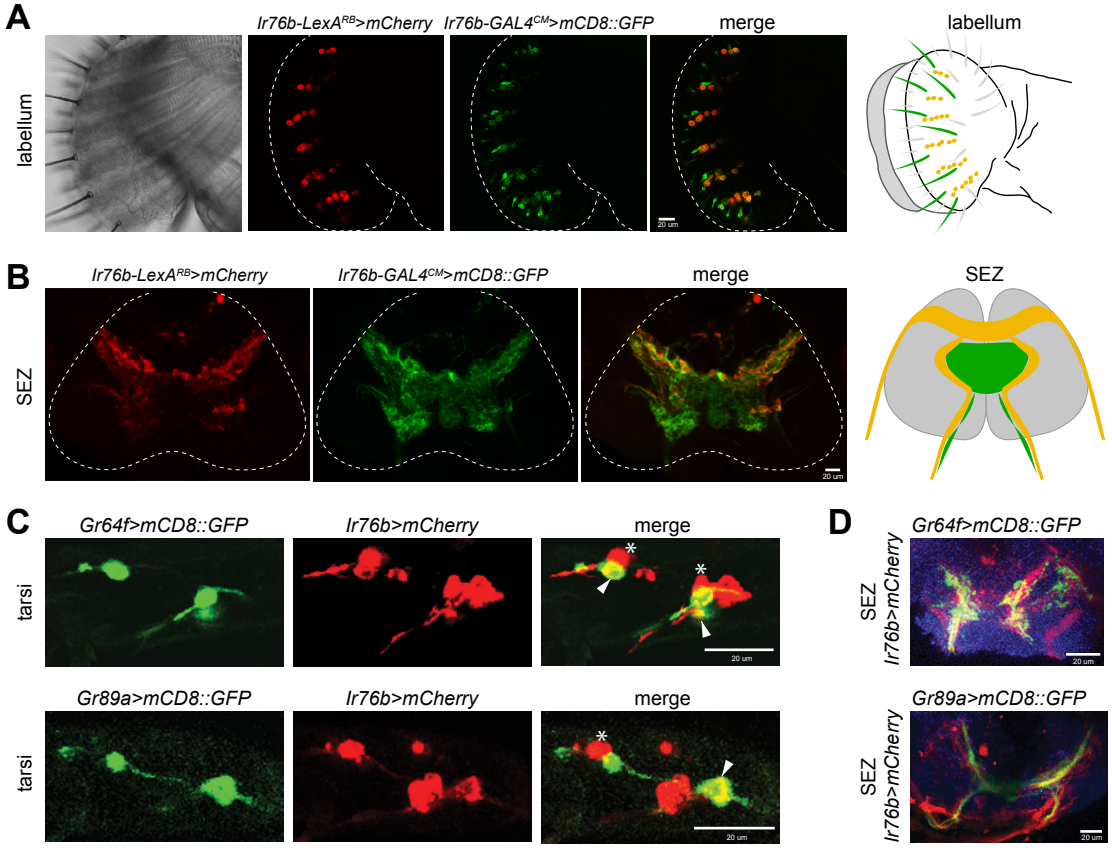
(B) Mean PI of mated female flies for 1% yeast extract (pink; tested against 5 mM sucrose, blue; left) and 5 mM sucrose (pink; tested against water, blue; right) obtained from binary feeding tests. Genotypes were as follows: w^{1118} (wild type), $Ir76b^{05}/Ir76b^{05}$ ($Ir76b^{05}$), $Ir76b^{05}$ precise excision ($Ir76b^{rev}$), $Ir76b^{1}/Ir76b^{1}$ ($Ir76b^{1}$), $Ir76b^{2}/Ir76b^{2}$ ($Ir76b^{2}$), *Ir76b-GAL4*^{RB}/*Ir76b-GAL4*^{RB}; $Ir76b^{2}$, *UAS-Ir76b*/$Ir76b^{2}$, *UAS-Ir76b* ($Ir76b^{2}$ rescue). *n*=6–10 (yeast extract) and *n*=8–12 (sucrose). For each stimulus, different letters indicate significantly different groups, $P<0.05$, one-way ANOVA with Tukey's *post hoc* test.

(C) Behavioral responses of mated females of $Ir76b^{05}$ mutant and precise excision revertant ($Ir76b^{rev}$) genotypes to indicated amino acids (25 mM, pink) tested in binary choice tests with 5 mM sucrose (blue). *n*=5–12. $^{**}P<0.01$, $^{***}P<0.001$, two-way ANOVA with pairwise comparison.

(D) Behavioral responses of mated females of $Ir76b^{2}$ mutants and transgenic rescue flies (*Ir76b-GAL4*^{RB}; $Ir76b^{2}$, *UAS-Ir76b*) to indicated amino acids (25 mM, pink) tested in binary choice tests with 5 mM sucrose (blue). *n*=5–9. $^{***}P<0.001$, two-way ANOVA with pairwise comparison.

(E) Mean PI of mated females for 1% yeast extract (pink; tested against 5mM sucrose, blue) obtained from binary feeding tests. Genotypes were as follows: w^{1118} (wild type); *Sp/CyO; Ir76b*^{1}*, Ir76b-GAL4* (*GAL4* control); *UAS-AgIr76b; Ir76b*^{1} (*UAS* control); *UAS-AgIr76b; Ir76b*^{1}*, Ir76b-GAL4* (*GAL4>UAS* rescue). *n*=6-18. The different letters indicate significantly different groups, *P*<0.05, one-way ANOVA with Tukey's *post hoc* test.

(F) Behavioral responses of mated females to indicated amino acids (25 mM, pink) in binary choice tests with 5 mM sucrose (blue). Genotypes were as in (E). *n*=6–8. The different letters indicate significantly different groups, *P*<0.05, one-way ANOVA with Tukey's *post hoc* test. Dotted lines delineate groups for ANOVA. Error bars indicate S.E.M.


A
Ir76b-LexA^RB>mCherry
Ir76b-GAL4^CM>mCD8::GFP
merge
labellum
labellum
B
Ir76b-LexA^RB>mCherry
Ir76b-GAL4^CM>mCD8::GFP
merge
SEZ
SEZ
C
Gr64f>mCD8::GFP
Ir76b>mCherry
merge
tarsi
Gr89a>mCD8::GFP
Ir76b>mCherry
merge
tarsi
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Gr89a>mCD8::GFP
SEZ
Ir76b>mCherry

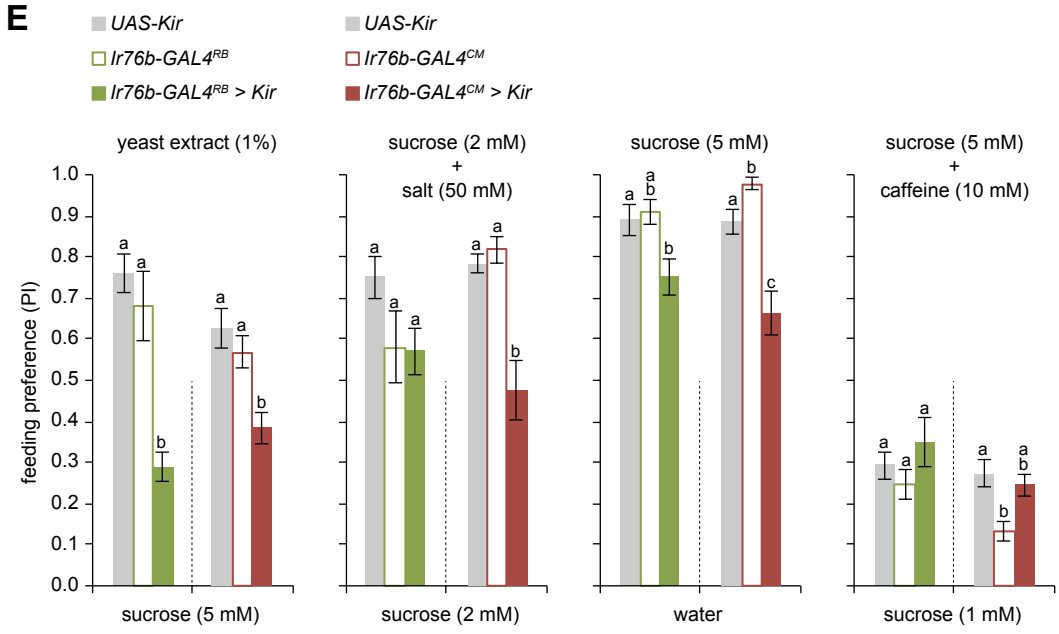

E
UAS-Kir
Ir76b-GAL4^RB
Ir76b-GAL4^RB > Kir
UAS-Kir
Ir76b-GAL4^CM
Ir76b-GAL4^CM > Kir
yeast extract (1%)
sucrose (2 mM) + salt (50 mM)
sucrose (5 mM)
sucrose (5 mM) + caffeine (10 mM)
feeding preference (PI)
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
sucrose (5 mM)
sucrose (2 mM)
water
sucrose (1 mM)

Figure 2.9. Different subsets of *Ir76b-GAL4* neurons mediate behavioral responses to amino acids and salt

(A) Confocal images (left) and schematic (right) of *Ir76b-LexA*^{RB} (red) and *Ir76b-GAL4*^{CM} (green) in the labellum. Neurons labeled exclusively in green innervate taste hairs. Genotype was *Ir76b-LexA*^{RB}/*UAS-mCD8::GFP*; *Ir76b-GAL4*^{CM}/*lexAop-mCherry::HA*. Scale bar, 20 μM.

(B) Confocal images (left) and schematic (right) of axonal projections of *Ir76b-LexA*^{RB} (red) and *Ir76b-GAL4*^{CM} (green) neurons in the subesophageal zone (SEZ) visualized using anti-HA (red) and anti-GFP (green). Genotype as in (A). Scale bar, 20 μM.

(C) Confocal images of *Ir76b-LexA*^{RB} (red) and *Gr64f-GAL4* or *Gr89a-GAL4* (green) neurons in the tarsi. Genotypes were *Ir76b-LexA*^{RB}/*Gr64f-GAL4*; *UAS-mCD8::GFP*/*lexAop-mCherry::HA* and *Ir76b-LexA*^{RB}/*Gr89a-GAL4*; *UAS-mCD8::GFP*/*lexAop-mCherry::HA.* Arrowheads mark *Ir76b*$^{+}$ cells that are also positive for *Gr64f* or *Gr89a*. Asterisks mark *Ir76b*$^{+}$ that do not overlap with the indicated markers. Scale bar, 20 μM.

(D) Confocal images of axonal projections of *Ir76b-LexA*^{RB} (red) and *Gr64f-GAL4* or *Gr89a-GAL4* (green) neurons in the SEZ visualized using anti-HA (red), anti-GFP (green) and anti-nc82 (blue). Genotypes as in (C). Scale bar, 20 μM.

(E) Mean PI of mated females of indicated genotypes from binary feeding tests. Stimuli presented in each set of binary choice trials are listed above (pink) and below (blue) the graphs. n=6–11. For each stimulus, different letters indicate significantly different groups, P<0.05, one-way ANOVA with Tukey's *post hoc* test. Dotted lines delineate separation of groups for ANOVA. Genotypes were: *UAS-Kir2.1*/*UAS-Kir2.1* (*UAS-Kir*); *Ir76b-*

GAL4^{RB}/*CyO*; *TM2*/*TM6b* (*Ir76b-GAL4*^{RB}); *Ir76b-GAL4*^{RB}/+; *UAS-Kir2.1*/*TM2* (*Ir76b-GAL4*^{RB} > *Kir*); *Ir76b-GAL4*^{CM}/*TM6b* (*Ir76b-GAL4*^{CM}); *Ir76b-GAL4*^{CM}/*UAS-Kir2.1* (*Ir76b-GAL4*^{CM} > *Kir*). Error bars indicate S.E.M.

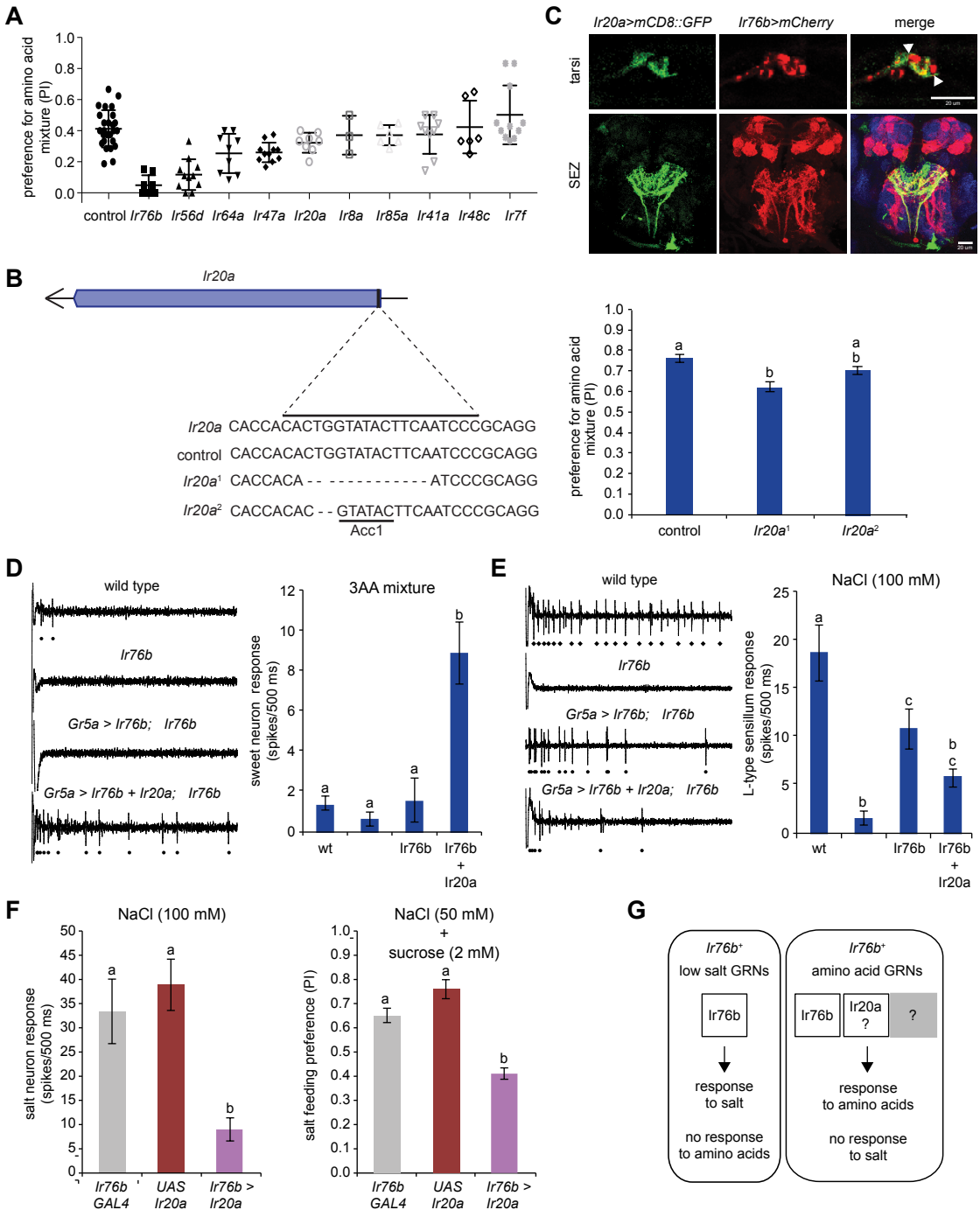

A
preference for amino acid mixture (PI)
1.0
0.8
0.6
0.4
0.2
0.0
control Ir76b Ir56d Ir64a Ir47a Ir20a Ir8a Ir85a Ir41a Ir48c Ir7f
C
Ir20a>mCD8::GFP
Ir76b>mCherry
merge
tarsi
SEZ
B
Ir20a
Ir20a CACCACACTGGTATACTTCAATCCCGCAGG
control CACCACACTGGTATACTTCAATCCCGCAGG
Ir20a1 CACCACA - - - - - - - - - - - ATCCCGCAGG
Ir20a2 CACCACAC - - GTATACTTCAATCCCGCAGG
Acc1
preference for amino acid mixture (PI)
control
Ir20a1
Ir20a2
D
wild type
Ir76b
Gr5a > Ir76b; Ir76b
Gr5a > Ir76b + Ir20a; Ir76b
3AA mixture
sweet neuron response (spikes/500 ms)
wt
Ir76b
Ir76b + Ir20a
E
wild type
Ir76b
Gr5a > Ir76b; Ir76b
Gr5a > Ir76b + Ir20a; Ir76b
NaCl (100 mM)
L-type sensillum response (spikes/500 ms)
wt
Ir76b
Ir76b + Ir20a
F
NaCl (100 mM)
salt neuron response (spikes/500 ms)
Ir76b GAL4
UAS Ir20a
Ir76b > Ir20a
NaCl (50 mM) + sucrose (2 mM)
salt feeding preference (PI)
Ir76b GAL4
UAS Ir20a
Ir76b > Ir20a
G
Ir76b+
low salt GRNs
Ir76b
response to salt
no response to amino acids
Ir76b+
amino acid GRNs
Ir76b
Ir20a ?
?
response to amino acids
no response to salt

Figure 2.10. *Ir76b* is not sufficient for conferring amino acid response

(A) Scatter plots showing PIs of mated female flies for 1% yeast extract–amino acids supplemented with 25 mM each of serine, threonine and phenylalanine. Genotypes were *elav-GAL4*/*UAS-Ir-RNAi*; *UAS-Dcr2* or *elav-GAL4*; *UAS-Ir-RNAi*/*UAS-Dcr2*. F1 progeny from *elav-GAL4*; *UAS-Dcr2* crossed to w^{1118} were used as control. n=6–26 except for Ir8a (n=3). *P<0.05, **P<0.01, ***P<0.001, Mann-Whitney U tests versus the control. Error bars indicate S.D.

(B) Schematic for generation of *Ir20* mutants using CRISPR/Cas9-mediated gene disruption (left). Mean PI of mated females of the indicated genotypes for 1% yeast extract–amino acids supplemented with 25 mM each of serine, threonine and phenylalanine. n=11–17. Different letters indicate statistically different groups, P<0.05, one-way ANOVA. Error bars indicate S.E.M.

(C) Confocal images of *Ir76b-LexA*^{RB} (red) and *Ir20a-GAL4* (green) neurons in tarsi (top) and SEZ (bottom). Axonal projections in the SEZ were visualized using anti-HA (red), anti-GFP (green) and anti-nc82 (blue). Genotype was *Ir76b-LexA*^{RB}/*Ir20a-GAL4*; *UAS-mCD8::GFP*/*lexAop-mCherry::HA.* Scale bar, 20 μM.

(D) Representative traces (left) and mean responses (right) obtained from L-type labellar sensilla of the first 500 ms upon stimulation with a mixture of 100 mM serine, 50 mM phenylalanine and 100 mM threonine (3AA mixture). Black dots indicate action potentials assigned to the sweet taste neuron and counted.

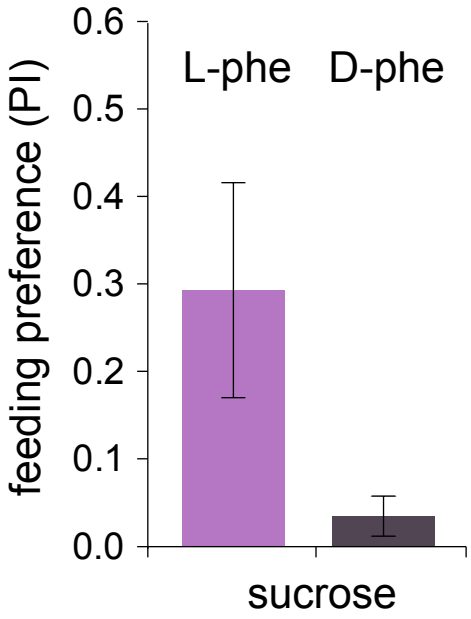
Genotypes: w^{1118} (wild type); *Sp*/*CyO*; $Ir76b^{2}$ (Δ*Ir76b*); *Gr5a-GAL4;* $Ir76b^{2}$*, UAS-Ir76b* (*Gr5a > Ir76b*; Δ*Ir76b*); *Gr5a-GAL4/UAS-Ir20a;* $Ir76b^{2}$*, UAS-Ir76b/*$Ir76b^{2}$ (*Gr5a > Ir76b + Ir20a*; Δ*Ir76b*). *n*=9–18 sensilla from 3–4 flies. For each stimulus, different letters indicate significantly different groups, $P<0.05$, one-way ANOVA. Error bars indicate S.E.M.

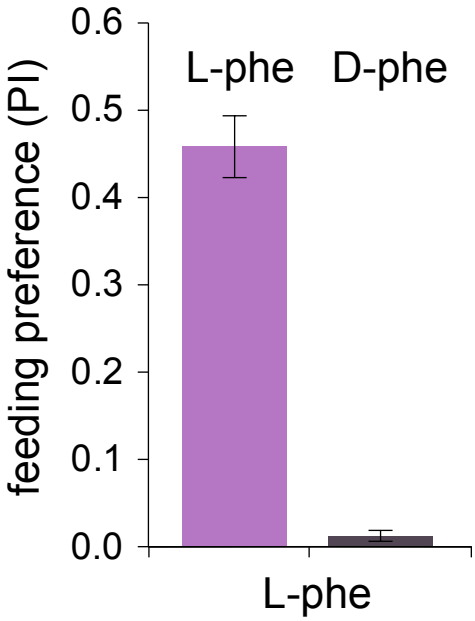
(E) Representative traces (left) and mean responses (right) obtained from L-type labellar sensilla of the first 500 ms upon stimulation with 100 mM NaCl. Only spikes of the larger amplitude were counted, representing the salt neuron in wild type (diamonds), and the sweet neuron in the $Ir76b^{1}$ mutant background (dots). Genotypes as in (C). *n*=9–18 sensilla from 3–4 flies. For each stimulus, different letters indicate significantly different groups, $P<0.05$, one-way ANOVA. Error bars indicate S.E.M.

(F) Mean responses in the first 500 ms upon stimulation obtained from L-type sensilla with a stimulus of 100 mM NaCl (left). *n*=10–11 sensilla from 3 flies. Mean PI of mated females (7–10 days old) for 50 mM NaCl mixed with 2 mM sucrose (tested against 2 mM sucrose) obtained from binary choice tests (right). Results are pooled from pink/blue dye swap experiments. *n*= 12–14. Genotypes were: *Ir76b-GAL4*^{CM}/*TM6b* (*Ir76b-GAL4*); *UAS-Ir20a*/*CyO*; *Dr*/*TM3* (*UAS-Ir20a*); and *UAS-Ir20a*/+; *Ir76b-GAL4*^{CM}/*TM3* (*Ir76b > Ir20a*). For each stimulus, different letters indicate significantly different groups, $P<0.05$, one-way ANOVA. Error bars indicate S.E.M.

(G) Cartoon illustrating possible mechanisms by which distinct subsets of $Ir76b^{+}$ taste neurons mediate salt and amino acid taste.

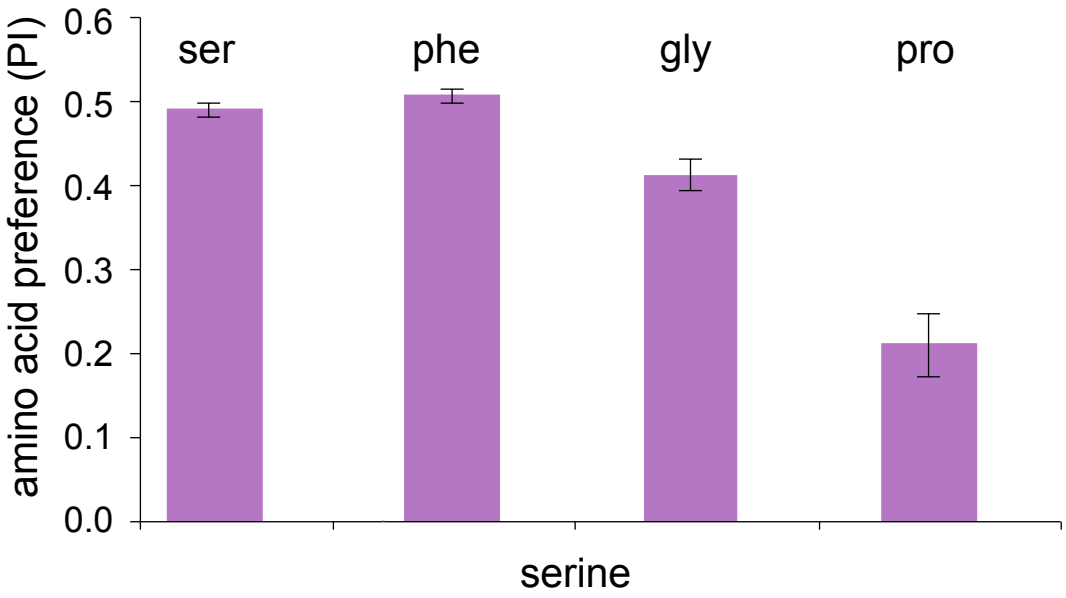
SUPPLEMENTARY FIGURES


0.6
0.5
0.4
0.3
0.2
0.1
0.0
feeding preference (PI)
L-phe
D-phe
sucrose


0.6
0.5
0.4
0.3
0.2
0.1
0.0
feeding preference (PI)
L-phe
D-phe
L-phe

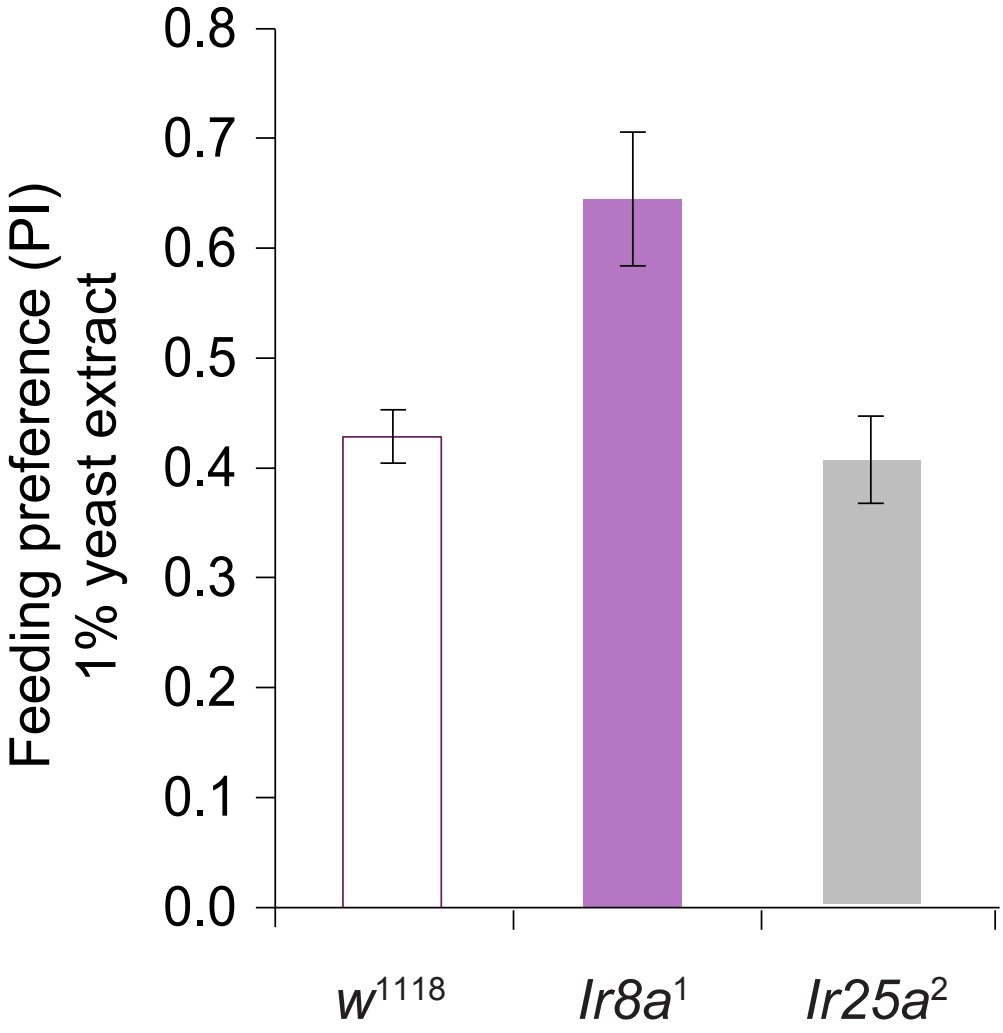
Supplementary Figure 2.S1: Amino acid preference depends on isomeric form.

Mean PI obtained from binary feeding tests with choices between 25mM of L- or D-phenylalanine (pink dye), as indicated, and 5mM sucrose (blue dye; left), and from binary feeding assays in which either 25mM L- or D- phenylalanine, as indicated, were pooled for D-phe/L-phe experiments. *n*=6-9. Results are shown for mated females, genotype was w^{1118}.


amino acid preference (PI)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
ser
phe
gly
pro
serine

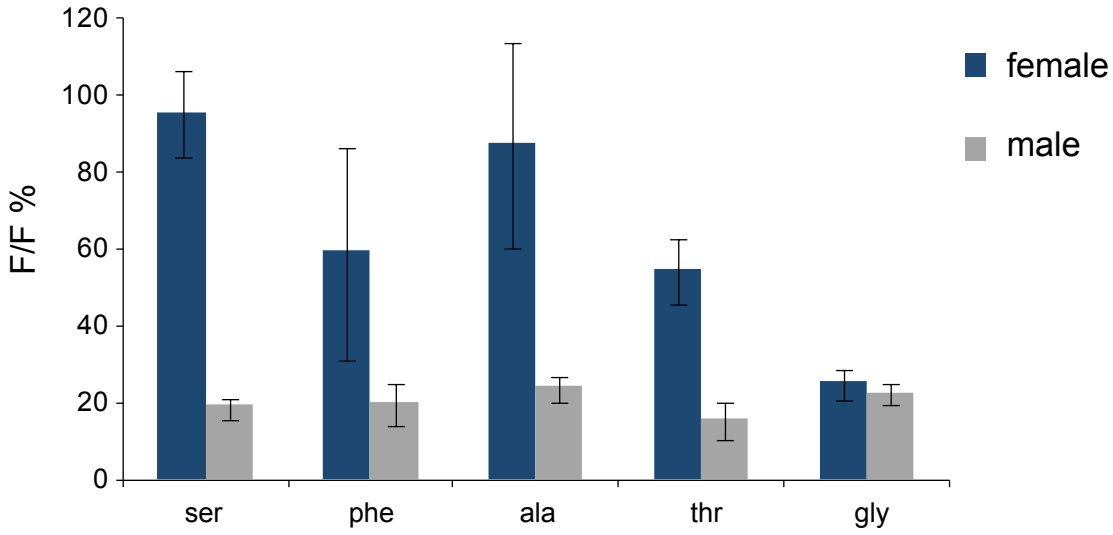
Supplementary Figure 2.S2: Amino acid preference depends on identity.

Mean PI obtained from binary feeding tests with choices between 25mM of the indicated amino acid (pink dye) and 25 mM serine (blue dye). *n*=6-11. Results are shown for mated females, genotype was w^{1118}.


Feeding preference (PI)
1% yeast extract
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
w1118
Ir8a1
Ir25a2

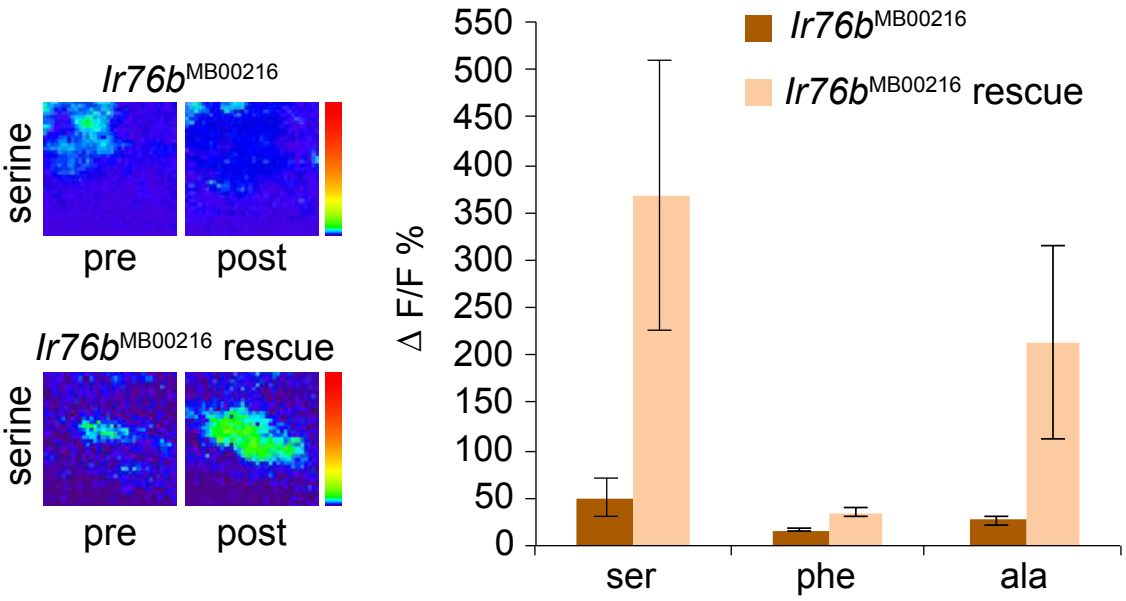
Supplementary Figure 2.S3: Preference for yeast extract is not reduced in *Ir8a* or *Ir25a* mutants.

Mean PI of mated females for 1% yeast extract (pink dye) tested against 5 mM sucrose (blue dye) in binary feeding assays. Genotypes were as follows: w^{1118} (wild type), *Ir8a1;Bl/CyO (Ir8a1)* and *Ir25a2/Ir25a2* (*Ir25a2*). *n*=6-10.


120
100
80
60
40
20
0
F/F %
ser
phe
ala
thr
gly
female
male

Supplementary Figure 2.S4: Amino acid sensitivity in tarsal neurons displays sexual dimorphism.

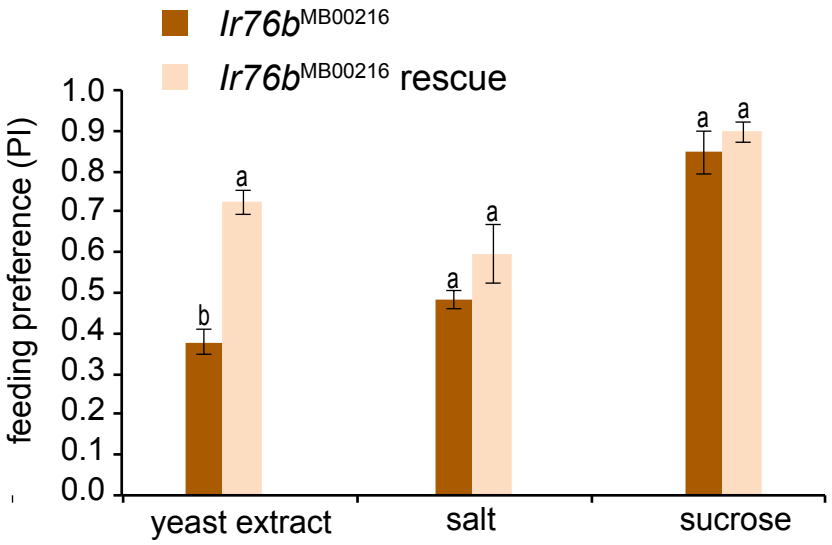
Mean percent changes in GCaMP3 fluorescence in *Ir76b-GAL4* tarsal neurons of female and male flies, as indicated, upon application of individual amino acids. Amino acids were tested at 100mM, except phenylalanine at 50mM. *n*=8-36 for females and 3-11 for males. Genotype was w^{1118}.


Ir76b MB00216
serine
pre
post
Ir76b MB00216 rescue
serine
pre
post
Ir76b MB00216
Ir76b MB00216 rescue
Δ F/F %
550
500
450
400
350
300
250
200
150
100
50
0
ser
phe
ala

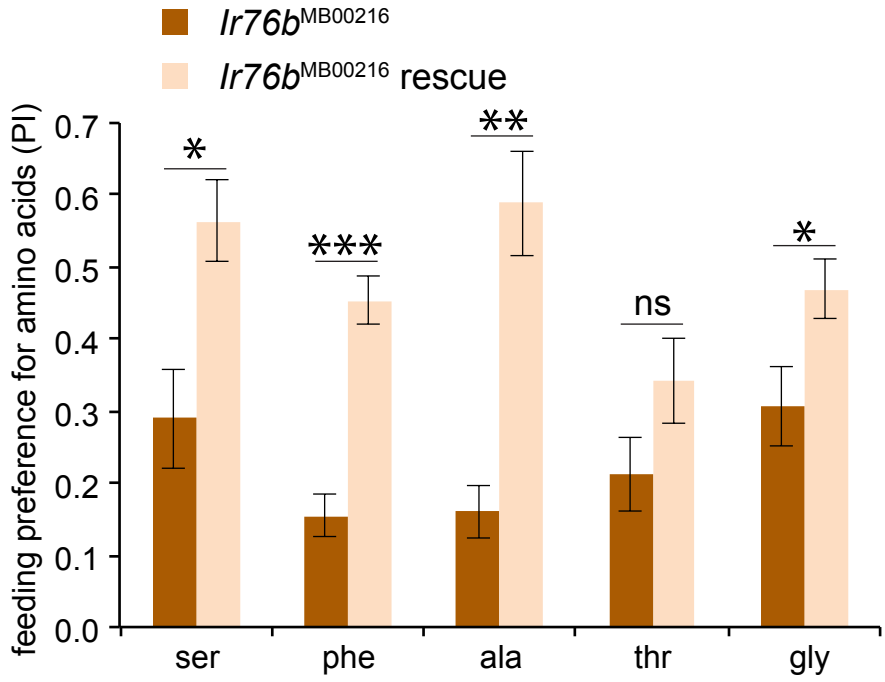
Supplementary Figure 2.S5: *Ir76b* is necessary for taste neuron response to amino acids.

Images of calcium activity on application of 100mM serine(left) and mean percentage changes in fluorescence in tarsal cells of *Ir76b*^{MB00216} and *Ir76b*^{MB00216} rescue (*Ir76b-GAL4/Ir76b-GAL4; Ir76b*^{MB00216}, *UAS-Ir76b/ Ir76b*^{MB00216}, *UAS-GCaMP3*) flies. Amino acids were tested at 100 mM except for phenylalanine which was tested at 50mM. n=9 cells from 7-8 *Ir76b*^{MB00216} flies, n=5–15 cells from 3–5 *Ir76b*^{MB00216} rescue flies.

A


Ir76b MB00216
Ir76b MB00216 rescue
feeding preference (PI)
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
a
b
a
a
a
a
yeast extract
salt
sucrose

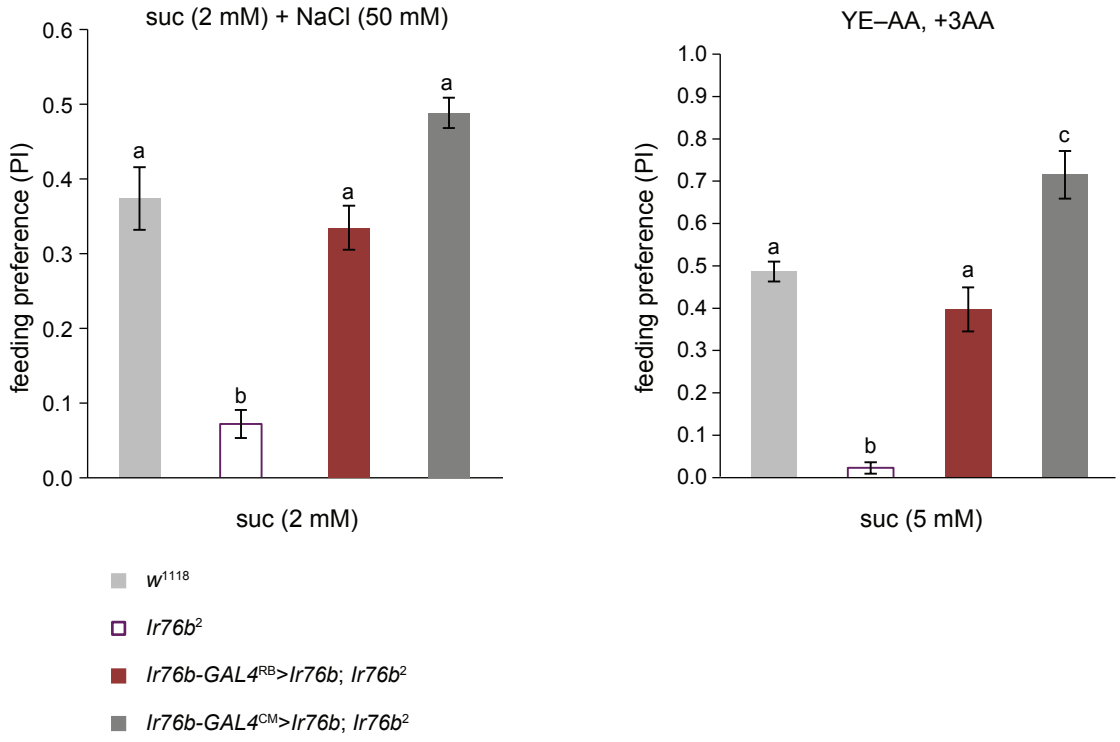
B


Ir76b MB00216
Ir76b MB00216 rescue
feeding preference for amino acids (PI)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
*
***
**
ns
*
ser
phe
ala
thr
gly

Supplementary Figure 2.S6: Ir76b is necessary for behavioral responses to yeast extract and amino acids.

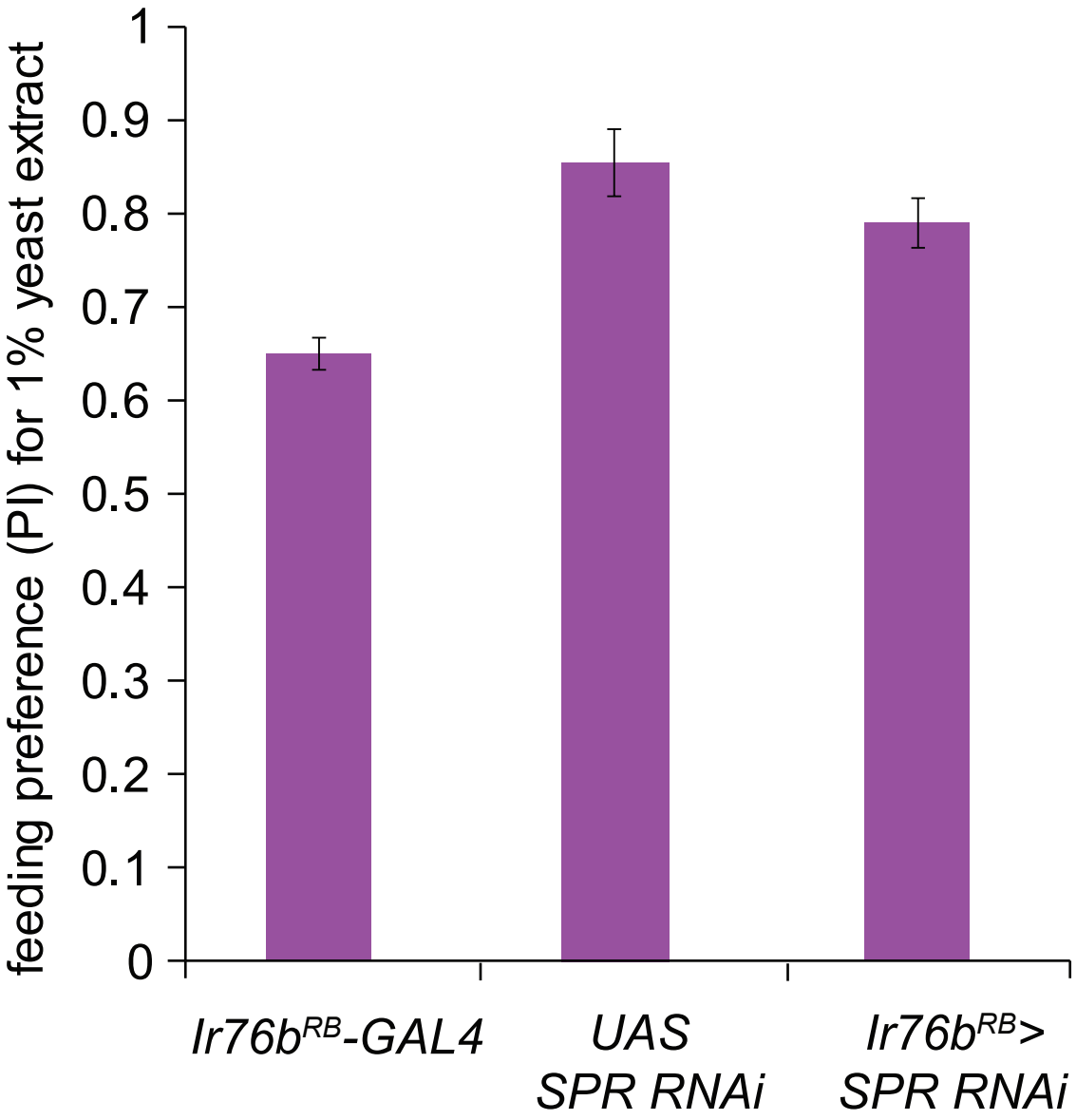
(A) Mean PI of mated female flies of *Ir76b* mutant (*Ir76b*^{MB00216}) *and Ir76b-GAL4;* *Ir76b*^{MB00216}*, UAS-Ir76b* (*Ir76b*^{MB00216} rescue). Yeast extract (1%) was tested against 5 mM sucrose; salt (50 mM NaCl) was tested in a mixture with sucrose (2 mM) against 2 mM sucrose alone; and sucrose (5 mM) was tested against water. n=6–23. For each stimulus, bars with different letters are significantly different, $P < 0.05$, one-way ANOVA with Tukey's post hoc test.

(B) Behavioral responses of *Ir76b*^{MB00216} mutants and *Ir76b-GAL4; Ir76b*^{MB00216}*, UAS-Ir76b* flies (*Ir76b*^{MB00216} rescue) to indicated amino acids (25 mM) tested in binary choice tests with 5 mM sucrose. *n*=7–13. *P < 0.05, **P < 0.01, ***P < 0.001, Mann-Whitney test.


suc (2 mM) + NaCl (50 mM)
feeding preference (PI)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
a
b
a
a
suc (2 mM)
YE–AA, +3AA
feeding preference (PI)
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
a
b
a
c
suc (5 mM)
w1118
Ir76b2
Ir76b-GAL4RB>Ir76b; Ir76b2
Ir76b-GAL4CM>Ir76b; Ir76b2

Supplementary Figure 2.S7: Both Ir76b-GAL4RB and Ir76b-GAL4CM rescue appetitive response to salt and amino acids.

Mean PI of males and females (pooled) for 50mM NaCl mixed with 2mM sucrose (pink dye) tested against 2 mM sucrose (blue dye), and of mated females for 1% yeast extract without amino acids supplemented with 25mM of serine, phenylalanine and threonine (YE-AA, +3AA, pink dye; tested against 5mM sucrose, blue dye). Genotypes were as follows: w^{1118} (wild type), $Ir76b^{2}/Ir76b^{2}$ ($Ir76b^{2}$), $Ir76b\text{-}GAL4^{RB}/Ir76b\text{-}GAL4^{RB}; Ir76b^{2}$, $UAS\text{-}Ir76b/Ir76b^{2}$, *UAS-Ir76b* ($Ir76b\text{-}GAL4^{RB}>Ir76b; Ir76b^{2}$), $Ir76b^{2}$, $UAS\text{-}Ir76b/Ir76b^{2}$, $Ir76b\text{-}GAL4^{CM}$ ($Ir76b\text{-}GAL4>Ir76b; Ir76b^{2}$). *n*=6-18. For each experimental condition, different letters indicate significantly different groups, P<0.05, one-way ANOVA with Tukey's *post hoc* test.


feeding preference (PI) for 1% yeast extract
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Ir76b^RB-GAL4
UAS SPR RNAi
Ir76b^RB> SPR RNAi

Supplementary Figure 2.S8:

Mean PI of female flies for 1% yeast extract (pink dye) when tested against 5mM sucrose (blue dye) in binary choice assay. Genotypes are as indicated. *n*= 6-9.

Chapter 3: Independent signaling pathways engender compensatory changes in taste sensitivity upon macronutrient deprivation.

ABSTRACT:

The taste system in animals plays an important role in food evaluation and choice, but the extent to which its function is modulated under different dietary conditions is not known. We utilized a macronutrient imbalance paradigm to assess the role of the taste system in engendering compensatory changes in feeding in *Drosophila melanogaster*. Flies were fed on iso-caloric diets of sugar and yeast-extract and tested for behavioral sensitivity to sugar and amino acids in two independent assays. We then tested consequences of depleting either macronutrient and found that flies increased their behavioral sensitivity to sugar and decreased their preference for amino acids upon dietary sugar deprivation. Conversely, when deprived of yeast extract, flies increased their preference for amino acids and reduced their behavioral sensitivity to sugar. Recordings from taste hairs revealed corresponding changes in responses of sweet taste neurons: increased sensitivity upon sugar deprivation and decreased sensitivity upon sugar abundance. Interestingly, changes in preference and sensitivity persisted for some time after flies were returned to a balanced diet, suggesting that changes in gene expression may be involved. Signaling via *Dop2R* is involved in increasing cellular and behavioral sensitivity to sugar and reducing behavioral sensitivity to amino acids upon sugar deprivation. On the other hand, the decrease in cellular and behavioral sensitivity to sugar under conditions of sugar abundance (yeast deprivation) relies on *dilp5*.

3.2 INTRODUCTION:

Drosophila melanogaster, like other animals, require different macronutrients like sugars and amino acids for survival. Flies use their gustatory system to detect these nutrients and feed on food substances containing them. The feeding behavior of flies is regulated by nutritional requirements of the flies which changes with changing physiological states and nutritional availability. Modifications in feeding behavior aids in maintaining nutritional homeostasis despite the altered nutritional requirements. For instance, in female flies, mating causes an increased preference for amino acids which is required to meet the higher demand for protein (Carvalho et al., 2006; Ganguly et al., 2017; Vargas et al., 2010). Nutritional availability is another factor that alters feeding preferences in flies (Inagaki et al., 2012; Liu et al., 2017; Marella et al., 2012). Sugars and amino acids are two important macronutrients in flies and decreased availability of any or both of them can lead to nutritional imbalances. Feeding behavior of flies therefore undergo changes to ensure that flies feed more on the required nutrients to maintain nutritional homeostasis. Starvation, for instance, causes increased sugar preference in flies (Inagaki et al., 2012; Marella et al., 2012). Both these studies also report that the increased behavioral sensitivity to sugar is mediated by dopamine, a biogenic amine controlling feeding and several other behaviors in flies. A recent publication by Liu et al., 2017, shows that amino acid deprivation increases intake of yeast and decreases intake of sugar in flies and this is mediated by a subgroup of dopaminergic neurons (DA-WED neurons).

However, whether compensatory changes in taste preferences upon nutrient deprivation is also correlated with altered sensitivities of the peripheral taste neurons have not been systematically studied. One research group however reported changes in sweet neuron activity when the flies are fed with sucralose, a non-nutritious sugar (Wang et al., 2016). According to them sucralose feeding leads to upregulation of insulin receptor (*InR*) and increased insulin signaling eventually leads to altered sweet neuron activity through an NPF dependent mechanism. The same study also demonstrated changes in appetite and behavioral sensitivity for sucrose upon feeding the flies with sucralose. Since the sucralose fed flies are either sugar/calorie deficient or starved (Park et al., 2017) it suggests that nutrient deprivation can lead to compensatory changes in sweet taste neuron activity which might affect behavior and feeding pattern of the flies. Based on the above studies, it is possible to hypothesize that biogenic amines like dopamine and insulin like peptides play a significant role in modulating taste neuronal sensitivity.

Here, we investigated compensatory changes in the peripheral gustatory system following macronutrient deprivation. We fed the flies with different isocaloric diets lacking in either of the two macronutrients viz. sugar or amino acids and tested them for changes in feeding preferences as well as neuronal activity. Flies deprived of a macronutrient exhibited compensatory changes in their food preference, behavioral sensitivity as well as taste neuron sensitivity. We find that expression of *Dop2R* a dopamine receptor in GABAergic neurons is required for compensatory changes following sugar deprivation while compensatory changes following feeding with sugar enriched diet depends on *dilp5* a *Drosophila* insulin like peptide.

It is to be noted that since mated females are known to have a strong preference for amino acids (Ganguly et al., 2017; Ribeiro and Dickson, 2010; Vargas et al., 2010), although we tested both genders, we only discussed about our results in the male flies.

3.3 MATERIALS AND METHODS:

Fly stocks: Flies were raised at a temperature of 22^{o}C -25^{o}C on standard cornmeal-dextrose media. Wild type flies were *w^{1118}* (BL 5905) unless otherwise mentioned. *elav-GAL4* (BL 8765), *Dop2R RNAi^{1}* (BL 26001), *Dop2R RNAi^{2}* (BL 36824), *Dop2R* mutant^{1} (BL 52025), *Dop2R* mutant^{2} (BL 52157), *GABA-A-R* RNAi^{1} (BL 31286), *GABA-A-R* RNAi^{2} (BL 31662), *GABA-A-R* RNAi^{3} (BL 52903), *GABA-B-R1* RNAi^{1} (BL 28353), *GABA-B-R1* RNAi^{2} (BL 51817), *GABA-B-R2* RNAi^{1} (BL 27699), *GABA-B-R2* RNAi^{2} (BL 50608), *GABA-B-R3* RNAi^{1} (BL 26729), *GABA-B-R3* RNAi^{2} (BL 42752), *GABA-B-R3* RNAi^{3} (BL 50622), *dilp5* RNAi (BL 33683), *UAS-TNTG* (BL 28828), *Ir76b1* (BL 51309) and *Gr64f-GAL4* (BL 57669) were obtained from *Drosophila* Bloomington Stock Center. *Gad1-GAL4*, *Tdc2-GAL4*, *Ple-GAL4*, *TRH-GAL4*, *v-Glut-GAL4*, *dilp2* (BL 30881), *dilp3* (BL 30882), *dilp5* (BL 30884), *dilp2-GAL4*, *n-syb-GAL4* and *UAS-InR^{DN}* flies were kindly shared by Dr. Naoki Yamanaka, University of California, Riverside.

Chemicals: Sucrose (S7903), Yeast extract (Y1625), L-serine (84959), L-phenylalanine (P-5482), L-threonine (89179), yeast nitrogen base without amino acids and ammonium sulfate (Y1251), D(+) glucose (G8270) were obtained from Sigma. Drosophila agar type II (66-103) and tegosept (20-258) were obtained from Apex Bioresearch Products (66-103). Propionic acid (UN3463) was obtained from Acros organics, yellow cornmeal (43-375) was obtained from Quaker. For behavior experiments tastants were mixed in water while they were mixed in 30mM TCC (Sigma, T0252) for electrophysiological recordings.

Preparation of food: Following are the compositions per 100 vials for each kind of food:

	Standard	Sugar deprived/ Yeast enriched	Sugar enriched/ Yeast deprived
D(+) glucose (gm)	100	0	126.67
Yeast extract (gm)	50	239.5	0
Cornmeal (gm)	70	70	70
Drosophila agar (gm)	6	6	6
Propionic acid (ml)	6	6	6
Tegosept (ml)	12	12	12
milliQ water (autoclaved) (ml)	1025	1025	1025

First little amount of water was mixed with the Drosophila agar and poured on the cooking utensil (glass beaker or conical flask) placed on a hot plate. A magnetic bead was used to stir the food continuously. A slurry of water and cornmeal is prepared and is poured into the agar once it melts. Subsequently sugar, yeast extract and the rest of the water is added. Once the food comes to a rolling boil, the hot plate is turned off and the food is allowed to cool down. Once the food reaches a temperature below 80^{o}C, propionic acid and tegosept are added. The food is now dispensed into vials using a serological pipette.

Binary Choice Assay: Binary choice assay was performed as explained in Chapter 2.

Proboscis Extension Response (PER) Assay: For PER assays flies were starved for 24 hours prior to the experiment. For PER with labellar stimulation flies were starved for 24 hours prior to the experiment. They were then inserted into p-200 tips which are truncated such that only the head of the fly protrudes outside. The flies were water satiated before starting the experiment. They were also allowed to drink water between the stimuli or every time there is a positive response to ensure that the positive response is towards the tastant and not water. Full and partial extensions were scored as 1 and 0.5 respectively, while a score of 0 was awarded when the flies failed to extend their proboscis.

For PER with tarsal stimulation flies were anesthetized with CO2 and attached on a glass slide on their backs using nail polish. The flies were then kept in a humid chamber for 2-2.5 hours to let them recover fully. The flies were water satiated prior to the experiment and also allowed to drink water between two stimuli. They were also tested with water after every positive response to ensure that the response was because of the tastant. Full extensions were scored as 1 and partial extensions were scored as 0.5. No extension was scored as 0.

Electrophysiological recordings: Extracellular tip recordings from labellar and tarsal sensilla were performed as explained in Chapter 2.

Library construction, sequencing and sequencing data analysis: For library preparation, flies fed with appropriate food were taken and their taste tissues and brain dissected. For proboscis and tarsi, tissues were collected from 150 flies while brain was dissected out from 16 flies. RNA was extracted from the tissues using standard Trizol-chlorofom extraction method. From the RNA, c-DNA library was prepared using Illumina

Tru-Seq RNA sample kit v2. Libraries of the taste tissues and brains from the flies fed either with the sugar enriched diet or the sugar deprived diet and the standard diet were multiplexed and then subjected to 1*51*7 cycle Hi-Seq2500 run in UCR Genomics core facility. The base-calling was done at the core-facility. Alignment was done at UCR bioinformatics core facility using a Bioconductor pipeline. Subsequent analysis of differentially expressed genes using EdgeR and GO-term analyses were done at UCR bioinformatics core facility. Significant changes in expression was determined based on log2(Fold Change) >1(upregulated) or < 1(downregulated) and both p value and false discovery rate (FDR) <0.5.

Statistical analyses: One-way or two-way ANOVA and Mann-Whitney tests were performed using SPSS. Student's t-tests were performed in MS Excel. For every figure, error bars indicate standard error of mean (S.E.M.).

3.4 RESULTS:

Sugar deprivation leads to increased preference, behavioral sensitivity and neuronal response to sugar.

The strategy we adopted to study changes in fly taste following macronutrient deprivation have been summarized in **Figure 3.1**. We prepared three different approximately isocaloric diets: standard diet containing both yeast extract and glucose, sugar deprived diet enriched in yeast extract and sugar(glucose) enriched diet deprived of yeast extract. The flies were raised in bottles at identical temperature and humidity to keep conditions constant during development. Subsequently 0-2 days old flies were collected and fed with one of the above-mentioned diets for 1-7 days.

The flies were then tested for changes in their food preference using binary choice assay giving the flies a choice between sugar and yeast extract.

We also conducted proboscis extension response assays and extracellular tip recordings from labellar taste hairs to account for any changes in their behavioral and neuronal sensitivity respectively. Since female flies have a sexually dimorphic high preference for yeast extract, we mainly targeted female flies in our experiments.

We observed that female flies fed with sugar deprived food exhibits a higher preference for sucrose when given a choice between 5mM sucrose and 1% yeast extract as compared to flies that have been fed with a standard diet. We observed significant changes in preference after just one day of sugar deprivation following which with further days of feeding with sugar deprived diet their sucrose preference stays higher than the flies fed with standard diet (**Figure 3.2A**). Since just 1 day of sugar deprivation was sufficient to induce changes in dietary preferences we typically used this paradigm for studying sugar deprivation induced compensatory changes in the subsequent experiments.

In order to understand if the observed change in preference is specific for sucrose or is applicable for any sugar, we deprived the flies of sugar for 1 day and subsequently tested their dietary preferences in a binary choice assay providing them a choice between 1% yeast extract and 5mM of glucose and fructose respectively. In both the cases the sugar deprived flies exhibited increased preference for the sugar (i.e. glucose or fructose) suggesting that sugar deprivation increases the preference for sugars in general (**Figure 3.2B**).

To test the possibility that changes in behavioral preference are an outcome of altered behavioral sensitivity to sugars, we measured proboscis extension responses elicited by stimulating the tarsal segments by a range of sucrose concentrations. We found that the flies deprived of sugar had a higher sensitivity to the sucrose solutions than the flies fed with the standard diet (**Figure 3.2C**).

To investigate whether sugar deprivation also leads to altered neuronal sensitivity to sugar we measured responses of labellar sweet taste neurons from the L hairs for a range of sucrose concentrations using single sensillum recordings. We found that sweet neurons in flies fed on the sugar-deprived diet showed higher responses to sugar as compared to those that were fed on the standard diet (**Figure 3.2D**).

As expected, in sugar deprived flies there was an increase in sweet neuron activity in the tarsi (**Figure 3.S1A**) and an increase in PER response on labellar stimulation (**Figure 3.S1B**) to a range of sucrose concentrations. This suggested that in both of the main taste organs viz. labellum and tarsus, there is an increase in behavioral sensitivity and neuronal responses to sugar upon sugar deprivation. Since the results of the single sensillum recordings and PER tests were consistent in both the organs, we elected to conduct single sensillum recordings and PER tests on labella and tarsi respectively for ease of experimentation.

Compared to the standard diet the sugar deprived diet consisted of greater amounts of yeast extract to keep the total calories constant. Hence, the sugar deprived diet could also be described as yeast enriched diet. Previous studies have reported that yeast is a primary source of amino acids for flies and the preference towards yeast is caused by the amino acids present in it (Ganguly et al., 2017).

Hence, to investigate if the resulting nutritional imbalance causes any changes in the preference of the flies for amino acids, we fed the flies with the sugar deprived or yeast enriched diet for 1 day and subsequently tested them for their preferences for a sucrose and amino acids (serine, phenylalanine, threonine) mixture tested against a similar concentration of sucrose in binary choice assays. The amino acids were so chosen since a previous study had demonstrated that a mixture of these three amino acids is sufficient to emulate feeding preference elicited by yeast extract in binary choice assays (Ganguly et al., 2017). The sugar deprived or yeast enriched flies had a lower preference for the sucrose, amino acid mixture compared to the flies fed with standard diet (**Figure 3.S2**).

Thus, feeding the flies with a sugar deprived, yeast enriched diet not only increases their feeding preference, behavioral sensitivity and neuronal sensitivity for sugar but also decreases their feeding preferences for amino acids.

Sugar enriched diet decreases preference, behavioral sensitivity and neuronal response to sugar.

To explore the outcome of the opposite paradigm i.e. feeding the flies with a sugar enriched diet, 0-2 days old flies were fed with this diet and subsequently tested for changes in feeding preferences to 5mM sucrose in binary choice assays with 1% yeast extract (**Figure 3.1**). Initially the flies fed on sugar enriched diet did not show significant changes in sucrose preferences. However, on feeding the flies with sugar enriched diet for 4 days or more there was a significant decrease in their preferences for sucrose (alternatively increased preference for yeast extract) (**Figure 3.3A**). Thus, although the feeding preferences were modified when the flies were fed with sugar enriched diet as

well, the time course of the changes was much different than when the flies were fed with sugar deprived diet. A possible explanation is that since sugar is a major source of energy for the flies, lack of it leads to more drastic changes in the fly's physiology leading to faster changes in preferences and sensitivity.

Since changes were visible only after the flies were fed with the sugar enriched diet for 4 days while investigating outcomes of sugar enriched diets in the later experiments we typically fed the flies with sugar enriched diet for 4 days.

To investigate changes in behavioral sensitivity following feeding the flies with sugar enriched diet we measured their proboscis extension responses upon tarsal stimulation with a range of sucrose concentrations. Flies fed with sugar enriched diet had decreased proboscis extension responses i.e. decreased behavioral sensitivity to sucrose compared to flies fed with standard diet (**Figure 3.3B**).

To explore the possibility that changes in feeding preferences and behavioral sensitivity were accompanied by changes in neuronal responses we conducted extracellular tip recordings from their labellar taste hairs. Flies fed with a sugar enriched diet exhibited decreased activity of the sugar neurons as compared to flies fed with standard diet, when tested with similar concentrations of sucrose (**Figure 3.3C**).

Since the sugar enriched diet did not consist of yeast extract, the flies fed with this food were thus deprived of yeast extract or amino acids. Hence to test if feeding flies with this diet causes any changes in their feeding preferences to amino acids as well, we conducted binary choice assays giving them a choice between sucrose mixed with three amino acids (serine, phenylalanine and threonine) and the same concentration of sucrose. Flies fed with the sugar enriched or yeast deprived diet had a higher preference

for the sugar-amino acid mixture compared to flies fed with standard diet (**Figure 3.S3A**). To further verify these results, we conducted a series of binary choice assays giving the flies a choice between sucrose and yeast extract without amino acids or yeast extract without amino acid supplemented with three amino acids (serine, threonine and phenylalanine). Although yeast deprived (sugar enriched) and standard fed flies had little difference in their preferences for yeast extract without amino acids, the yeast deprived flies had a significantly higher preference for yeast without amino acids supplemented with the three amino acid mixture than flies fed with standard diet (**Figure 3.S3B**).

Thus, flies fed with a sugar enriched (or yeast extract deprived) diet not only had decreased feeding preference, behavioral sensitivity and neuronal sensitivity to sugar but also a higher preference for amino acids.

Since *Ir76b*, an ionotropic receptor mediates amino acid taste response in flies (Croset et al., 2016; Ganguly et al., 2017), it was interesting to test how the mutants perform in a binary choice assay between sugar and yeast extract. Interestingly the *Ir76b* mutant flies (*Ir76b*^{1}) fed with sugar enriched (yeast deprived) diet had no changes in their preferences for yeast extract (i.e. no changes in preferences for sugar) compared to the mutant flies fed with standard diet (**Figure 3.S3C**). That the sugar preferences in the Ir76b1 flies also remained unchanged was somewhat unexpected. A possible explanation is that the preferences for sugar and amino acids are somewhat antagonistic to each other, and when preference for one of them increases, to keep the total caloric intake constant, the preference for the other goes down. Here since amino acid preference was unchanged so was the sugar preference.

Altered feeding preferences resulting from macronutrient deprivation persists for some time even after the flies are transferred to standard diet after deprivation

In order to investigate upto what extent the altered preferences upon macronutrient deprivation persists we transferred the flies fed with both sugar deprived and sugar enriched diet to standard diet after 1 day and 4 days of feeding respectively.

Flies fed with sugar deprived diet maintained an increased feeding preference for sucrose even after they were fed with standard diet for 1 day following sugar deprivation (**Figure 3.4A**). However, after the flies were fed with standard diet for 2 days following sugar deprivation, their feeding preference for sucrose became identical to that of the flies fed with standard diet all along (**Figure 3.4A**).

We had already demonstrated that an increase in sugar feeding preference in the sugar deprived flies is also associated with increased behavioral sensitivity and neuronal responses to sugar. Hence it was important to investigate if the increased behavioral sensitivity and neuronal activity to sugar also persists for some time. To test that we first conducted PER assays with tarsal stimulation with a range of sucrose concentrations. For 1mM, 5mM and 30mM sucrose concentrations the sugar deprived flies did no longer have increased PER responses after they were transferred to standard diet (**Figures 3.4B and 3.S4A**). Only for 10mM sucrose the sugar deprived flies still had an increased sugar sensitivity even after they were subsequently fed with standard diet for 1 day but after two days of feeding with standard diet they no longer had increased sugar sensitivity compared to flies fed with standard diet all along (**Figures 3.4B and 3.S4A**).

To test if the increased neuronal sensitivity persists we performed single sensillum recording from labellar L hairs with a range of sucrose concentrations. The flies deprived

of sugar no longer had an increased neuronal response to 10mM sucrose after they were fed with standard diet for 1 day (**Figures 3.4C and 3.S4B**). However, the sugar deprived flies maintained an increased neuronal response to 30mM and 100mM sucrose even after they were fed with standard diet for 1 day (**Figures 3.4C and 3.S4B**). Surprisingly, after the sugar deprived flies were fed with standard diet for 2 days, they had slightly lower neuronal responses for all the different sucrose concentrations tested than flies fed with standard diet all along (**Figure 3.4C and 3.S4B**).

The results suggest that sugar sensitivity is differently regulated in the tarsi and the proboscis. While the increased sensitivity in proboscis upon sugar deprivation mostly persists even after the flies are fed with standard diet for 1 day after the deprivation, in case of tarsi this was not observed. However, sugar deprived flies still had an increased sugar preference after they were fed with standard diet for 1 day. This leads to two possible explanations. Firstly, it is possible that proboscis contributes more to taste preferences than tarsi. Secondly, there could also be changes in the way the brain perceives sugar stimuli, leading to the above phenotypes.

Decreased sugar preferences in flies fed with sugar enriched diet persisted for two days even after they were fed with standard diet for 2 days following sugar enriched diet (**Figure 3.4D**). After the flies were fed with standard diet for 3 or more days following sugar enriched diet, they however had a feeding preference for sugar similar to flies that have been fed with standard diet all along (**Figure 3.4D**).

We next tested for changes in behavioral sensitivity and the results were consistent with the preference assays. Flies that were fed with sugar enriched diet continued to exhibit a decreased behavioral sensitivity for sugars (relative to flies fed with standard

food all along), as observed from PER assays with tarsal stimulation, even after they were fed with standard diet for upto 2 days (**Figures 3.4E and 3.S4C**). Surprisingly, the extent of the decrease was even greater when the flies were transferred to standard diet following sugar enriched diet (**Figures 3.4E and 3.S4C**). After feeding the sugar enriched diet flies with standard diet for 3 days there was a reduction in the relative loss of behavioral sensitivity (**Figures 3.4E and 3.S4C**). The sugar enriched flies fed with standard diet for 3 days showed an increased behavioral sensitivity to 1mM and 5mM sucrose concentrations compared to flies fed with standard diet all along (**Figures 3.4E and 3.S4C**).

Decreased neuronal responses to sucrose in sugar enriched diet flies persisted for some time even after they were subsequently fed with standard diet (**Figures 3.4F and 3.S4D**). Flies that were fed with standard diet for 1 day following sugar enriched diet still had decreased neuronal response to sugar across all the concentrations tested. Interestingly the extent of the loss in neuronal sensitivity was even greater in them as compared to flies fed with sugar enriched diet for 4 days (**Figures 3.4F and 3.S4D**). Flies fed with standard diet for 2 days after sugar enriched diet had reduced neuronal responses to 10mM and 100mM sucrose (**Figures 3.4F and 3.S4D**). However, consistent with the preference and PER assays, the neuronal sensitivity of the flies fed with standard diet for 3 days after sugar enriched diet were similar to flies that were fed with standard diet all along (**Figures 3.4F and 3.S4D**).

Thus, altered taste preferences and sensitivity caused in flies due to macronutrient deprivation persist for some time even after the flies are transferred to standard diet before eventually reaching to the level of flies fed with standard diet. This suggests that changes in gene expression might underlie changes upon nutrient deprivation.

Dop2R function is required for increased feeding preference and neuronal sensitivity for sugar following sugar deprivation.

We first surveyed existing literature for genes that have already been assigned with a role in modulating taste preferences or sensitivity following changes in nutritional status. Several groups have previously demonstrated that dopamine pathway is essential in changes following nutrient deprived conditions (Inagaki et al., 2012; Liu et al., 2017; Marella et al., 2012; Wang et al., 2016). Inagaki et al., 2012 suggested that hunger leads to increased dopamine secretion onto the gustatory receptor neuron leading to increased behavioral sensitivity. According to this study, DopEcR a dopamine receptor is required for increased behavioral sensitivity to sucrose following starvation but the effect of DopEcR is more vital for 6 hours following starvation following which other factors come into play. Marella et al., 2012 reports that increased dopamine secretion increases the probability for proboscis extension responses but unlike the previous study they suggest Dop2R another dopamine receptor to be mediating this process. Interestingly, a recent study by Liu et al., 2017 implicated *Dop2R* in increased yeast preference upon yeast deprivation in *Drosophila*. According to this study, yeast deprivation increases preference for yeast and decreases preference for sucrose and *Dop2R* plays a role in changed yeast preferences but not sugar preference following yeast deprivation. In our sugar deprivation/yeast enriched paradigm, changes were observed in preferences for both sugar and yeast. Hence, we determined *Dop2R* to be a suitable candidate to be tested for a role in altered preferences and sensitivity following sugar deprivation.

To test the role of *Dop2R* in altered preferences upon feeding the flies with sugar deprived diet, we silenced *Dop2R* across all the neurons by crossing two different *Dop2R* RNAi lines with *elav-GAL4* a pan-neuronal driver. A copy of dicer was added to intensify the effects of the RNAi. In both the cases, when *Dop2R* was silenced, flies fed with sugar deprived diet did not show an increased preference to sugar compared to the flies that were fed with standard diet (**Figure 3.5A**). A significant increase in preference for sugar upon sugar deprivation was however observed in the co-tested *GAL4* controls (**Figure 3.5A**).

Dop2R RNAi^{1} was chosen for future silencing experiments since the preference of the control lines had a preference much closer to that observed in the control lines when fed with standard diet (**Figure 3.5A**).

We next tested two different *Dop2R* mutants for their feeding preferences following sugar deprivation. Both the lines failed to show increase in feeding preferences to sucrose upon sugar deprivation (**Figure 3.5B**). We selected *Dop2R* mutant^{1} for future experiments (**Figure 3.5B**). Surprisingly, the mutant flies still had an increased behavioral sensitivity, i.e. increased PER upon tarsal stimulation with a range of sucrose concentrations, to sugar upon sugar deprivation (**Figure 3.5C**). We next recorded from the labellar sweet neurons in the *Dop2R* mutant^{1}. Increase in sweet neuron activity in sugar deprived mutant flies was not observed for 10mM sucrose; for 30mM and 100mM there was still significant increase in sweet neuron response but the change in sensitivity was much reduced compared to the co-tested wild type flies (**Figures 3.5D and 3.S5**). Thus, *Dop2R* plays a role in increased sugar preferences and neuronal response to sugar upon sugar deprivation. Although the outcome of the PER experiments was surprising, a possible explanation could be that the sweet neurons in the proboscis and

tarsi are `differentially regulated and dopamine is involved in regulation of the activity of the labellar sweet neurons but not the tarsal ones.

Previous studies reported that *Dop2R* function is required for increased sugar sensitivity after starvation (Inagaki et al., 2012; Marella et al., 2012), whereas we observed the same in flies when they were fed with a sugar deprived diet. Hence there was a possibility that the sugar deprived flies were actually starved.

To test that we mixed red dye to yeast extract/sugar deprived food. After one day in that food all flies showed presence of red dye in their gut indicating that they were actually feeding on the food.

We next elected to check if the starvation resistances of the sugar deprived flies were different from that of wet starved flies after one day of sugar deprivation and starvation respectively. The wet starved flies started dying between 10-15 hours while the sugar deprived flies did not show any mortality till 30-35 hours (**Figure 3.S6A**). The starved flies also had 50% mortality earlier than the sugar deprived flies (**Figure 3.S6A**). Thus, the sugar deprived flies had higher starvation resistance than starved flies.

We also compared mortality rates in flies fed with sugar deprived diet with flies. The sugar deprived flies had a much higher rate of survival, attaining 50% mortality after approximately 16 days while all the wet starved flies were dead in less than three days (**Figure 3.S6B**). Also, flies fed with sugar deprived food for 1 day, when tested in a binary choice assay, without the 24hours starvation, still had an increased sugar preference compared to flies fed with standard diet or 24 hours wet-starved flies (**Figure 3.S6C**).

Thus, evidently the sugar deprived flies were not starved, indicating that *Dop2R* mediated changes in food preferences and sensitivity occurs not only in starvation but also when the flies are deprived of a sugar.

***Dop2R* function is required in the GABAergic neurons for increase in sucrose preference and sensitivity upon sugar deprivation.**

To identify the subset of neurons in which *Dop2R* function is required for compensatory changes upon sugar deprivation, we used a panel of drivers to silence *Dop2R* in different subsets of neurons and examined feeding preference of flies fed on standard and sugar-deprived diets. Interestingly, silencing of *Dop2R* in GABAergic neurons abolished change in sugar feeding preference upon sugar deprivation (**Figure 3.6A**) while both the UAS and GAL4 controls which were co -tested showed significant increases in sugar preference upon sugar deprivation (**Figure 3.6A**).

Among the other lines tested, silencing *Dop2R* only in dopaminergic neurons appeared to show a defect in increasing sugar preference upon sugar deprivation (**Figure 3.6B**). Silencing in octopaminergic, serotoninergic and glutamatergic neurons had no appreciable effects (**Figure 3.6B**). Silencing *Dop2R* in the primary sweet taste neurons, using Gr64f-GAL4 driver, did not have an appreciable effect either (**Figure 3.S7**).

Expectedly, silencing *Dop2R* in the GABAergic neurons also abolished increased sweet neuron activity atleast for 30mM and 100mM sugar whereas for 10mM sucrose the change was much less than that of the co-tested control flies (**Figure 3.6C**).

Previous studies have reported that sweet neurons have GABA receptors (GABAR) and their pre-synaptic activity could be modulated by GABA secreted from GABAergic interneurons leading to altered behavioral sensitivity to sugar (Chu et al., 2014).

Since altered preferences upon sugar deprivation required *Dop2R* function specifically in the GABAergic neurons, we hypothesized that signaling through *Dop2R* could be modulating GABA secretion from the GABAergic neurons, controlling the amount of GABA reaching the GABAR present in the sugar neurons, thereby regulating their excitability, ultimately leading to altered behavioral sensitivity and feeding preferences. In order to test this hypothesis, we silenced all the different GABA receptors individually in the sweet taste neurons and compared the feeding preferences of the flies fed with both standard and sugar deprived diets. Although the *GAL4* control flies had increased sugar preference upon sugar deprivation, several of the UAS-RNAi lines used (*GABA-B-R1* RNAi^{2} and *GABA-B-R3* RNAi^{3}) did not have an increased preference for sugar upon sugar deprivation deeming them unsuitable for the screen (**Figures 3.6D and 3.S8A**). In one out of three RNAi lines used to silence *GABA-A-R* the change in sugar preference in the sugar deprived flies was slightly reduced compared to both controls, whereas no such effect was observed in case of the two other RNAi lines (**Figures 3.6D and 3.S8A**). No effect was observed on silencing *GABA-B-R1* and *GABA-B-R2* (**Figures 3.6D and 3.S8A**). However, while *GABA-B-R3* was silenced, for one of the RNAi lines (RNAi^{2}), the increase in sugar preference upon sugar deprivation was abolished (**Figures 3.6D and 3.S8A**). On silencing using the other RNAi line the change was highly reduced compared to the the *UAS* control but not to the *GAL4* control (**Figures 3.6D and 3.S8A**). Thus, apparently, expression of *GABA-B-R3* is required for increased sugar preference upon sugar deprivation but other GABA receptors were not.

However, additional experiments are required to validate the role of GABA-B-R3 in increased sugar preference on sugar deprivation.

Since flies fed with sugar deprived or yeast enriched diet also has reduced preferences for amino acids compared to flies fed with standard diet, we next tested whether *Dop2R* function is required for altered amino acid preferences as well. Interestingly, both the *Dop2R* mutants did not have decreased amino acid preference upon feeding with sugar deprived or yeast enriched diet (**Figure 3.S8B**).

We next tested if *Dop2R* plays a role in altered taste preferences following feeding with sugar enriched diet. Both the Dop2R mutants when fed with sugar enriched diet showed a decreased preference for sugar, like the control flies that were co-tested (**Figure 3.S8C**).

As increased preference and sensitivity for sugar in sugar deprived flies persists even after the flies were fed with standard diet following sugar deprivation (**Figure 3.3**), it is highly probable that feeding with sugar deprived flies altered transcription of genes involved in metabolism and feeding regulation.

In order to identify them we performed RNAseq experiments and compared taste tissue and brain transcriptomes of flies fed on standard and sugar deprived diets and found several differentially expressed candidates. However, the results obtained are only from one trial and additional trials are required to validate the differentially expressed genes.

***dilp5* is required for decreased feeding preference, behavioral sensitivity and neuronal sensitivity to sugar upon feeding with sugar enriched diet.**

As already demonstrated, decreased feeding preferences and behavioral and neuronal sensitivities for sugar upon feeding with sugar enriched diet persists even after the flies were subsequently fed with standard diet. This suggests that changes in transcriptions of genes due to feeding with sugar enriched diet. Since sweet neuron activity was altered on feeding with sugar enriched food there was a possibility that the expression of the sugar receptors was altered. Another possibility was changes in the transcription of neuropeptides and/or hormones related with feeding regulation in the brain.

To test that we performed RNAseq experiments and compared taste tissue and brain transcriptomes of flies fed on standard and sugar enriched diet. We did not find any receptor with known roles in sugar or amino acid taste being differentially regulated in the taste tissues. However, two insulin like peptides *dilp3* and *dilp5* were significantly downregulated in the brain of the flies fed on sugar enriched diet (**Figure 3.7A and Table 1**). *dilp2*, though not significantly downregulated, had slightly diminished level in the brains of the sugar enriched flies as well (**Figure 3.7A and Table 1**This is consistent with previous studies which reported that dilp5 is differentially expressed with altered protein or amino acid concentration in food (Okamoto and Nishimura, 2015; Post and Tatar, 2016). Incidentally dilp2, dilp3 and dilp5 are secreted from the same group of cells namely the insulin producing cells (IPCs) in the brain. A recent study shows that IPCs can directly sense the amino acid leucine and secrete dilp2 and dilp5 in response (Manière et al., 2016).

We tested *dilp2*, *dilp3* and *dilp5* mutants for changes in sugar preference on feeding with sugar enriched food. Only in the *dilp5* mutants, but not the *dilp2* and *dilp3* mutants, there was no decrease in feeding preference to sucrose upon feeding with sugar enriched food (**Figure 3.7B**). *dilp5* mutant flies also did not show a decrease in behavioral sensitivity and sweet neuron activity upon feeding with sugar enriched food (**Figures 3.7C and 3.7D**). In fact, we observed the opposite with the mutant showing the opposite effect, at least at some concentrations (**Figures 3.7C and 3.7D**). Since dilp5 is secreted from the IPCs in the brain, to further ascertain the role of *dilp5* in decreased sugar preference we inhibited vesicle secretion from the IPCs by expressing tetanus toxin (*TNTG*) in them using *dilp2-GAL4* driver. dilp2-GAL4 driver was chosen since among *dilp*s 2-5, *dilp2* showed the least change in transcription when the flies were fed with sugar enriched diet. Expectedly, when *TNTG* was expressed in the IPCs the flies did not have decreased sugar preferences upon feeding with sugar enriched diet (**Figure 3.7E**).

We next silenced *dilp5* specifically in the IPCs using *UAS-dilp5* RNAi. When *dilp5* was silenced, the flies did not show a decrease in sugar preference on feeding with sugar enriched diet (**Figure 3.7F**). Taken together, these results suggest that *dilp5* secreted from the IPCs in the fly brain mediates decrease in feeding preference, behavioral sensitivity and neuronal activity to sugar in flies following sugar enrichment.

We also examined the role of *dilp5* in increased amino acid preference upon feeding with the yeast deprived (or sugar enriched) diet. The *dilp5* mutant flies still had increased preference for amino acids on feeding with sugar enriched diet (**Figure 3.S9A**).

Thus, *dilp5* mediates only sugar taste but not amino acid taste upon feeding with sugar enriched or yeast deprived diet.

We next tested *dilp5* mutants for changes in sugar preference upon feeding with sugar deprived diet. *dilp5* mutants still had increased preferences for sugar upon sugar deprivation (**Figure 3.S9B**) suggesting that *dilp5* does not play a role in altered feeding preferences following sugar deprivation.

3.5 DISCUSSION:

Flies fed with a sugar deprived, yeast enriched food had increased preference for sugar as well as a decreased preference for amino acids. These flies also had increased behavioral sensitivity for sugar and increased sweet neuron activity. Likewise, flies fed with sugar enriched, yeast deprived food had decreased preferences for sugar but increased preference for amino acids. They also had reduced behavioral and neuronal sensitivity for sugar. This modulation of food preferences based on nutrient imbalance is essential for survival and reproductive success of the flies. While sugar is required as energy source, for storage and synthesis of several components in the body, too much feeding of sugar is associated with disease conditions like diabetes and obesity (Musselman et al., 2011). On the other hand, amino acids are essential as they are the building blocks of proteins and are also required for fecundity, but feeding flies only with yeast, the ethologically relevant source of amino acid decreases life-span (Skorupa et al., 2008). Hence deprivation of sugar coupled with high yeast increased and decreased preferences for amino acids and sugar respectively, and the vice versa.

We also demonstrate that the time course of the compensatory changes taking place is different for different macronutrient deprivation. While sugar deprivation causes changes in one day, yeast deprivation takes 4 days to cause observable phenotypes. Sugar is the primary energy source and is more essential for survival. Hence sugar deprivation elicits more acute responses. Deprivation of amino acids is however not met with such a reaction because firstly the flies still have an ample supply of calorific sugars i.e. they are not energy deficient and secondly, flies can synthesize some amino acids in their body. However, on the long run deprivation of amino acids are bound to cause problems like decrease in fecundity, and hence eventually preference of amino acids increases.

DA-WED neurons have been suggested to control sugar and amino acid feeding antagonistically (Liu et al., 2017). Flies need to keep their caloric intake approximately constant. So, while they ingest more amounts of one nutrient to counter deficiencies, automatically they need to decrease their intake of the other nutrient. Thus, intake of sugar and amino acids appear to be like the two arms of a balance, where while one arm is raised, the other arm goes down. This serves to maintain nutritional homeostasis.

Most previous studies conducted on outcomes of nutritional deprivation studied changes in feeding viz. food preference, appetite or PERs and implied modulatory changes occurring in neurons in the central nervous system due to nutrient deprivation as the underlying causes for these changes. Behavior is, however, a much downstream activity. First, the primary taste neurons detect a stimulus. Information about the stimulus is subsequently conveyed to higher brain centers where the information is evaluated and decision-making takes place which is subsequently reflected in the behavioral output.

A change in behavior could be an outcome of changed neuronal activity of the primary taste neurons causing the same stimulus to be perceived differently in different conditions. It can well be an outcome of modulations in neurons further upstream in the brain, causing the same information to be evaluated differently for feeding related decision-making. A change in feeding behavior could be caused by both occurring at the same time as well.

Here we demonstrate changes in sweet neuron activity as outcome of different diet paradigms. This is a particularly novel and interesting finding. It suggests that primary sensory neurons themselves are more plastic than is believed and can themselves undergo changes to suit the changing physiological needs of the organism.

We find *Dop2R* a dopamine receptor to be playing an important role in compensatory changes following feeding with sugar deprived or yeast enriched diet. Interestingly *Dop2R* is required both for altered preferences of both sugar and amino acids. However, Dop2R was not observed to be important in compensatory changes following feeding with yeast deprived, sugar enriched diet. The findings are apparently conflicting with a previous study which reported that *Dop2R* is required for increased yeast preference but not for decreased sugar preference following yeast deprivation (Liu et al., 2017).

We further demonstrate that *Dop2R* function is required in the GABAergic neurons for increased activity of the sugar neurons. Previous studies have reported increased dopamine secretion following starvation (Inagaki et al., 2012). Although we did not measure changes in dopamine levels in flies fed with sugar deprived food it might be reasonable to assume that they undergo an increased dopamine secretion as well.

Dop2R leads to a Gi mediated signaling pathway and could lead to decrease in GABA secretion from GABAergic neurons. GABAergic interneurons are known to decrease pre-synaptic output in sweet taste neurons via GABA-B-R2 receptors (Pool et al., 2014). Hence sweet neuron activities are likely to be controlled by GABA. So, any decrease in GABA secretion would decrease GABA-mediated inhibition of the sweet neurons allowing them to be more sensitive to sugar. However, no impact was observed in flies where *GABA-B-R2* was silenced in the sweet neurons. A small effect of silencing *GABA-B-R3* was however noted. More experiments are required to validate the role of GABA-B-R3 receptors on feeding with sugar deprived diet. It is possible that several different subtypes of GABA receptors are involved and hence silencing only one of them did not cause a drastic effect.

How *Dop2R* affects amino acid feeding preference was not clear from the study. There is a possibility that *Dop2R*+ neurons downstream to the DA-WED neurons (Liu et al., 2017) could also play an important role in this. Also, whether *Dop2R* function in the GABAergic neurons is required for amino acid taste modulation as well was not tested. Further investigations are required to explain how *Dop2R* modulates amino acid preferences.

From RNAseq experiments we found downregulation of *dilp5* and *dilp3* in the brains of flies fed with sugar enriched/yeast deprived diet. This is consistent with some previous studies which reported that *dilp5* is upregulated and downregulated by increasing and decreasing yeast concentration respectively in their diet (Okamoto and Nishimura, 2015; Post and Tatar, 2016). Preliminary RNAseq experiments indeed suggested upregulation of dilp5 in the brains of flies fed with sugar deprived, yeast enriched diet.

Mutant analysis revealed that *dilp5* but not *dilp3* is required for decrease in sucrose preference in flies fed with sugar enriched diet. However, *dilp5* was not required for increased amino acid preferences in these flies. This was surprising because *dilp5* secretion is regulated mainly by amino acid availability. However, since amino acid and sugar preferences are in all likelihood antagonistic in nature, it probably indirectly modulates amino acid feeding by altering sugar preferences. Further investigation is required to identify the mechanisms by which *dilp5* modulates feeding behavior and sweet neuron activity.

Drosophila Insulin Receptor InR has been shown to be the receptor for dilp5 in flies. So, an obvious assumption would be to test the role of *InR* in changes following sugar enriched diet. Preliminary studies indeed show that expressing a dominant negative form of *InR* reduce compensatory changes in feeding preferences upon feeding with sugar enriched diet (**Figure S10**). However more experiments using RNAi lines and mutants is required to verify the role of *InR* in this phenotype and also to investigate the neuronal subset where *InR* function is required for this phenotype.

It is important to note that compensatory changes stemming from the two different diet paradigms employs two different pathways which are apparently mutually exclusive. While sugar deprived, yeast enriched diet employs a dopaminergic pathway, an insulin mediated pathway is required for compensatory changes following sugar enriched, yeast deprived diet. Both pathways eventually lead to relative changes in sugar and amino acid preferences. Hence the requirement for two different pathways in response to two different diet paradigms is really intriguing. A possible explanation could be that the temporal pattern of the changes varies when dopaminergic pathway is used as opposed to that of the insulin mediated pathway.

Since the time-course requirements of occurrence of compensatory changes vary in the two paradigms, the pathways are likewise chosen.

Both dopamine and insulin are found in all higher animals including human where both of them play important role in controlling feeding and metabolism. It might be important to study if nutritional imbalances cause changes at the level of the taste cells in mammals and if they are similarly regulated by these two pathways.

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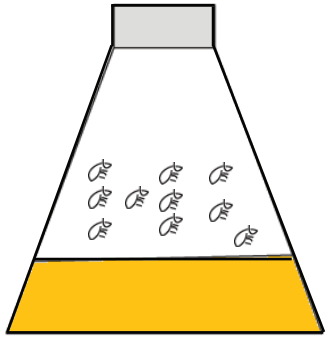
Wang, Q.-P.P., Lin, Y.Q., Zhang, L., Wilson, Y.A., Oyston, L.J., Cotterell, J., Qi, Y., Khuong, T.M., Bakhshi, N., Planchenault, Y., et al. (2016). Sucralose Promotes Food Intake through NPY and a Neuronal Fasting Response. Cell Metab. *24*, 75–90.



0 – 2 days

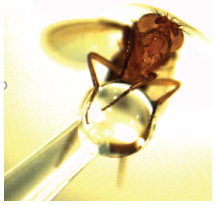
Sugar deprived/Yeast enriched

Feeding preference

Standard

Daily, 1 – 7 days

Taste sensitivity



Sugar enriched/Yeast deprived

Neuronal Response

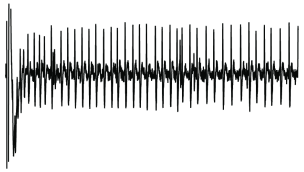


Figure 3.1: Schematics representing the experimental strategy.

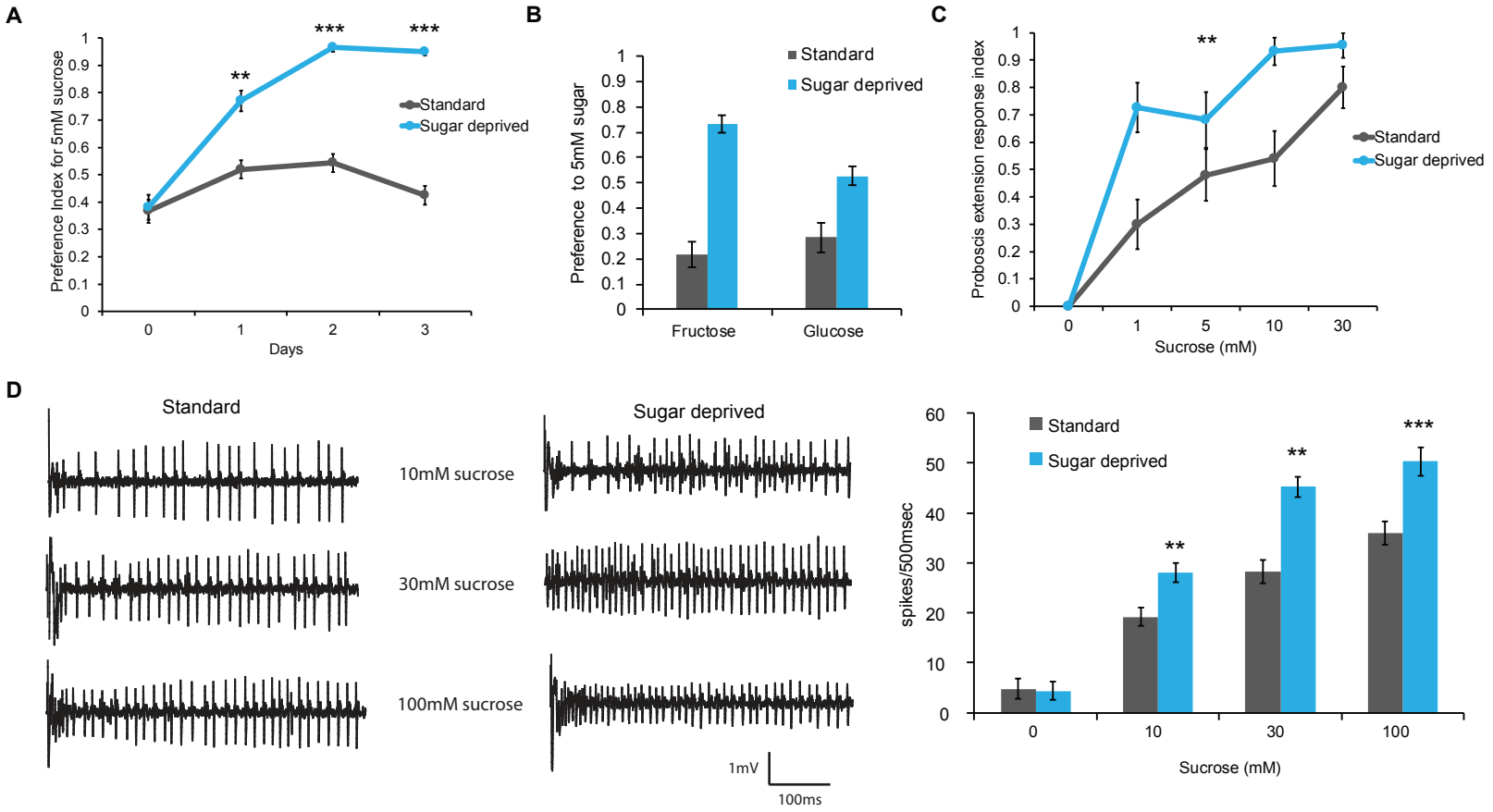

A
Preference Index for 5mM sucrose
Days
Standard
Sugar deprived
**
***
***
B
Preference to 5mM sugar
Standard
Sugar deprived
Fructose
Glucose
C
Proboscis extension response index
Sucrose (mM)
Standard
Sugar deprived
**
D
Standard
Sugar deprived
10mM sucrose
30mM sucrose
100mM sucrose
1mV
100ms
spikes/500msec
Sucrose (mM)
Standard
Sugar deprived
**
**
***

Figure 3.2: Dietary sugar deprivation increases feeding preference, behavioral sensitivity and taste neuron responses to sugar.

A) Mean preference indices of mated female flies to 5mM sucrose (blue dye) tested against 1% yeast extract (pink dye) in a binary choice assay. The flies tested were fed either with a sugar deprived diet or a standard diet for the indicated number of days. n= 6-17. *p<0.05, **p<0.01, ***p<0.001, 2-way ANOVA with pairwise comparison. Error bars indicate S.E.M.

B) Mean preference indices of mated female flies to 5mM glucose or fructose (blue dye) tested against 1% yeast extract (pink dye) in binary choice assay. The flies tested were fed with standard food or sugar deprived food for 1 day. n=7-10. Error bars indicate S.E.M.

C) Proboscis extension response index of mated female flies when their tarsi was stimulated by the indicated concentrations of sucrose. Flies were fed either with standard diet or with sugar deprived diet for 1 day. n= 25 for standard diet flies and 22 for sugar deprived flies. *p<0.05, **p<0.01, 2-way ANOVA with pairwise comparison. Error bars indicate S.E.M.

D) Representative traces (left) and mean neuronal responses for the first 500ms obtained from labellar L-hairs of mated female flies upon stimulation with the indicated concentration of sucrose. Flies were fed either with standard diet or sugar deprived diet for 1 day. n=13-20 sensilla from 3-4 flies. *p<0.05, **p<0.01, ***p<0.001, Mann-Whitney U tests. Error bars indicate S.E.M.

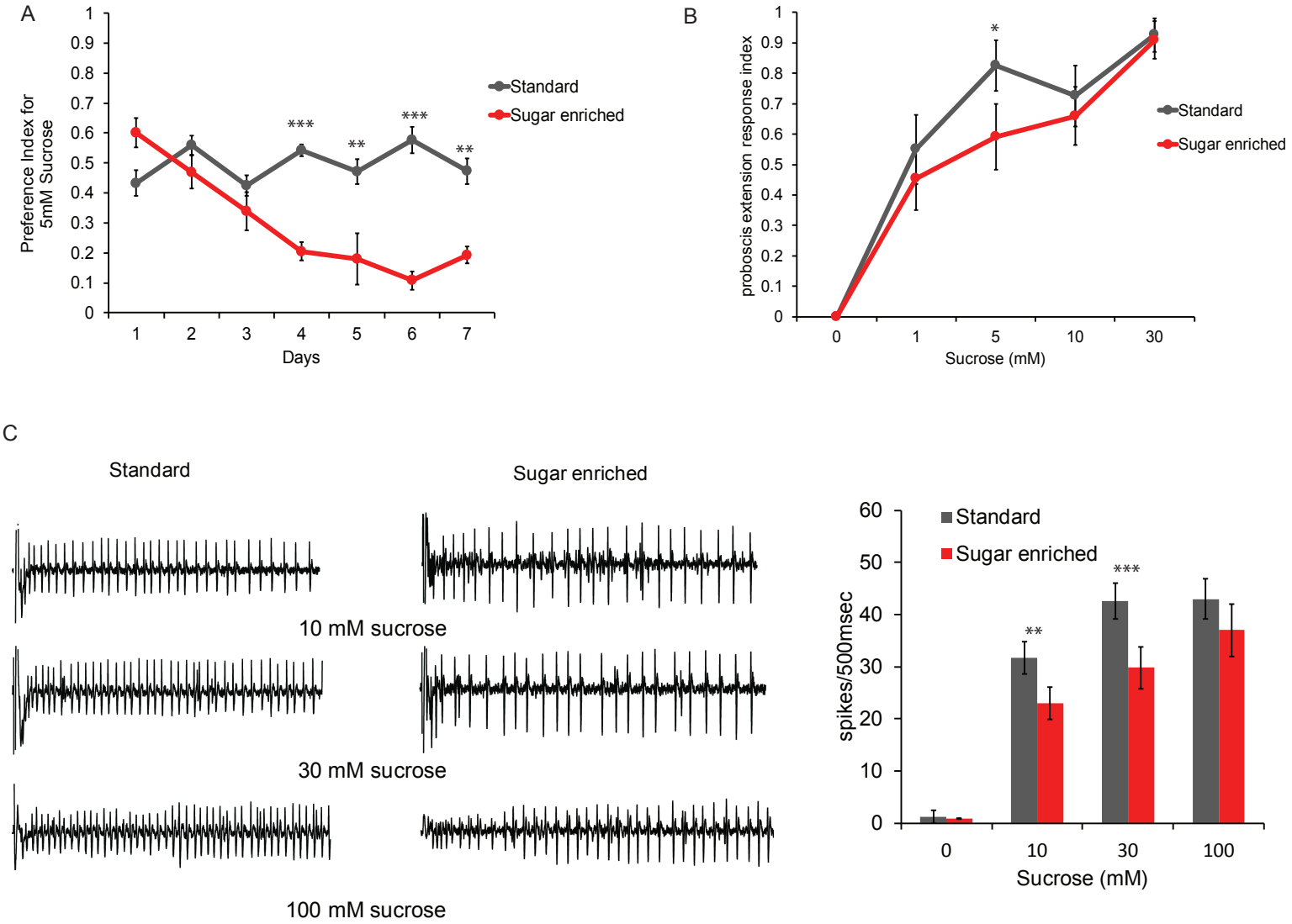

A
Preference Index for 5mM Sucrose
Days
Standard
Sugar enriched
***
**
***
**
B
proboscis extension response index
Sucrose (mM)
Standard
Sugar enriched
*
C
Standard
Sugar enriched
10 mM sucrose
30 mM sucrose
100 mM sucrose
spikes/500msec
Sucrose (mM)
Standard
Sugar enriched
**
***

Figure 3.3: Dietary sugar enrichment decreases feeding preference, behavioral sensitivity and taste neuron responses to sugar.

A) Mean preference (P.I.) of mated female flies, fed either with standard diet or sugar enriched diet for the indicated number of days, for 5mM sucrose (blue dye) tested against 1% yeast extract in a binary choice assay. n=6-17. *p<0.05, **p<0.01, ***p<0.001, 2-way ANOVA with pairwise comparison. Error bars indicate S.E.M.

B) Proboscis extension response index obtained from mated female flies upon tarsal stimulation with the indicated concentrations of sucrose. The flies were fed either with standard diet or sugar enriched diet for 4 days. n=20 and 22 for flies fed with standard diet and sugar enriched diet respectively. *p<0.05, 2-way ANOVA with pairwise comparison. Error bars indicate S.E.M.

C) Representative traces (left) and mean neuronal responses for the first 500ms obtained from labellar L-hairs of female flies upon stimulation with the indicated concentration of sucrose. Flies were fed either with standard diet or sugar enriched diet for 4 days. n≥20 sensilla from 4 flies. Error bars indicate S.E.M.

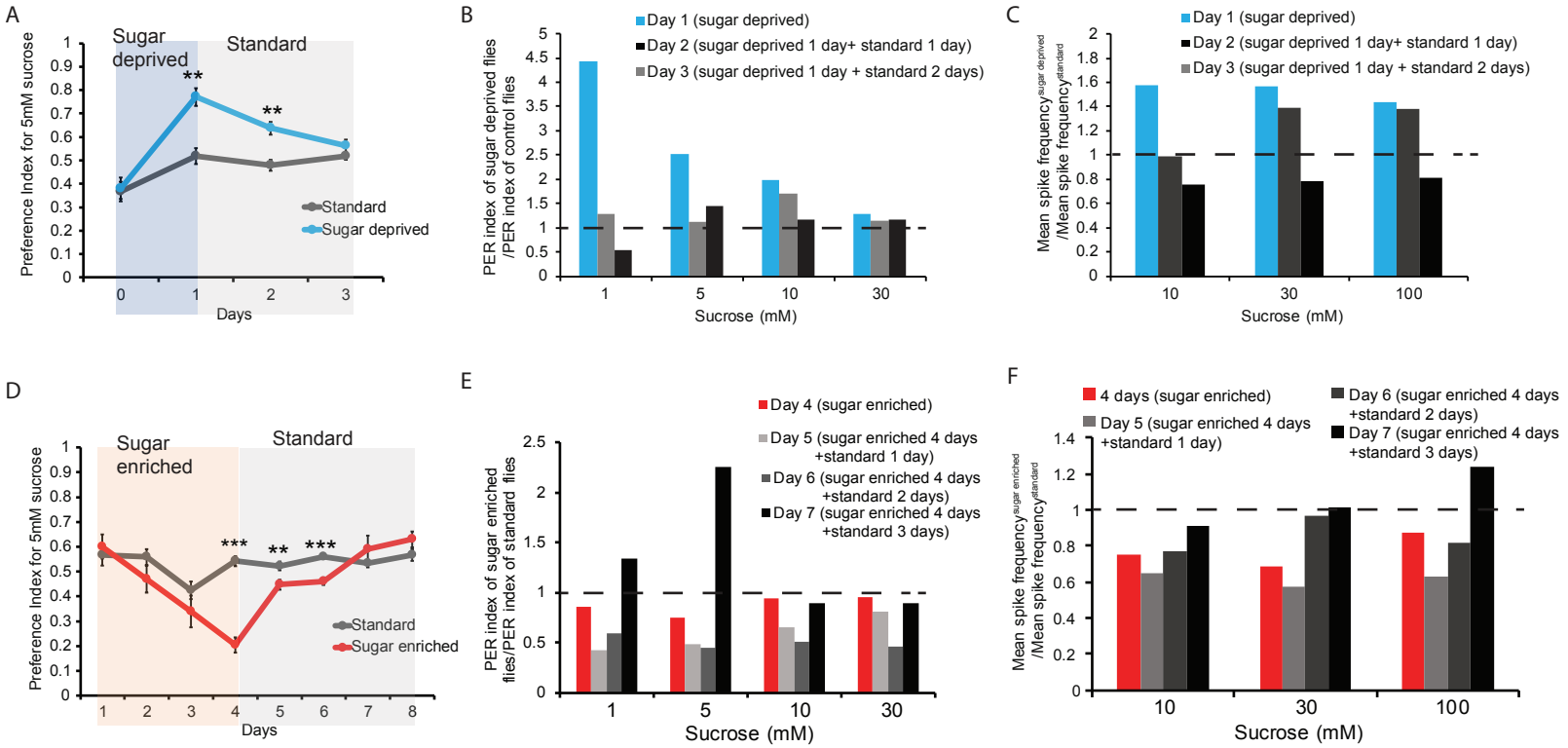

A
Sugar deprived
Standard
**
**
Preference Index for 5mM sucrose
Standard
Sugar deprived
Days
B
Day 1 (sugar deprived)
Day 2 (sugar deprived 1 day+ standard 1 day)
Day 3 (sugar deprived 1 day + standard 2 days)
PER index of sugar deprived flies /PER index of control flies
Sucrose (mM)
C
Day 1 (sugar deprived)
Day 2 (sugar deprived 1 day+ standard 1 day)
Day 3 (sugar deprived 1 day + standard 2 days)
Mean spike frequency sugar deprived /Mean spike frequency standard
Sucrose (mM)
D
Sugar enriched
Standard
***
**
***
Preference Index for 5mM sucrose
Standard
Sugar enriched
Days
E
Day 4 (sugar enriched)
Day 5 (sugar enriched 4 days +standard 1 day)
Day 6 (sugar enriched 4 days +standard 2 days)
Day 7 (sugar enriched 4 days +standard 3 days)
PER index of sugar enriched flies/PER index of standard flies
Sucrose (mM)
F
4 days (sugar enriched)
Day 5 (sugar enriched 4 days +standard 1 day)
Day 6 (sugar enriched 4 days +standard 2 days)
Day 7 (sugar enriched 4 days +standard 3 days)
Mean spike frequency sugar enriched /Mean spike frequency standard
Sucrose (mM)

Figure 3.4: Compensatory changes upon macronutrient deprivation persists even after the deprivation diet condition is removed.

A) Mean preference indices of mated female flies for 5mM sucrose (blue dye) tested against 1% yeast extract (pink dye) in binary choice assays in the indicated time points. The blue line indicates flies that were fed with sugar deprived diet for 1-day (blue box) and subsequently fed with standard diet (grey box). The grey line indicates flies that were fed with standard diet all along. n= 6-15. *p<0.05, **p<0.01, ***p<0.001, Student's T-test. Error bars indicate S.E.M.

B) Ratio of the mean proboscis extension response(PER) index obtained from test (sugar deprived) flies to that obtained from control (standard) flies upon tarsal stimulation with the indicated concentrations of sucrose at the indicated time points. All results are from mated female flies. The test flies were fed with a sugar deprived diet for 1 day following which they were fed with standard diet whereas the control flies were fed with standard diet all along. n=19-63. The dotted line indicates a ratio of 1 i.e. a point where the PER indices of the flies fed with the two different diets are equal.

C) Ratio of mean neuronal response obtained from the test (sugar deprived) flies to that obtained from control (standard) flies upon single-sensillum tip recording from labellar L hairs with indicated sucrose concentrations, at the indicated timepoints. All results are from mated females. The test flies were fed with a sugar deprived diet for 1 day following which they were fed with standard diet. The control flies were fed with standard diet all along. n=8-25 sensilla from 2-5 flies. The dotted line indicates a ratio of 1 i.e. a point where the neuronal responses of the flies fed with the two different diets are equal.

D) Mean preference indices of mated female flies for 5mM sucrose (blue dye) tested against 1% yeast extract (pink dye) in binary choice assays in the indicated time points. The red line indicates flies that were fed with sugar enriched diet for 4 days (red box) and subsequently fed with standard diet (grey box). The grey line indicates flies that were fed with standard diet all along. n= 6-17. *p<0.05, **p<0.01, ***p<0.001, Student's T-test. Error bars indicate S.E.M.

E) Ratio of the mean proboscis extension response(PER) index obtained from test (sugar enriched) flies to that obtained from control (standard) flies upon tarsal stimulation with the indicated concentrations of sucrose at the indicated time points. All results are from mated female flies. The test flies were fed with a sugar enriched diet for 4 days following which they were fed with standard diet whereas the control flies were fed with standard diet all along. n=16-24. The dotted line indicates a ratio of 1 i.e. a point where the PER indices of the flies fed with the two different diets are equal.

F) Ratio of mean neuronal response obtained from the test (sugar enriched) flies to that obtained from control (standard) flies upon single-sensillum tip recording from labellar L hairs with indicated sucrose concentrations, at the indicated timepoints. All results are from mated females. The test flies were fed with a sugar enriched diet for 4 days following which they were fed with standard diet. The control flies were fed with standard diet all along. n=15-29 sensilla from 3-6 flies. The dotted line indicates a ratio of 1 i.e. a point where the neuronal responses of the flies fed with the two different diets are equal.

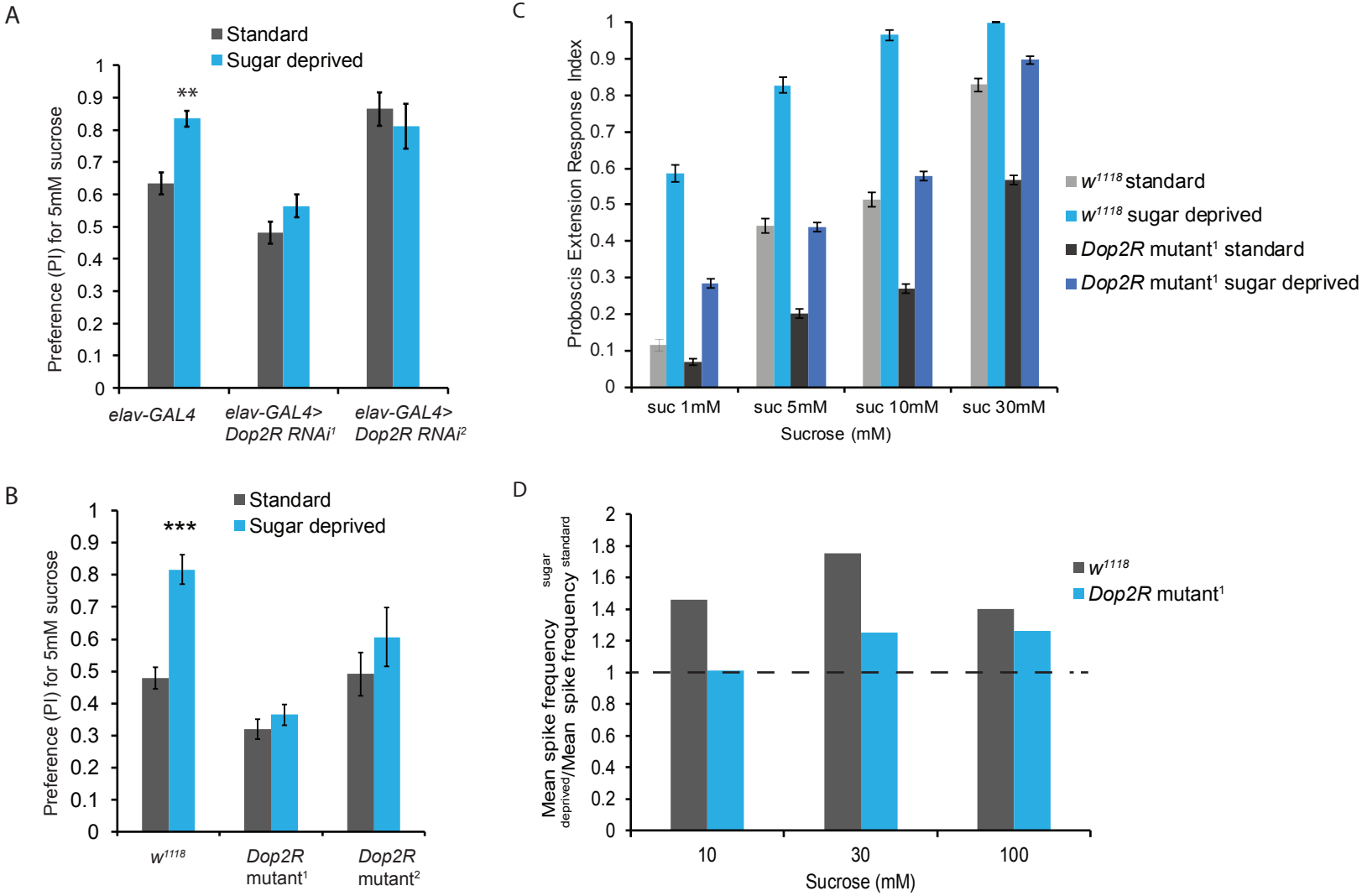

A
Standard
Sugar deprived
**
Preference (PI) for 5mM sucrose
elav-GAL4
elav-GAL4> Dop2R RNAi1
elav-GAL4> Dop2R RNAi2
B
Standard
Sugar deprived
***
Preference (PI) for 5mM sucrose
w1118
Dop2R mutant1
Dop2R mutant2
C
Proboscis Extension Response Index
suc 1mM
suc 5mM
suc 10mM
suc 30mM
Sucrose (mM)
w1118 standard
w1118 sugar deprived
Dop2R mutant1 standard
Dop2R mutant1 sugar deprived
D
Mean spike frequency sugar deprived/Mean spike frequency standard
w1118
Dop2R mutant1
10
30
100
Sucrose (mM)

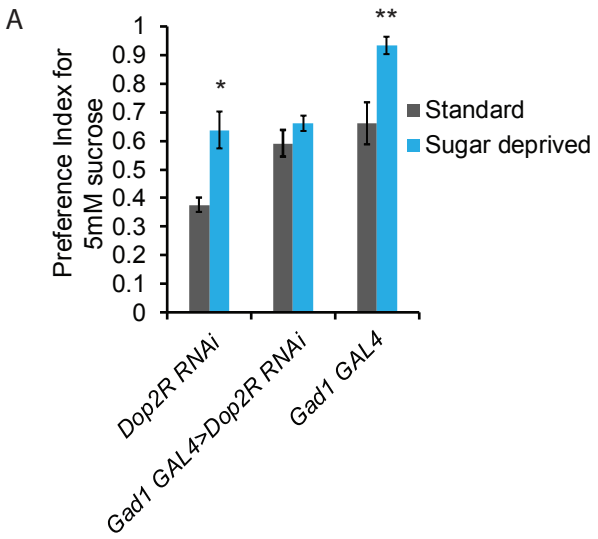
Figure 3.5: *Dop2R* is necessary for compensatory changes upon feeding with sugar deprived diet

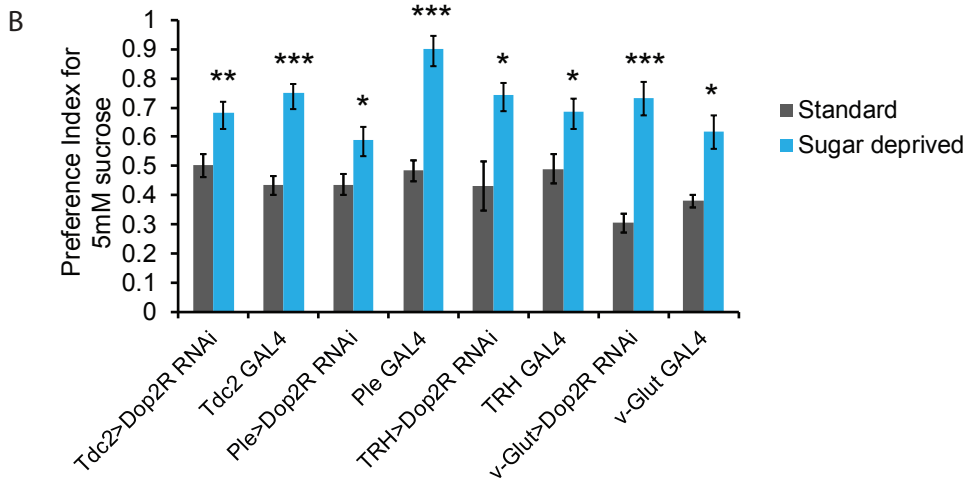
A) Mean preference indices of mated female flies to 5mM sucrose (blue dye) testesd against 1% yeast extract (pink dye) in a binary choice assay. Flies were either fed on sugar deprived diet or on standard diet for 1 day. Genotypes tested were *elav-GAL4* (n=3-7), *elav>Dop2R* RNAi1 (n=9) and *elav>Dop2R* RNAi2 (n=2-4). *p<0.05, **p<0.01, ***p<0.001, Student's T-test. Statistical comparisons were made between sugar deprived and standard flies of the same genotypes. Error bars indicate S.E.M.

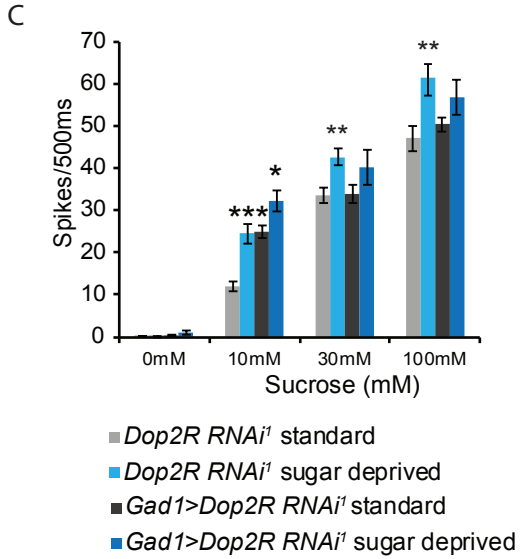
B) Mean preference indices of mated female flies to 5mM sucrose (blue dye) tested against 1% yeast extract (pink dye) in a binary choice assay. Flies were either fed on sugar deprived diet or on standard diet for 1 day. Genotypes tested were w^{1118} , *Dop2R* mutant^{1} (BL 52025) and *Dop2R* mutant^{2} (BL 51817). n= 5-10. *p<0.05, **p<0.01, ***p<0.001, Student's T-test. Statistical comparisons were made between sugar deprived and standard flies of the same genotypes. Error bars indicate S.E.M.

C) Mean proboscis extension response (PER) indices of mated female flies upon tarsal stimulation with the indicated concentrations of sucrose. Flies were either fed on sugar deprived diet or on standard diet for 1 day. Genotypes tested were w^{1118} and *Dop2R* mutant^{1} (BL 52025). n= 26-35 flies. Error bars indicate S.E.M.

D) Ratio of mean neuronal response obtained from mated female flies deprived of sugar for 1 day to those fed with standard diet for 1 day, upon single sensillum recording from labellar L sensilla with indicated concentrations of sucrose. Genotypes tested were w^{1118} and *Dop2R* mutant^{1} (BL 52025). n=16-29 sensilla from 5-6 flies.


A
Preference Index for 5mM sucrose
Standard
Sugar deprived
Dop2R RNAi
Gad1 GAL4>Dop2R RNAi
Gad1 GAL4


B
Preference Index for 5mM sucrose
Standard
Sugar deprived
Tdc2>Dop2R RNAi
Tdc2 GAL4
Ple>Dop2R RNAi
Ple GAL4
TRH>Dop2R RNAi
TRH GAL4
v-Glut>Dop2R RNAi
v-Glut GAL4


C
Spikes/500ms
0mM
10mM
30mM
100mM
Sucrose (mM)
Dop2R RNAi1 standard
Dop2R RNAi1 sugar deprived
Gad1>Dop2R RNAi1 standard
Gad1>Dop2R RNAi1 sugar deprived

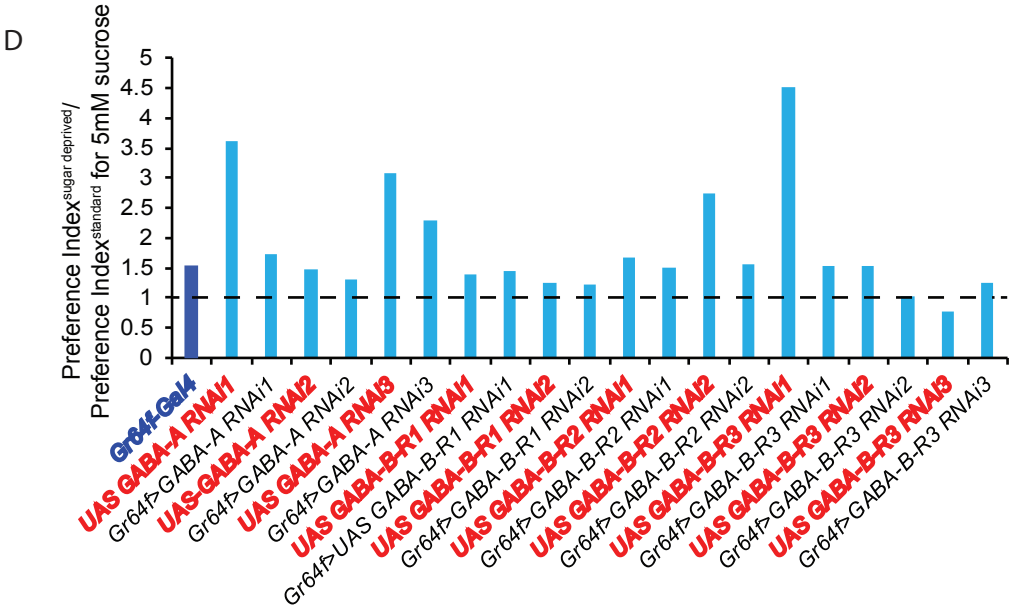

D
Preference Index^Sugar deprived / Preference Index^Standard for 5mM sucrose
Gr64f-GeL4
UAS GABA-A RNAi1
UAS-GABA-A RNAi2
UAS GABA-A RNAi3
UAS GABA-B-R1 RNAi1
UAS GABA-B-R1 RNAi2
UAS GABA-B-R2 RNAi1
UAS GABA-B-R2 RNAi2
UAS GABA-B-R3 RNAi1
UAS GABA-B-R3 RNAi2
UAS GABA-B-R3 RNAi3
Gr64f>GABA-A RNAi1
Gr64f>GABA-A RNAi2
Gr64f>GABA-A RNAi3
Gr64f>UAS GABA-B-R1 RNAi1
Gr64f>GABA-B-R1 RNAi2
Gr64f>GABA-B-R2 RNAi1
Gr64f>GABA-B-R2 RNAi2
Gr64f>GABA-B-R3 RNAi1
Gr64f>GABA-B-R3 RNAi2
Gr64f>GABA-B-R3 RNAi3

Figure 3.6: *Dop2R* function is required in the GABAergic neurons for compensatory changes upon feeding with sugar deprived diet

A) Mean preference indices of mated female flies to 5mM sucrose (blue dye) tested against 1% yeast extract (pink dye). Flies were either fed with sugar deprived diet or with standard diet for 1 day. Genotypes tested were *UAS-Dop2R* RNAi^{1} (BL 26001) (n=5), *Gad1> Dop2R* RNAi^{1} (n=10) and *Gad1* GAL4 (n=5). *p<0.05, **p<0.01, ***p<0.001, Student's T-test. Statistical comparisons were made between sugar deprived and standard flies of the same genotypes. Error bars indicate S.E.M.

B) Mean preference indices of mated female flies to 5mM sucrose (blue dye) tested against 1% yeast extract (pink dye). Genotypes tested were *Tdc2> Dop2R RNAi^{1}, Tdc2 GAL4, Ple> Dop2R RNAi^{1}, Ple GAL4, TRH> Dop2R RNAi^{1}, TRH GAL4, v-Glut> Dop2R RNAi^{1}, v-Glut GAL4*. Flies were either fed with sugar deprived diet or with standard diet for 1 day. n=4-5 for the GAL4 controls and 5-11 for the GAL4-UAS lines. *p<0.05, **p<0.01, ***p<0.001, Student's T-test. Statistical comparisons were made between sugar deprived and standard flies of the same genotypes. Error bars indicate S.E.M.

C) Mean neuronal responses obtained from mated female flies upon single sensillum recording from labellar L sensilla with indicated concentrations of sucrose. Genotypes tested were UAS-*Dop2R* RNAi^{1} (BL 26001) and *Gad1> Dop2R* RNAi^{1}. n=12-16 sensilla from 3-4 flies. *p<0.05, **p<0.01, ***p<0.001, Student's T-test. For each concentration, statistical comparisons were made between sugar deprived and standard flies of the same genotypes. Error bars indicate S.E.M.

D) Ratio of the mean preference index of the mated female flies deprived of sugar for 1 day to that of mated female flies fed with standard food for 1 day, for 5mM sucrose

(blue dye) when tested against 1% yeast extract (pink dye) in a binary choice assay. Genotypes of the flies are as indicated. n=10 for the GAL4 control, 2-6 for the UAS control lines and 6-8 for the GAL4-UAS flies.

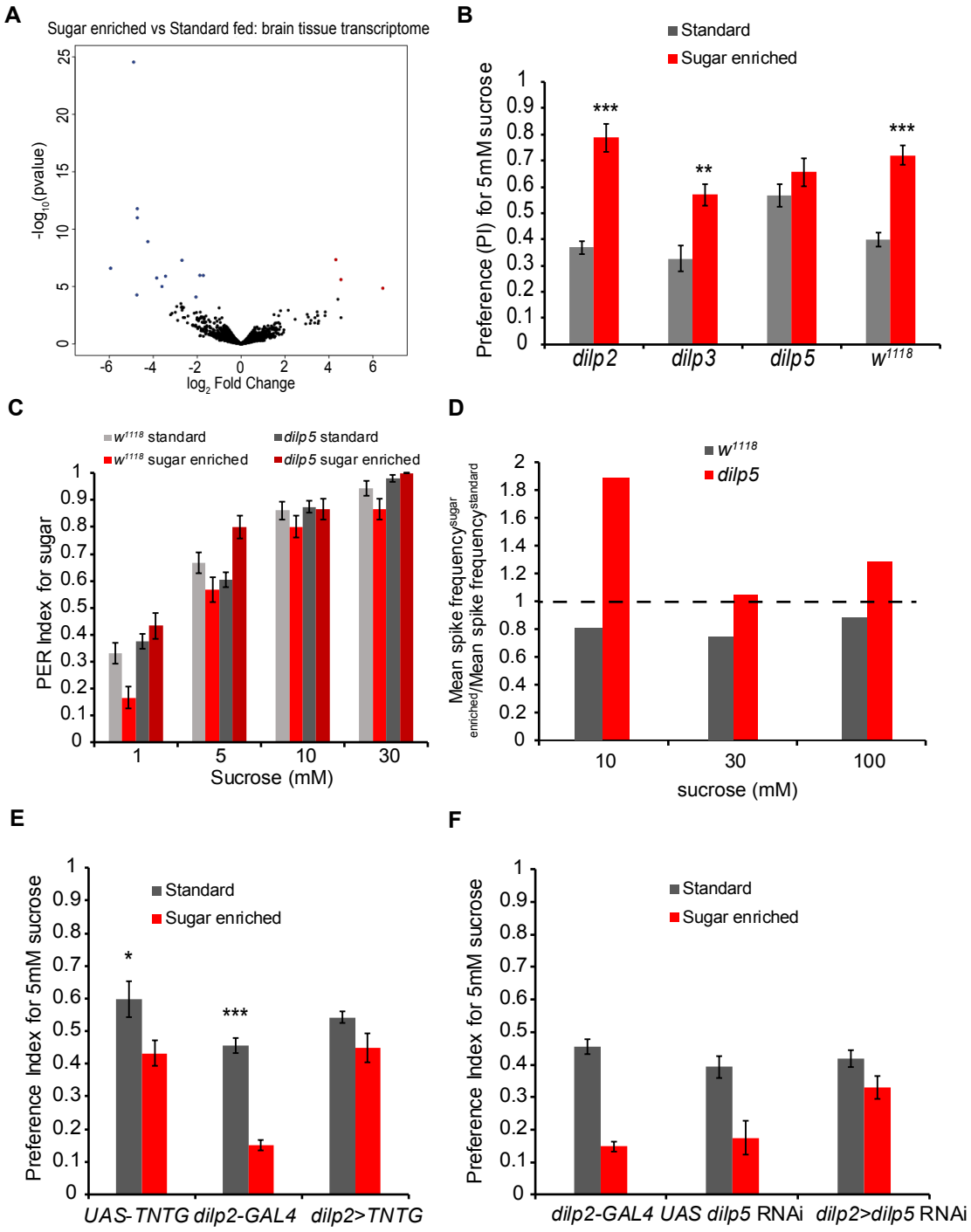

A
Sugar enriched vs Standard fed: brain tissue transcriptome
-log10(pvalue)
log2 Fold Change
B
Standard
Sugar enriched
Preference (PI) for 5mM sucrose
dilp2
dilp3
dilp5
w1118
C
w1118 standard
w1118 sugar enriched
dilp5 standard
dilp5 sugar enriched
PER Index for sugar
Sucrose (mM)
D
w1118
dilp5
Mean spike frequency sugar enriched/Mean spike frequency standard
sucrose (mM)
E
Standard
Sugar enriched
Preference Index for 5mM sucrose
UAS-TNTG
dilp2-GAL4
dilp2>TNTG
F
Standard
Sugar enriched
Preference Index for 5mM sucrose
dilp2-GAL4
UAS dilp5 RNAi
dilp2>dilp5 RNAi

Figure 3.7: *dilp5* is needed for compensatory changes upon feeding with sugar enriched diet

A) Volcano plot comparing the results of RNAseq from brain tissues of mated female flies fed with sugar deprived diet for 1 day to mated female flies fed with standard diet for the same period. The red and blue dots represent genes that are significantly upregulated and downregulated respectively in flies while deprived of sugar. Significance is determined based on $\log_2$(Fold Change)>or <1 and both p value and false discovery rate < 0.05.

B) Mean preference indices of mated female flies for 5mM sucrose (blue dye) tested against 1% yeast extract (pink dye) in binary choice assays. Genotypes tested were mutants for the Drosophila insulin like peptides *dilp2*, *dilp3* and *dilp5* and wild type (w^{1118}). Flies were either fed with sugar enriched diet or with standard diet for 4 days. n=6-9. *p<0.05, **p<0.01, ***p<0.001, Student's T-test. Statistical comparisons were made between sugar deprived (yeast enriched) and standard flies of the same genotypes. Error bars indicate S.E.M.

C) Mean proboscis extension response (PER) indices obtained from mated female flies upon tarsal stimulation with the indicated concentrations of sucrose. Genotypes tested were w^{1118} and *dilp5* mutants. Flies were fed either with sugar enriched diet or with standard diet for 4 days. n= 15-24. Error bars indicate S.E.M.

D) Ratio of mean neuronal response obtained from mated female flies fed with sugar enriched diet for 4 days to those fed with standard diet for 4 days, upon single sensillum recording from labellar L sensilla with indicated concentrations of sucrose. Genotypes tested were w^{1118} and *dilp5* mutants. n=19-30 sensilla from 4-6 flies. The

dotted line indicates a ratio of 1 i.e. a point where the neuronal responses of the flies fed with the two different diets are equal.

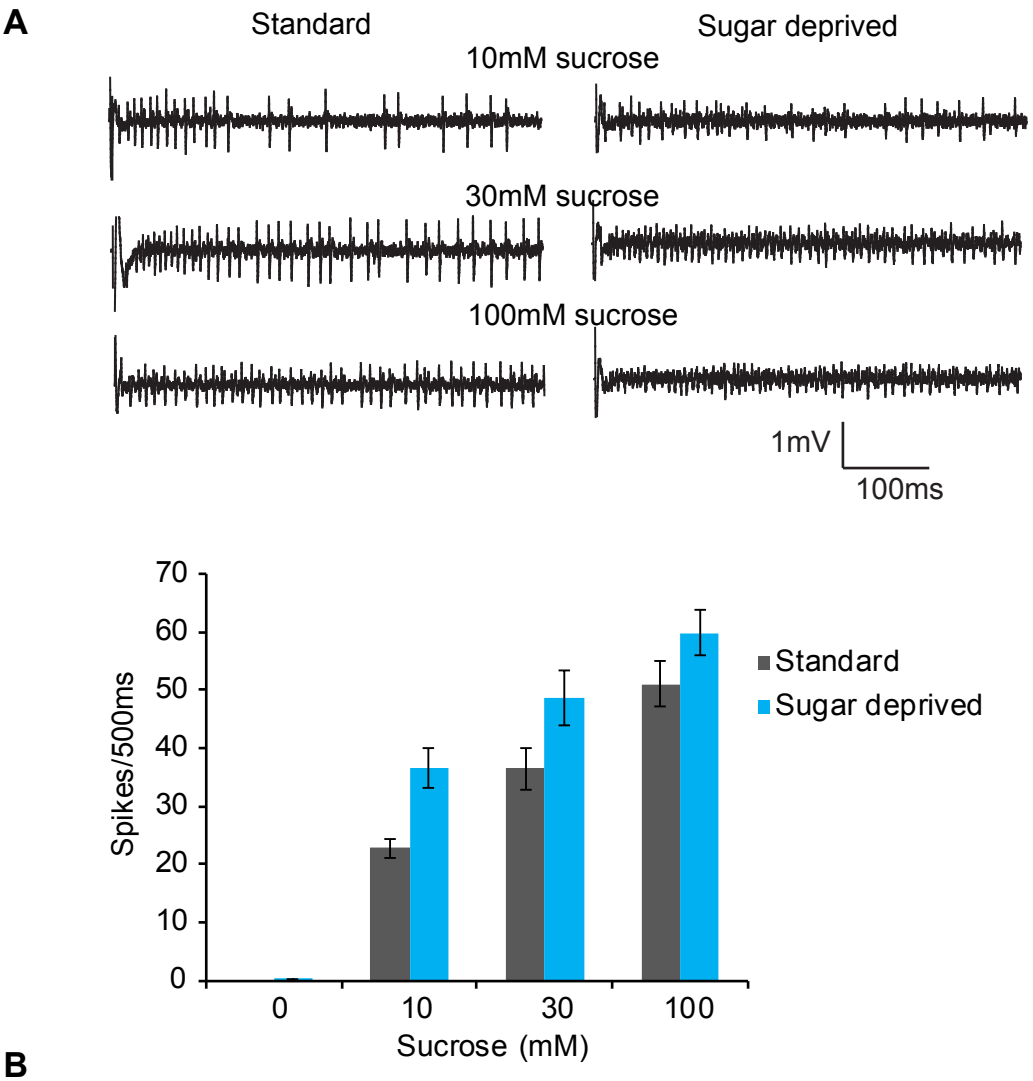
E) Mean preference indices of mated female flies for 5 mM sucrose (blue dye) tested against 1% yeast extract (pink dye) in binary choice assays. Flies were fed either with sugar enriched diet or with standard diet for 4 days. Genotypes tested were *UAS-TNTG*, *dilp2 GAL4* and *dilp2>TNTG*. n= 9-17. *p<0.05, **p<0.01, ***p<0.001, Student's T-test. Statistical comparisons were made between sugar deprived (yeast enriched) and standard flies of the same genotypes. Error bars indicate S.E.M.

F) Mean preference indices of mated female flies to 5mM sucrose (blue dye) tested against 1% yeast extract in binary choice assays. Flies were fed either with sugar enriched diet or with standard diet for 4 days. Genotypes tested were *dilp2 GAL4* (n=9-10), *UAS-dilp5 RNAi* (BL 33683) (n=3-5) and *dilp2>dilp5 RNAi* (n=7-9). Error bars indicate S.E.M.

Table 3.1:

Insulin like peptide	log_2FC (Fold Change)	p value	FDR (False Discovery Rate)
dilp2	-0.60026	0.039803	0.999818
dilp3	-1.72251	1.08E-06	0.001137
dilp5	-1.88204	1.06E-06	0.001137

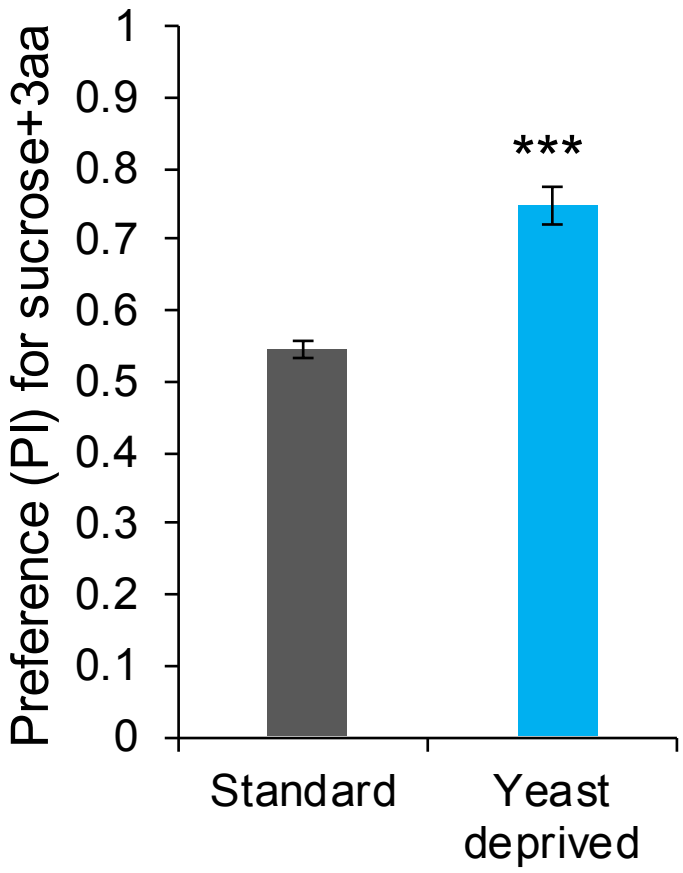
SUPPLEMENTARY FIGURES


A
Standard
Sugar deprived
10mM sucrose
30mM sucrose
100mM sucrose
1mV
100ms
Spikes/500ms
70
60
50
40
30
20
10
0
0
10
30
100
Sucrose (mM)
Standard
Sugar deprived
B
PER index to sucrose
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
1
5
10
30
Sucrose (mM)
Standard
Sugar deprived

Supplementary Figure 3.S1:

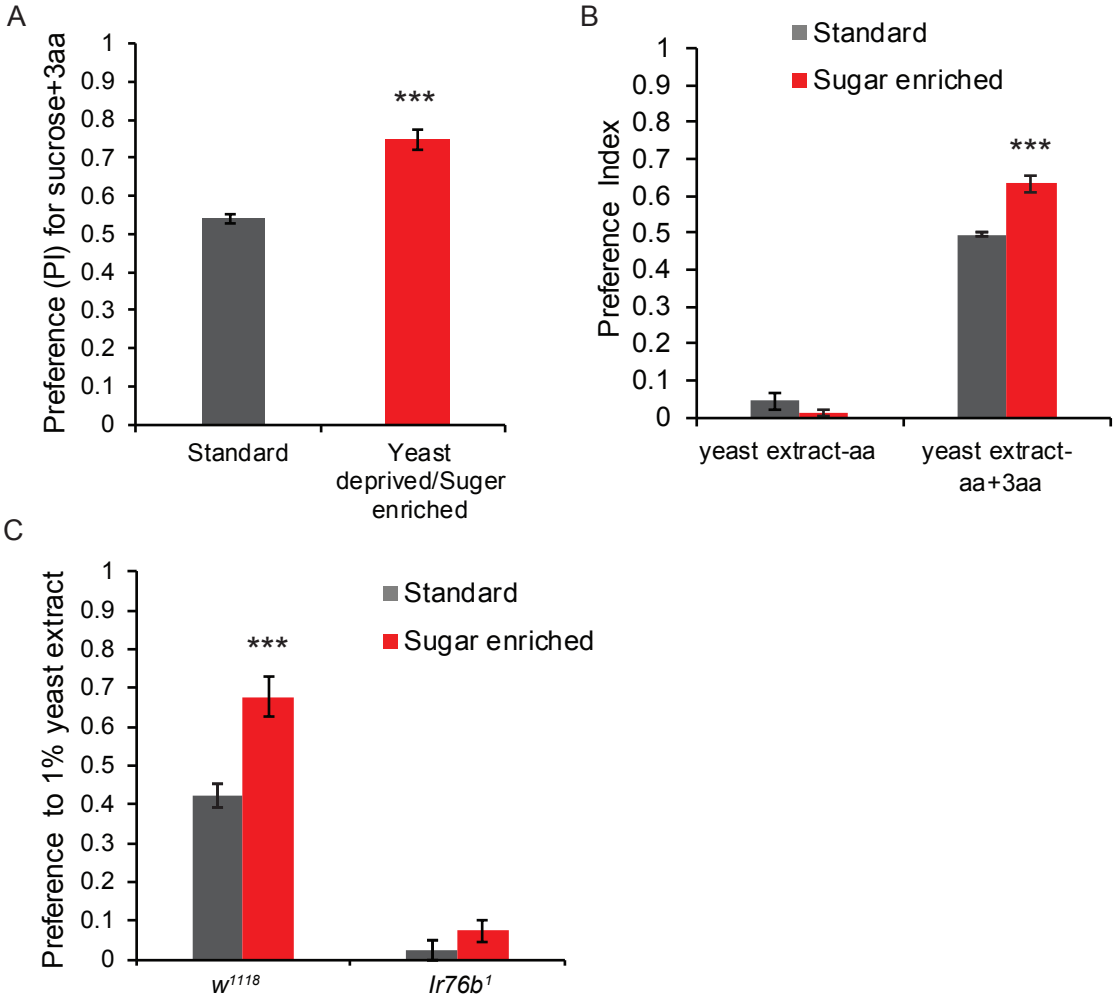
A) Representative traces (top) and mean neuronal responses (bottom) for the first 500ms obtained from tarsal taste bristles of mated female flies upon stimulation with the indicated concentration of sucrose. Flies were fed either with standard diet or sugar deprived diet for 1 day. n=9-10 sensilla from 3-4 flies.Error bars indicate S.E.M.

B) Proboscis extension response index of mated female flies on labellar stimulation with the indicated concentrations of sucrose. Flies were fed either with standard diet or with sugar deprived diet for 1 day. n= 48 and 43 for flies fed with standard and sugar deprived diet respectively. Error bars indicate S.E.M.


Preference (PI) for sucrose+3aa
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
***
Standard
Yeast deprived

Supplementary Figure 3.S2:

Mean preference (PI) of mated female flies fed with the indicated diet for 1 day to 2mM sucrose laced with 25mM of each of serine, phenylalanine and threonine (pink dye) tested with 2mM sucrose (blue dye) in a binary choice assay. $n \geq 5$. *$p<0.05$, **$p<0.01$, ***$p<0.001$, Student's T-test. Error bars indicate S.E.M.

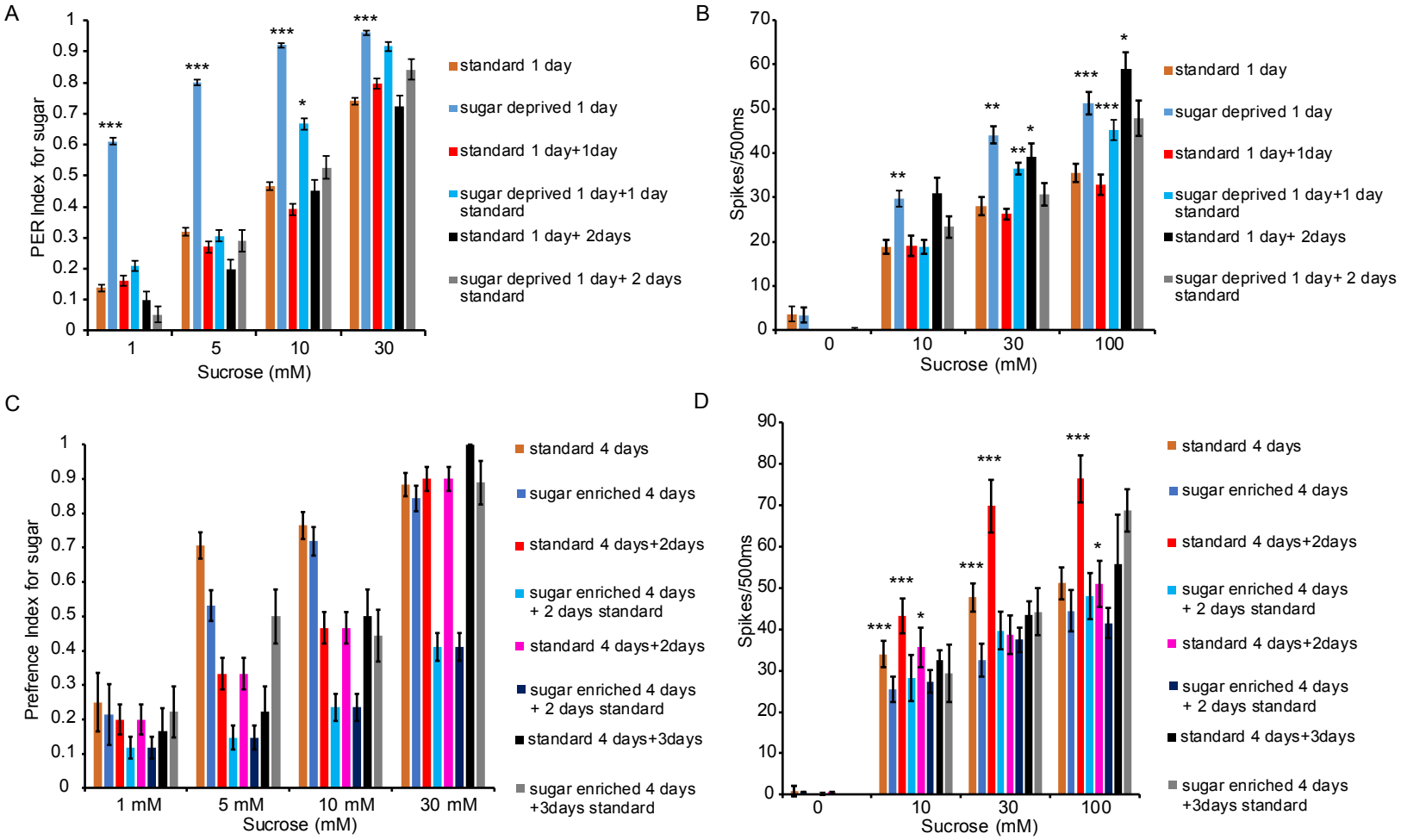

A
Preference (PI) for sucrose+3aa
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
***
Standard
Yeast deprived/Suger enriched
B
Standard
Sugar enriched
Preference Index
***
yeast extract-aa
yeast extract-aa+3aa
C
Preference to 1% yeast extract
Standard
Sugar enriched
***
w1118
Ir76b1

Supplementary Figure 3.S3:

A) Mean preference index of mated female flies fed with the indicated diet for 4 days to 2mM sucrose mixed with 25mM of each of serine, phenylalanine and threonine (pink dye) tested against 2mM sucrose (blue dye) in a binary choice assay. n=7-10. *p<0.05, **p<0.01, ***p<0.001, Student's T-test. Error bars indicate S.E.M.

B) Mean preference index of mated female flies to 1% of the indicated tastants (pink dye) tested against 5mM sucrose (blue dye) in binary choice assay. Flies were fed either with standard diet or with sugar enriched diet for 4 days. n=8-17. *p<0.05, **p<0.01, ***p<0.001, Student's T-test. Error bars indicate S.E.M.

C) Mean preference index of mated female flies to 1% yeast extract (pink dye) tested against 5mM sucrose (blue dye) in binary choice assay. The genotypes tested were w^{1118} and $Ir76b^{1}$ and the flies were fed either with standard diet or with sugar enriched diet for 4 days. n=7-10. *p<0.05, **p<0.01, ***p<0.001, Student's T-test. Error bars indicate S.E.M.


A
PER Index for sugar
Sucrose (mM)
1
5
10
30
standard 1 day
sugar deprived 1 day
standard 1 day+1day
sugar deprived 1 day+1 day standard
standard 1 day+ 2days
sugar deprived 1 day+ 2 days standard
B
Spikes/500ms
Sucrose (mM)
0
10
30
100
C
Prefrence Index for sugar
1 mM
5 mM
10 mM
30 mM
standard 4 days
sugar enriched 4 days
standard 4 days+2days
sugar enriched 4 days + 2 days standard
standard 4 days+3days
sugar enriched 4 days +3days standard
D

Supplementary Figure 3.S4:

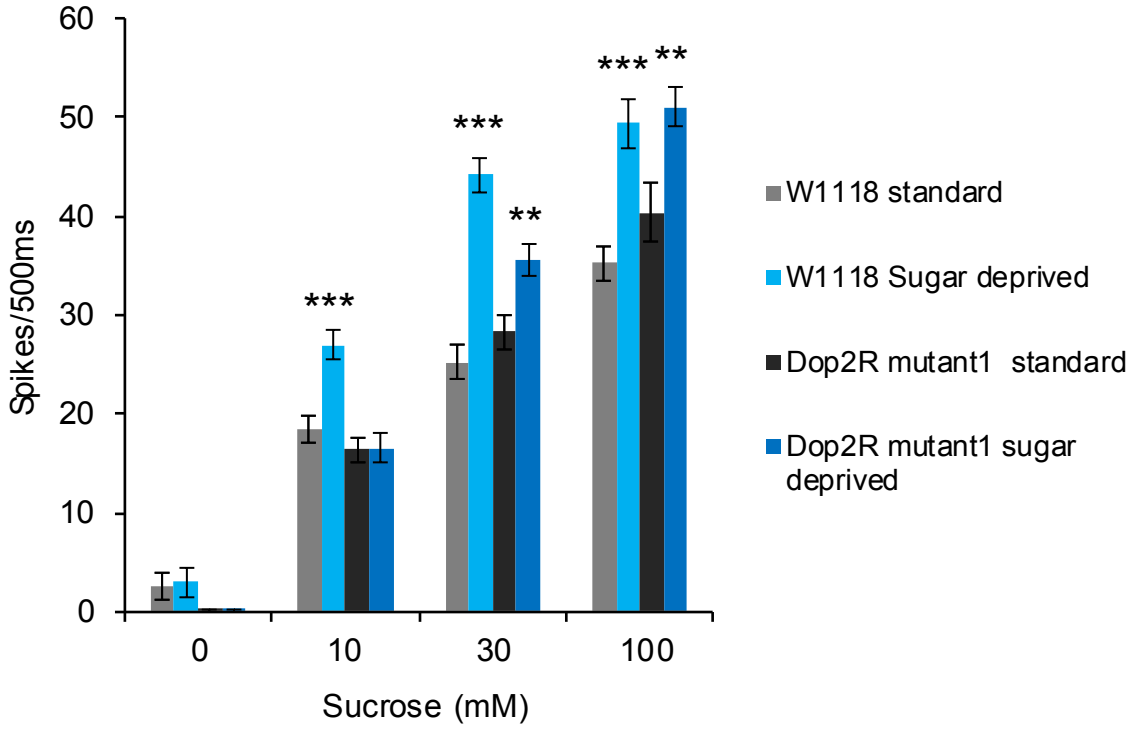
A) Mean proboscis extension response(PER) indices obtained from mated female flies upon tarsal stimulation with the indicated concentrations of sucrose at the indicated time points. Flies were either deprived of sugar for 1 day and fed with standard diet thereafter or were fed with standard diet all along. n=19-63. *p<0.05, **p<0.01, ***p<0.001, Student's T-test. For each concentration, statistical comparisons were made between sugar deprived and standard flies of identical time points. Error bars indicate S.E.M.

B) Mean neuronal responses obtained from mated female flies upon single sensillum recording from labellar L hairs with the indicated concentrations of sucrose at the indicated time points. Flies were either deprived of sugar for 1 day and fed with standard diet thereafter or were fed with standard diet all along. n=8-25 sensilla from 2-5 flies. *p<0.05, **p<0.01, ***p<0.001, Student's T-test. For each concentration, statistical comparisons were made between sugar deprived and standard flies of identical time points. Error bars indicate S.E.M.

C) Mean proboscis extension response(PER) indices obtained from mated female flies upon tarsal stimulation with the indicated concentrations of sucrose at the indicated time points. Flies were either fed with sugar enriched diet for 4 days and with standard diet thereafter or were fed with standard diet all along. n=16-24. *p<0.05, **p<0.01, ***p<0.001, Student's T-test. For each concentration, statistical comparisons were made between sugar deprived and standard flies of identical time points. Error bars indicate S.E.M.

D) Mean neuronal responses obtained from mated female flies upon single sensillum recording from labellar L hairs with the indicated concentrations of sucrose at the

indicated time points. Flies were either fed with sugar enriched diet for 4 days and with standard diet thereafter or were fed with standard diet all along. n=15-29 sensilla from 3-6 flies. *p<0.05, **p<0.01, ***p<0.001, Student's T-test. For each concentration, statistical comparisons were made between sugar deprived and standard flies of identical time points. Error bars indicate S.E.M.


60
50
40
30
20
10
0
Spikes/500ms
***
***
**
***
**
0
10
30
100
Sucrose (mM)
W1118 standard
W1118 Sugar deprived
Dop2R mutant1 standard
Dop2R mutant1 sugar deprived

Supplementary Figure 3.S5:

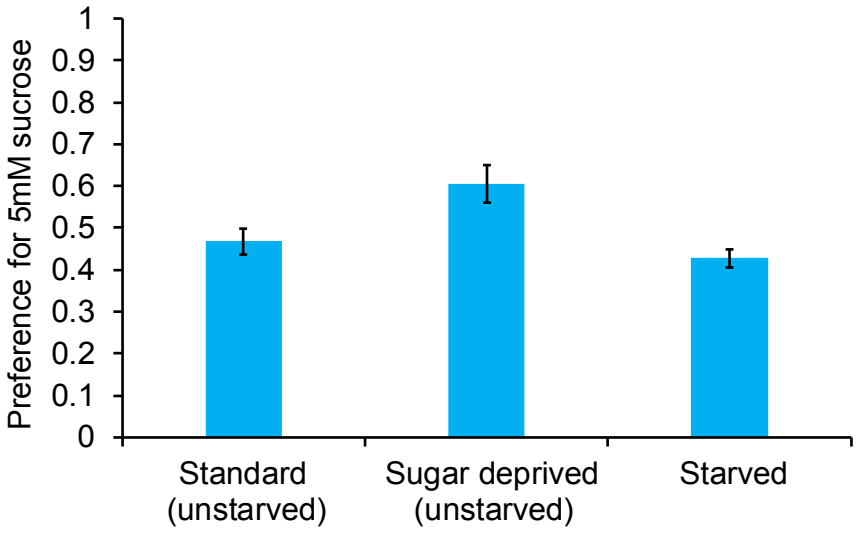
Mean neuronal responses obtained from mated female flies upon single sensillum recording from labellar L hairs with the indicated concentrations of sucrose. Flies were either fed with sugar deprived diet or with standard diet for 1 day. Genotypes tested were w^{1118} and *Dop2R* mutant[1] (BL 52025). n=16-29 sensilla from 5-6 flies. *p<0.05, **p<0.01, ***p<0.001, Student's T-test. Statistical comparisons were made between sugar deprived and standard flies of the same genotypes. Error bars indicate S.E.M.

A

B

Females	50% Mortality	100% mortality
starved	~67 hours (< 3 days)	~87 hours (<4 days)
Sugar-deprived	~ 16 days	~24 days

C

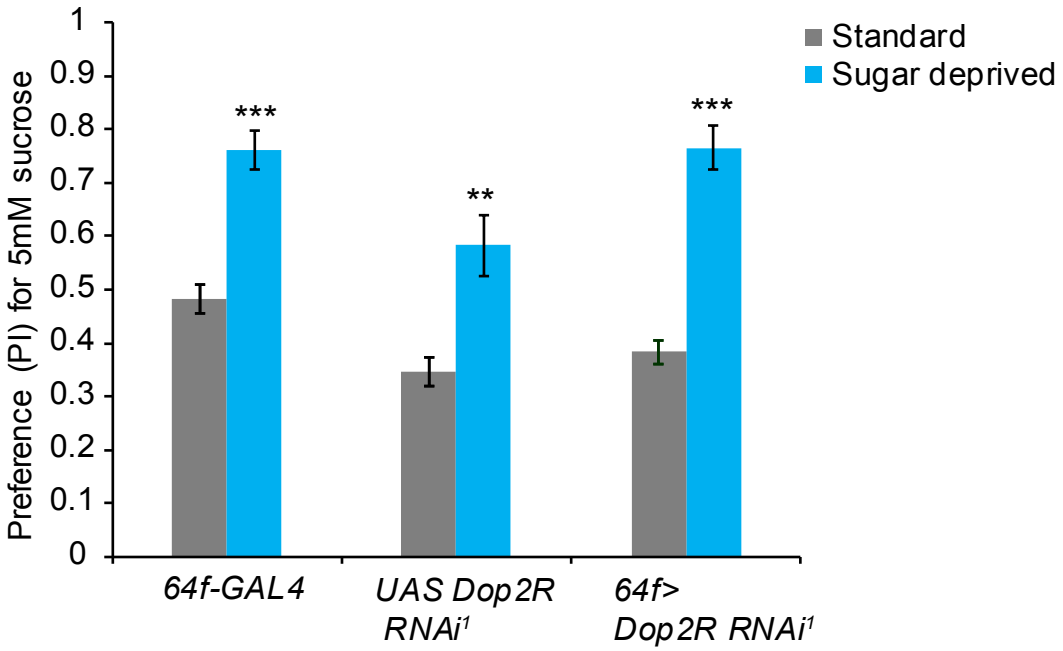


Supplementary Figure 3.S6:

A) Starvation resistance of wet starved flies and flies fed with sugar deprived diet in terms of percentage of dead flies. For this experiment flies were first wet starved for 24 hours or fed with sugar deprived diet for 24 hours. Subsequently, the flies were transferred into fresh starvation vials. Number of dead flies were counted approximately after every 5 hours (except for the last two data points counted at 6 and 7 hours respectively). Male and mated female flies were co-tested, the plot however represents mortality rate of only female flies. n=60 flies for both the conditions.

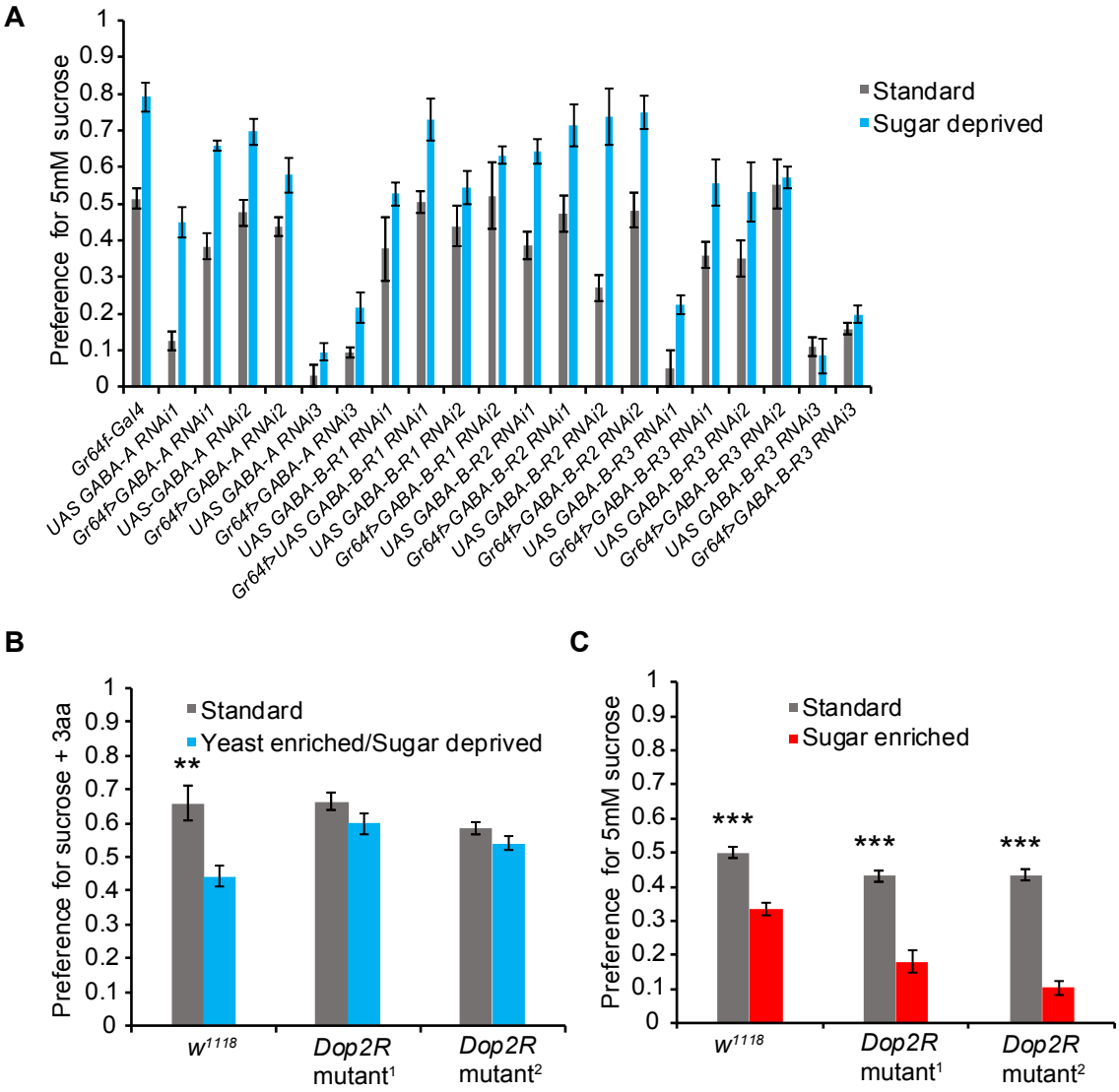
B) Table representing the mortality rate of mated female flies that are either wet starved or are fed with sugar deprived food in terms of percentage of flies dying in the conditions. n=50 flies for both conditions.

C) Mean preference indices of mated female flies to 5mM sucrose (blue dye) tested against 1% yeast extract (pink dye) in a binary choice assay. flies were either fed with standard diet for 1 day or fed with sugar deprived diet for 1 day or are wet starved for 1 day. Flies fed with standard diet and sugar deprived diets were not starved before the assay. n=5. Error bars indicate S.E.M.


Standard
Sugar deprived
Preference (PI) for 5mM sucrose
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
***
**
***
64f-GAL4
UAS Dop2R RNAi1
64f> Dop2R RNAi1

Supplementary Figure 3.S7:

Mean preference indices of mated female flies to 5mM sucrose (blue dye) tested against 1% yeast extract (pink dye) in a binary choice assay. Genotypes of the flies tested were as indicated. Flies were either fed with sugar deprived diet or with standard diet for 1 day. n=14-15 for the GAL4 control, 7 for the UAS control lines and 6 for the GAL4-UAS flies. n=5-6. *p<0.05, **p<0.01, ***p<0.001, Student's T-test. Statistical comparisons were made between sugar enriched and standard flies of the same genotypes. Error bars indicate S.E.M.

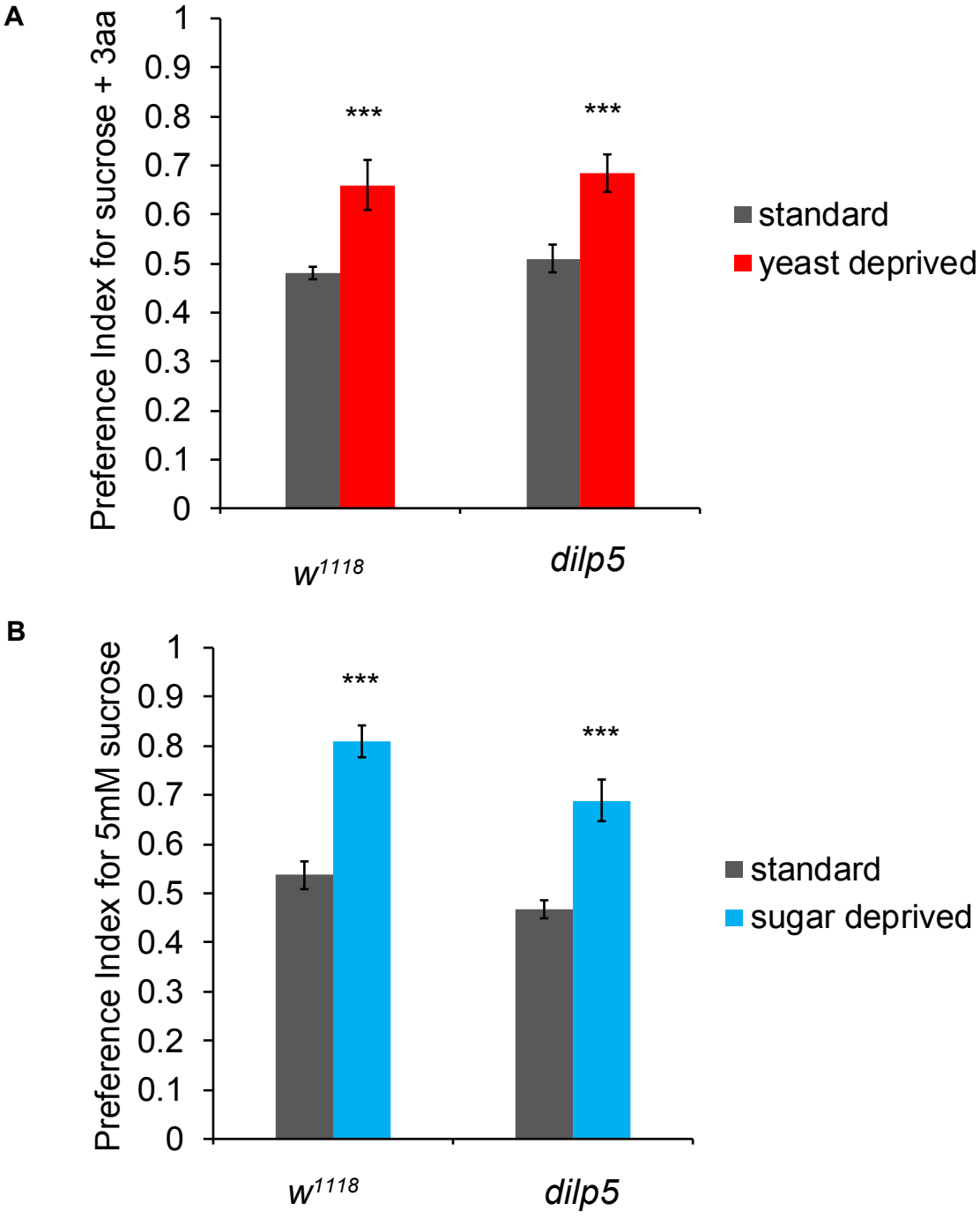

A
Preference for 5mM sucrose
Standard
Sugar deprived
Gr64f-Gal4
UAS GABA-A RNAi1
Gr64f>GABA-A RNAi1
UAS-GABA-A RNAi2
Gr64f>GABA-A RNAi2
UAS GABA-A RNAi3
Gr64f>GABA-A RNAi3
UAS GABA-B-R1 RNAi1
Gr64f>UAS GABA-B-R1 RNAi1
UAS GABA-B-R1 RNAi2
Gr64f>GABA-B-R1 RNAi2
UAS GABA-B-R2 RNAi1
Gr64f>GABA-B-R2 RNAi1
UAS GABA-B-R2 RNAi2
Gr64f>GABA-B-R2 RNAi2
UAS GABA-B-R3 RNAi1
Gr64f>GABA-B-R3 RNAi1
UAS GABA-B-R3 RNAi2
Gr64f>GABA-B-R3 RNAi2
UAS GABA-B-R3 RNAi3
Gr64f>GABA-B-R3 RNAi3
B
Preference for sucrose + 3aa
Standard
Yeast enriched/Sugar deprived
**
w1118
Dop2R mutant1
Dop2R mutant2
C
Preference for 5mM sucrose
Standard
Sugar enriched
***
***
***
w1118
Dop2R mutant1
Dop2R mutant2

Supplementary Figure 3.S8:

A) Mean preference indices of mated female flies to 5mM sucrose (blue dye) tested against 1% yeast extract (pink dye) in a binary choice assay. Genotypes of the flies tested were as indicated. Flies were either fed with sugar deprived diet or with standard diet for 1 day. n=10 for the GAL4 control, 2-6 for the UAS control lines and 6-8 for the GAL4-UAS flies. Error bars indicate S.E.M.

B) Mean preference indices of mated female flies to 2mM sucrose mixed with 25mM of each of serine, phenylalanine and threonine (pink dye) tested against 2mM sucrose (blue dye) in binary choice assays. Flies were either fed with sugar deprived (yeast enriched) diet or with standard diet for 1 day. Genotypes of the flies tested were w^{1118} , *Dop2R* mutant^{1} (BL 52025) and *Dop2R* mutant^{2} (BL 52517). *p<0.05, **p<0.01, ***p<0.001, Student's T-test. Statistical comparisons were made between sugar deprived (yeast enriched) and standard flies of the same genotypes. Error bars indicate S.E.M.

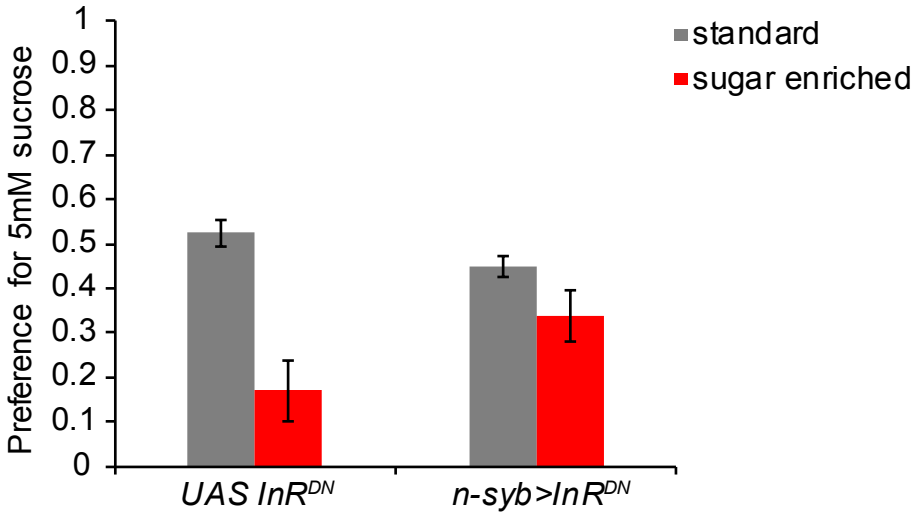
C) Mean preference indices of mated female flies to 5mM sucrose (blue dye) tested against 1% yeast extract (pink dye) in a binary choice assay. Genotypes of the flies tested were w^{1118} , *Dop2R* mutant^{1} (BL 52025) and *Dop2R* mutant^{2} (BL 52517). Flies were either fed with sugar enriched diet or with standard diet for 4 days. n=5-6. *p<0.05, **p<0.01, ***p<0.001, Student's T-test. Statistical comparisons were made between sugar enriched and standard flies of the same genotypes. Error bars indicate S.E.M.


A
Preference Index for sucrose + 3aa
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
***
***
standard
yeast deprived
w1118
dilp5
B
Preference Index for 5mM sucrose
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
***
***
standard
sugar deprived
w1118
dilp5

Supplementary Figure 3.S9:

A) Mean preference indices of mated female flies to 2mM sucrose mixed with 25mM of each of serine, phenylalanine and threonine (pink dye) tested against 2mM sucrose (blue dye) in binary choice assays. Genotypes tested were w^{1118} and *Δdilp5*. Flies were either fed with sugar enriched diet or with standard diet for 4 days. n=5-10. *p<0.05, **p<0.01, ***p<0.001, Student's T-test. Statistical comparisons were made between sugar deprived (yeast enriched) and standard flies of the same genotypes. Error bars indicate S.E.M.

B) Mean preference indices of mated female flies to 5mM sucrose (blue dye) tested against 1% yeast extract (pink dye) in binary choice assays. Genotypes tested were w^{1118} and *Δdilp5*. Flies were either fed with sugar deprived diet or with standard diet for 1 day. n=12-14. *p<0.05, **p<0.01, ***p<0.001, Student's T-test. Statistical comparisons were made between sugar deprived (yeast enriched) and standard flies of the same genotypes. Error bars indicate S.E.M.


standard
sugar enriched
Preference for 5mM sucrose
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
UAS InR^DN
n-syb>InR^DN

Supplementary Figure 3.S10:

Mean preference indices of mated female flies to 5mM sucrose (blue dye) tested against 1% yeast extract (pink dye) in binary choice assays. Genotypes are as indicated. Flies were either fed with sugar deprived diet or with standard diet for 4 days. n=5-6.

Chapter 4: Aversive stimuli inhibits sweet neuron activity in *Drosophila melanogaster*.

ABSTRACT:

Fruit flies have specialized gustatory receptor neurons to identify compounds with attractive (eg. sweet) and aversive (eg. bitter) taste. Stimulation of the attractive gustatory neurons elicit feeding which is arrested by activation of the aversive taste neurons. However, recent studies suggest that aversive stimuli like bitters or low pH can inhibit sweet neuron firing leading to cessation of feeding. Here we investigated if other classes of aversive stimuli viz ammonia or high salts can cause sweet neuron inhibition. Using single sensillum recording, we find that ammonia can inhibit firing of both sweet neurons and low salt neurons to their respective ligands. We identify high pH of ammonia to be instrumental in this inhibition. We further find that salts in high concentration are also capable of inhibiting sweet neuron firing to sugars.

INTRODUCTION

Drosophila uses its sense of taste to evaluate its environment and identify food sources as well as potentially harmful or toxic substances. While identification of nutritious food substances lead to feeding events, a fly generally chooses to avoid toxic or harmful substances. For this purpose, the fly has sensory neurons bearing different classes of receptors. Based on the presence of the kind of receptors a neuron can be specialized to identify only appetitive stimuli (viz. sugar, low salt) or only aversive stimuli (viz. bitter substances, acids) (Charlu et al., 2013; Dahanukar et al., 2007; Freeman and Dahanukar, 2015; Ling et al., 2014; Weiss et al., 2011). These taste receptor neurons are distributed widely on their proboscis, within the pharynx, on the tarsal segments of their legs as well as on their wings (Freeman and Dahanukar, 2015; LeDue et al., 2015; Ling et al., 2014; Raad et al., 2016; et al., 2014). Stimulation of the taste neurons for attractive taste induces feeding while stimulation of the taste neurons for aversive taste leads to cessation of feeding (Dahanukar et al., 2007; Freeman and Dahanukar, 2015; LeDue et al., 2015; Weiss et al., 2011; Zhang et al., 2013).

Since most naturally occurring food sources are a mixture of various compounds, flies potentially evaluate them by computing the relative weights of the signals coming from both the attractive and aversive channels before deciding whether to initiate a feeding event or terminate it. It is essential for an organism to avoid toxic or potentially harmful substances to survive. So, it is not surprising that flies deploy several pathways to make sure aversive compounds are not ingested. Firstly, direct activation of aversive taste neurons acts as an anti-feeding signal by itself (Charlu et al., 2013; Weiss et al., 2011) .

Secondly, activation of the bitter neurons leads to inhibition of sugar and water feeding by activating four GABAergic interneurons which inhibit consumption by gating food induced activation of feeding motor neurons (Pool et al., 2014).

Some recent studies have revealed a third pathway where aversive substances can directly inhibit sugar neurons (Charlu et al., 2013; French et al., 2015). Charlu et al., shows that low pH or acids can inhibit activation of sweet neurons in presence of sugars and can eventually prevent flies from feeding sugars laced with acids. French et al., demonstrates sweet neuron activity is inhibited while sugars are laced with certain bitter compounds and this leads to decreased behavioral sensitivity and feeding preference for these mixtures.

Bitter substances and acids encompass most of the aversive stimuli a fly encounters in its environment the others being ammonia and high salt (Alves et al., 2014; Hiroi et al., 2004; Kim et al., 2017; Menuz et al., 2017; Zhang et al., 2013).

Ammonia and amines occur commonly throughout different ecosystems. Rotting biomass like decomposing fruits and other plant parts produces ammonia and other amines (Davis et al., 2007; Kiss et al., 2006; Ough et al., 1981). Ammonia is also known to be present in the excreta of various animals (Borash et al., 2000; Botella et al., 1985). Urea present in the excreta of various animals can be converted to ammonia as well. Hence it acts as a chemosensory cue to a vast range of animals from nematodes to seabirds. It has been shown that ammonium acetate could be detected by both olfactory and gustatory systems of *C. elegans* (Frøkjaer-Jensen et al., 2008). Ammonia present in human sweat acts as an attractive cue for disease vectors like *Anopheles gambiae* (Meijerink et al., 2001; Smallegange et al., 2005) and *Aedes aegypti* (Geier et al., 1999)

who can sense ammonia through grooved peg sensilla present on their antennae. Ammonia emitted by *Rafflesia* could also act as an attractive cue for certain insects facilitating pollination (Davis et al., 2007).

Ammonia acts as an important cue in prey-finding and homing behavior of sea birds like petrels, albatrosses and shearwaters (Nevitt et al., 2006) Ammonia is also perceived by the olfactory system of other vertebrates like fishes (Barimo and Walsh, 2006) and mammals including humans (Wallrabenstein et al., 2013) and mice (Liberles and Buck, 2006) Amines present in mouse urine can act as social cues or pheromones and can be detected by olfactory epithelium (Liberles and Buck, 2006).

A conserved class of receptors known as trace amine associated receptors (TAAR) have been identified in mice to be responsible for smelling ammonia and amines (Liberles and Buck, 2006). In insects, endeavors to discover the amine or ammonia receptor have mostly used *Drosophila melanogaster*. Attraction towards ammonia appears to be fairly conserved in insects since, like mosquitoes, *Drosophila melanogaster* as well as a related species *Drosophila simulans* are attracted towards ammonia and certain amines in olfactory behavior assays. Ir92a an ionotropic receptor has been shown to be necessary for detection of ammonia and certain amines (Min et al., 2013) This study by Min *et al.* also maps ammonia elicited responses to VM1 glomeruli in antennal lobe and VM1-PNs going to the lateral horn. Another study by Menuz *et al.* reports the role of an ammonia transporter gene *Amt* in olfactory responses to ammonia (Menuz et al., 2014) This gene is expressed in the auxiliary cells surrounding the *Ir92a*-expressing ac1 neurons. Mutating this gene greatly decreases the neuronal firing frequency obtained from the ac1 neurons on stimulation with ammonia.

However, how these two contribute together to ammonia perception is not well understood.

Ammonia occurs in the decomposing biomass that flies feed upon. Ammonia is also excreted by fly larvae and is known to build up to considerable levels in over-crowded cultures (Borash et al., 2000; Botella et al., 1985) Ammonia being harmful to the body if ingested, is expected to elicit an aversive taste response in flies. Indeed, a recent study reveals that presence of ammonium salts inhibits feeding in flies (Menuz et al., 2017) . The study reveals that ammonium salts evoke strong neuronal responses from s sensilla while a weak response was obtained from L and I sensilla types as well. The responses in s sensilla is mediated by *Gr66a* neurons and was found to be responsible for the feeding aversion to ammonia. Although the study reveals that lacing sugars with ammonium salts inhibit feeding, whether ammonia has any influence on the sugar neurons was not explored.

Another stimulus that is highly aversive for flies is high concentrations of salt. Salt is essential for the upkeep of various physiological processes like maintaining neuronal excitability, transmembrane transport of organic compounds, as well as for maintaining proper osmolarity of body fluids. Hence all organisms need to ingest salts. Excessive ingestion of salt, however, is detrimental. An increase in the concentration of Na+ ion, for instance, can lead to hypertension, gastrointestinal cancer, osteoporosis and autoimmune diseases (Frisoli et al., 2012; He and MacGregor, 2009; Jörg et al., 2016; Kleinewietfeld et al., 2013; Tsugane et al., 2004) . Hence, to ensure optimum salt intake, organisms tend to prefer only low concentrations of salt and reject high concentrations (Alves et al., 2014; Hiroi et al., 2004; Kim et al., 2017; Oka et al., 2013; Zhang et al., 2013) .

Mice have feeding preference for low concentrations (<100mM) of sodium salts which is perceived by amiloride sensitive epithelial sodium channels (ENaC) present in taste cells (Chandrashekar et al., 2010) . Among the invertebrates, salt taste has been studied in the nematode *Caenorhabditis elegans*. One study suggested that *transmembrane channel-like-I* (*tmc-I*) a gene expressed in the ASH polymodal neurons is responsible for sodium salt evoked neuronal responses as well as avoidance behavior (Chatzigeorgiou et al., 2013) . However, a different reported that *tmc-I* is not required for salt induced behavioral responses in C. elegans (Wang et al., 2016). . Wang et al., suggested that sodium(Na^+) induced behaviroral responses in worms are mediated by G-protein signaling dependent activation of OSM-9, a TrpV channel.

In *Drosophila melanogaster*, the cellular and molecular basis for salt taste has been extensively studied. Flies have different neuronal classes expressing sensitivity to high and low concentrations of salt respectively (Freeman and Dahanukar, 2015; Hiroi et al., 2004; Zhang et al., 2013) *.* Flies have different receptors for molecular detection of high and low concentrations of salt. *Ir76b* an ionotropic receptor has been reported to be responsible for the detection of low concentrations of sodium salts (Zhang et al., 2013) . Interestingly, different other studies have demonstrated the roles of other receptors and genes in mediating high salt driven aversion in flies.

The defective proboscis extension response (dpr) locus, encoding a member of the Ig-superfamily expressed in certain primary neurons in the gustatory organs have been demonstrated to be necessary for high salt driven aversion in adult flies (Nakamura et al., 2002) . Another such study has reported the role of two members of Drosophila DEG-ENaC channel family (pickpocket receptors) namely *pickpocket 11* (*ppk11*) and *pickpocket 19* (*ppk19*), expressed widely in the taste organs of both larvae and adult

flies, in detection of sodium and potassium salts in both larvae and in adult flies (Liu et al., 2003) . According to the study, disrupting *ppk11* and *ppk 19* disrupts behavioral and neuronal responses to both low and high concentrations of salt in both larvae and adults. However, a different study refutes the role of the DEG-ENaC genes in low salt detection (Zhang et al., 2013) A different group have published that another gene named *serrano* (*sano*) can also mediate high salt mediated aversion in larvae (Alves et al., 2014) Alves et al., reported that *sano* is expressed in *Gr66a* neurons in the larvae and silencing *sano* as well as *ppk19* in *sano*-positive neurons can abolish high salt driven aversion in larvae. In most of the studies, where high salt driven aversion has been studied in adult flies, sugar mixed with high salt concentrations have been used. However, whether high salt can directly act on sweet neurons to contribute towards this aversion has not been explored.

Here we explore the possibility that like other aversive stimuli viz bitters and acids, both ammonia and high salt are also able to inhibit appetitive taste neuron firing. We demonstrate that ammonia can inhibit the responses of different classes of taste neurons to corresponding stimulants. We trace the basis of this inhibition to the high pH of ammonia solutions. We also test a few amines with high pH and find that they cause an identical degree of neuronal inhibition. We further report that high concentrations of different salts are also capable of inhibiting the sugar neurons to varying degree.

MATERIALS AND METHODS

Fly stocks: *Ir76b*[1] (BL 51309) flies were obtained from *Drosophila* Bloomington Stock Center. Flies were raised in standard cornmeal-dextrose medium at 22ºC-25ºC.

Tastants: Sucrose (S7903), ammonium hydroxide (NH_4OH) solution (44273), sodium hydroxide (S5881), 4-methylpiperidine (M73206), pyridine (P3776), di-methyl amine (426458), $MgSO_4$ (M5921) were all acquired from Sigma. Ammonium sulfate ($(NH_4)_2SO_4$) (A702) was obtained from Fisher Scientific. NaCl (7647-14-5) and KCl (6858) were obtained from Macron Fine Chemicals and Malinckrodt respectively.

For PER assays, the tastants were dissolved in water. For electrophysiological recordings, the tastants were dissolved in 30mM TCC (Sigma, T0252).

Electrophysiology: Single sensillum extracellular recordings were obtained from L hairs of the labellum as described by (Benton and Dahanukar, 2011). 5-7 days old mated female flies of appropriate genotype were used for recordings.

All the tastants were mixed in 30mM tri choline citrate electrolyte. For recordings with NH_4OH, the solutions were always freshly prepared just before the experiment. Neuronal responses were quantified by counting the number of spikes in the first 500 ms upon contact with the stimulus.

Proboscis Extension Response Assays:

The Proboscis Extension Response (PER) Assays with labellar stimulation were performed as previously described by (Charlu et al., 2013). 5-7 days old mated female flies, starved for 24 hours, were used for the experiment. Full extensions were scored as 1 whereas partial extensions were scored as 0.5. A score of zero was awarded in those

events where the flies failed to extend their proboscis in response to the tastant presented.

RESULTS

Ammonia can inhibit firing of sweet and salt neurons.

To investigate the effect of ammonia on taste neurons we first surveyed the labellar L-sensilla for responses evoked by 3% NH_4OH, a concentration that shows robust responses in olfactory neurons. However, consistent with previous reports (Menuz et al., 2017) , little or no firing of taste neurons was observed in response to 3% NH_4OH in the L-sensilla.

To further study if ammonia can interfere with a taste neuron's response to its known stimulant we elected to test mixtures of NH_4OH with sucrose, a strong activator of sweet-sensing neurons in the L sensilla. NH_4OH was observed to inhibit, in a concentration dependent manner, sugar-induced firing of the sweet neurons (Fig. 1A). Importantly, exposure to ammonia did not damage the neurons as the sugar neuron fired normally when stimulated with 30mM sucrose at the end.

Sweet neurons house sugar receptors which belong to the GR (gustatory receptor) family. Members of the GR class are also responsible for bitter taste detection in flies. But since it has already been reported that ammonia strongly activates bitter neurons in s hairs we could not test if bitter neuron firing to a known tastant is affected by ammonia contamination.

However, as already mentioned, appetitive low salt (NaCl) taste information in flies is conveyed by *Ir76b* (Zhang et al., 2013).To explore if ammonia induced neural inhibition is restricted to GR containing neurons only, we mixed 50 mM NaCl with 3% NH_4OH, a

concentration at which there was more than 80% inhibition of the sweet neurons. Interestingly, ammonia inhibited Ir76b mediated salt neuron activity as well (Fig. 1B). That the salt neuron was not damaged was evident from the normal activity of the neurons on subsequent stimulation with NaCl.

High concentrations of salt can inhibit sweet neuron firing in a concentration dependent manner

To test the possibility that high concentrations of salt can inhibit sweet neuron firing we elected to conduct single sensillum recording with an appropriate salt-sugar mixture.

We elected to use a mixture of 10mM salt and 500mM NaCl based on previous studies (et al., 2013; Zhang et al., 2013). High salt by itself can lead to neuronal firing across all classes to taste bristles and the resulting spikes are often indistinguishable from the sugar spikes. Testing the mixtures on wild type flies is highly unsuitable for these studies since our inability to confidently sort the two types of spike produced will not allow accurate measurement of neuronal inhibition if any. A previous study by Zhang et al., 2013, has reported that neuronal responses in labellar L hairs (but not in all s hairs) elicited by 500mM NaCl is greatly reduced in *Ir76b* mutant flies. To further verify the results, we tested 500mM NaCl on the labellar L-hairs of Ir76b mutant (*Ir76b*^{1}) flies, where we observed very little neuronal activity (Fig. 5A). Hence, we elected to test the sugar-NaCl mixture on labellar L taste hairs of *Ir76b*^{1} flies 10mM sucrose alone, when tested, elicited a normal response which was almost 50% diminished when it was laced with 500mM NaCl. An almost 100% recovery of the neurons upon subsequent stimulation with 10mM sucrose ruled out any possibilities of the neurons being damaged during recording (Figure 2A). To test if the observed inhibition of sugar neurons is

dependent on the salt concentration we stimulated the L hairs of *Ir76b*^{1} flies with10mM sucrose laced with a range of salt (NaCl) concentrations.

Increasing salt concentration led to greater degree of inhibition of the sugar neuron (Fig. 2B). Interestingly, approximately 34% reduction in firing frequency was observed even when the sucrose solution was mixed with a salt concentration as low as 100mM. Further increasing the salt concentration to 500mM and 1M caused approximately 57.7% and 65.5% inhibition respectively. An increase in firing frequency was observed while sucrose was tested on the same hairs at the end thereby ruling out any possibilities of neuronal damage causing the observed neuronal inhibition (Fig. 2B).

Ammonia mediated neuronal inhibition is an outcome of its high pH.

A recent study has reported that NH_4^+ ions are responsible for the neuronal responses evoked by ammonium salts (Menuz et al., 2017) . To test if presence of NH_4^+ ions in NH_4OH is required for the neuronal inhibition we laced sucrose solutions with 242 mM $(NH_4)_2SO_4$. The concentration was so chosen as to match the amount of NH_4^+ ions obtained from 3% NH_4OH when there is complete dissociation. Neuronal firing to sucrose, however, was not inhibited when sucrose was mixed with $(NH_4)_2SO_4$ suggesting that the neuronal inhibition was caused by some other mechanisms (Fig. 3A).

NH_4OH is highly basic in nature. When measured, 0.1%, 1% and 3% NH_4OH solutions had an approximate pH of 8.5, 10.5 and 12.5 respectively. Earlier studies have reported changes in neuronal sensitivity induced by pH changes (Fukuda and Loeschcke, 1977; Fukuda et al., 1980; et al., 2012; Ruusuvuori and Kaila, 2014) . To test if high pH is instrumental in the observed neuronal inhibition we used varying amounts of NaOH to make several 30mM sucrose solutions with a range of pH.

Interestingly, increasing pH resulted in increasing inhibition of the sucrose induced firing of the sweet neuron (Fig. 3B, 3C).

At pH 12.5 and 10.5 we observed inhibition in sweet neuron firing to sucrose comparable to that caused by 3% and 1% NH_4OH respectively. This implied that neuronal inhibition caused by ammonia is an outcome of high pH. Surprisingly, inhibition caused by pH 8 was much lower than that caused by 0.1% NH_4OH. Robust neuronal responses to 30mM sucrose obtained from the same hairs at the end of the experiment suggested that the observed neuronal inhibition was not due to neurons getting damaged.

To determine if the electrophysiological responses are behaviorally relevant we conducted PER (proboscis extension response) assays by labellar stimulation. Proboscis extension in response to 100mM sucrose was decreased both by addition of increasing concentration of NH_4OH as well as by increasing pH (Fig. 2D). This suggested that the observed neuronal inhibition is indeed leads to avoidance of food contaminated with ammonia or high pH.

Although the degree of inhibition at higher pH ranges matched the patterns seen in the electrophysiology experiments, we observed a much more robust inhibition of sugar induced PER at pH 8.5 than expected from the tip recordings.

High pH can explain neuroinhibitory effects of amines on taste neurons.

Amines are inorganic derivatives of ammonia. Different amines occur naturally in the ecological niche of the fly. Amines like dimethylamine occur naturally whereas others enter the ecosystem through industrial effluents. All amines are basic in nature and many of them are toxic to living organisms. Hence it is expected that flies would employ the same strategy of neuronal inhibition to avoid toxic amines.

To test whether high pH amines are also capable of causing the neuroinhibitory effect we elected to lace 30mM sucrose with 1% and 3% 4-methylpiperidine. 4-methylpiperidine is a derivative of piperidine which occurs naturally in pepper plants. The pH of 4-methylpiperidine solutions resembled that of NH_4OH solutions at similar concentrations. We recorded from the labellar L sensilla using the sucrose 4-methylpiperidine solutions. 30mM sucrose was tested on the same hairs before and after testing the sucrose-amine mixture to ensure that the hairs are not damaged.
Adding 4-methylpiperidine to 30mM sucrose produced a neuroinhibitory effect like that caused by NH_4OH at identical concentrations (Fig. 3A).
We also tested two other amines, pyridine and dimethylamine; 3% of both amines were mixed with 30mM sucrose and then the solutions were used for tip recordings. While the sucrose-pyridine mixture had a pH of approximately of 8, the sucrose-dimethylamine mixture had a pH of 12. In both the cases we observed a neuroinhibitory effect (Fig. 3B). While the neuroinhibitory effect observed in case of 3% dimethylamine is slightly higher than that observed at pH 12.5, mixing 30mM sucrose with 3% pyridine resulted in a neuroinhibitory effect much higher than that caused by pH 8, suggesting that additional factors might be involved there.

As before we tested 30mM sucrose before and after testing the sucrose-amine mixtures to ensure that the neuronal inhibition observed is not an artifact of the neurons getting damaged.

Sweet neuron can be inhibited by high concentrations of different salts.

To test if the inhibition of sweet neurons is a specific property of high concentrations of NaCl only, we elected to test mixtures of other salts viz. KCl and $MgSO_4$with 10mM sucrose on the labellar L hairs of *Ir76b*^{1} flies. To ensure that the salts by themselves did not elicit robust responses from the L-hairs we first checked their response to 500mM of KCl and $MgSO_4$ individually and in both cases, we observed little to no response (Fig. 5A). As expected these hairs also showed little response to 500mM NaCl (Fig. 5A).

Interestingly, both KCl and $MgSO_4$ elicited robust neuronal responses when tested upon the L-hairs of wild type (*w*^{1118}) flies. This was a rather surprising result since previous studies have reported that *Ir76b* is a NaCl specific receptor (Zhang et al., 2013). However, a different study has suggested that Ir76b can act as a co-receptor and can contribute towards different other tastes along with other Irs (Ganguly et al., 2017). Hence the possibility that Ir76b acts with other receptors to mediate responses of these other salts cannot be ruled out.

Both 500mM KCl and 500mM $MgSO_4$ inhibited sugar neuron activity in the L sensilla of *Ir76b*^{1} flies (Fig. 5C). While 500mM KCl produced an inhibition akin to that caused by 500mM NaCl, $MgSO_4$ caused a much higher degree of inhibition, suggesting that high concentration of different salts can produce different degree of sugar neuron inhibition.

DISCUSSION

Here we report that appetitive taste neurons could be inhibited by aversive stimuli other than bitters and acids. Using single sensillum recording, we demonstrate that both high pH and high salt concentration can also have a dampening effect on sweet neuron activity. It had been previously published that low pH inhibits sweet neuron activity (Charlu et al., 2013). Taken together with this result our findings on neuronal inhibition by high pH suggests that, for feeding, flies prefer the food to be at a neutral pH or within a narrow range around it. This could be important in ensuring that the flies choose the right food needed for survival.

Noxious pH could be nociceptive, causing pain sensations. High levels of acid in a fruit might suggest that it is still unripe while high levels of alkalinity suggest that the food might have got contaminated with excreta or different toxic amines. Interestingly, high pH also inhibits low salt neuron and thus can prevent feeding of any otherwise appetitive substance. It will be interesting to investigate if low pH can also inhibit low salt mediated responses.

Inhibition of feeding by noxious high pH is not unprecedented in animals. *C. elegans*, a nematode is known to avoid alkaline pH (Wang et al., 2016). Trigeminal neurons in rodents are also activated by a wide range of alkaline pH (Bryant, 2005). But unlike these cases where activation of neurons is associated with avoidance of high pH fruit flies are unique in exhibiting inhibition of neuronal activity.

The exact mechanism by which pH is sensed by the appetitive sense neurons is yet to be understood. One possibility is that there could be some pH sensor expressed in these cells that causes neuronal inhibition in response to noxious pH.

Another possibility is that the receptor might undergo changes in presence of noxious pH causing a decrease in its binding affinity to its ligands. One possible way to distinguish between these two possibilities could be to express individual sugar receptors in ab1c neurons in the antenna and record with sugar solutions of high pH.

Another significant finding that we report here is that high concentrations of salts can inhibit sweet neuron activity. Interestingly, different salts can inhibit sugar neurons to different extent. However, the mechanism by which high concentrations of salts can inhibit sweet neuron activity could not be understood from the study.

A larger panel of salts is required to investigate if the inhibitory property is restricted to certain salts only or is true for any salt.

Additionally, expressing the sugar receptors in ab1c neurons in the antenna and recording with salt-sugar mixtures might be useful in exploring whether this inhibition is through the receptor or through other properties of the neuron itself.

Although flies have separate neurons to identify different aversive stimuli, inactivation of sugar neurons act as a second line of defense to ensure that any toxic or potentially harmful stimuli is strongly rejected. No such phenomenon has yet been reported in any other group of animals. It is however extremely likely that this could be a more conserved phenomenon and needs to be studied in other animals carefully.

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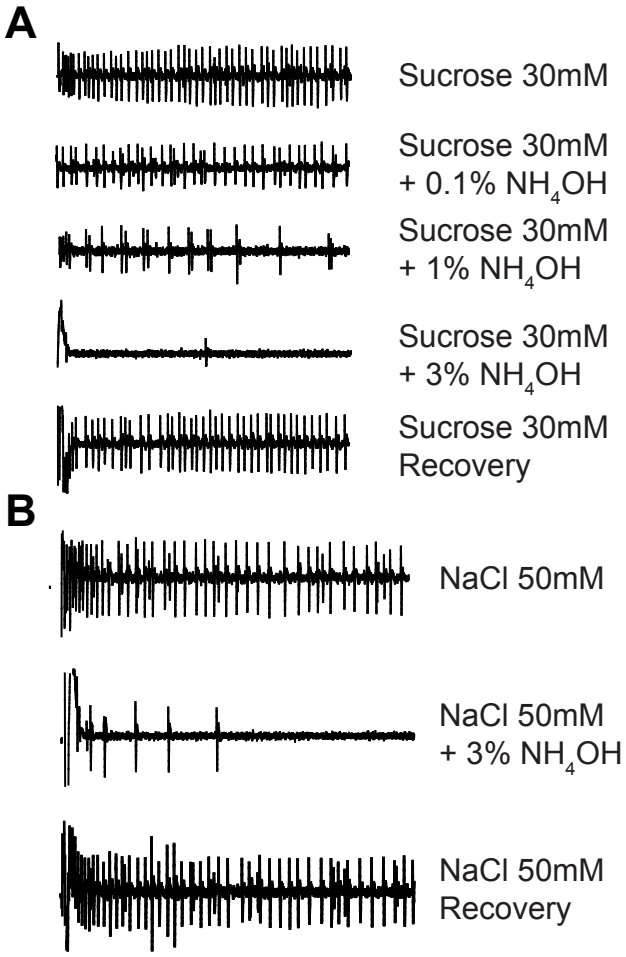
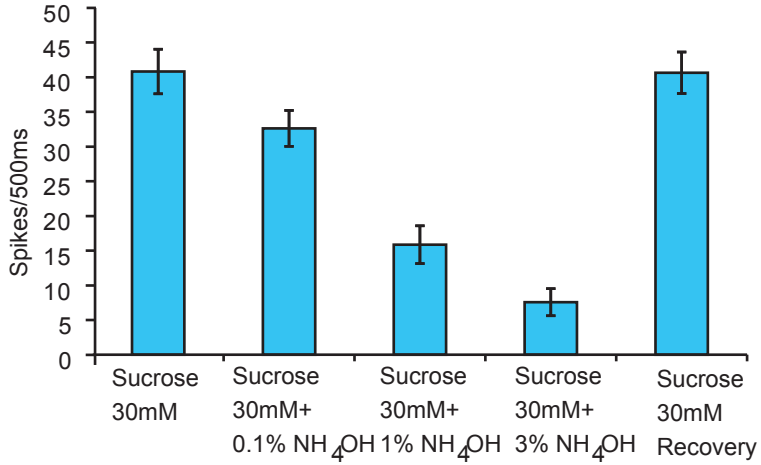

A
Sucrose 30mM
Sucrose 30mM
+ 0.1% NH4OH
Sucrose 30mM
+ 1% NH4OH
Sucrose 30mM
+ 3% NH4OH
Sucrose 30mM
Recovery
B
NaCl 50mM
NaCl 50mM
+ 3% NH4OH
NaCl 50mM
Recovery


Spikes/500ms
0
5
10
15
20
25
30
35
40
45
50
Sucrose 30mM
Sucrose 30mM+ 0.1% NH4OH
Sucrose 30mM+ 1% NH4OH
Sucrose 30mM+ 3% NH4OH
Sucrose 30mM Recovery

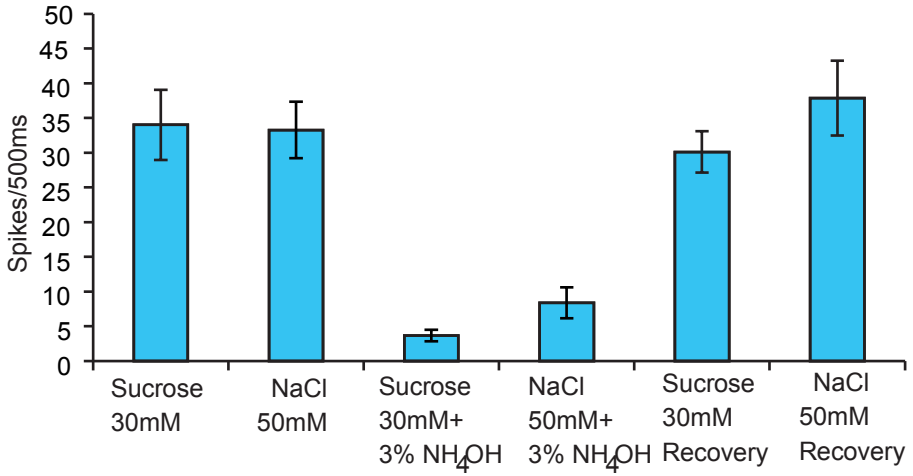

Spikes/500ms
0
5
10
15
20
25
30
35
40
45
50
Sucrose 30mM
NaCl 50mM
Sucrose 30mM+ 3% NH4OH
NaCl 50mM+ 3% NH4OH
Sucrose 30mM Recovery
NaCl 50mM Recovery

Figure 4.1: Ammonia inhibits activity of sweet and low salt taste neurons.

A) Representative traces (left) and mean responses recorded from labellar sensilla (L-type) for the first 500msecs when stimulated with the indicated tastants. 5-7day old wild type(CS) flies were used for the recordings. n= 11 sensilla from 3 flies. Error bars indicate S.E.M.

B) Representative traces (left) and mean responses for the first 500 msecs recorded from L-type labellar sensilla upon stimulation with the indicated tastants. 5-7day old wild type (CS) flies were used for the recordings. n= 8-10 sensilla from 3 flies. Error bars indicate S.E.M.

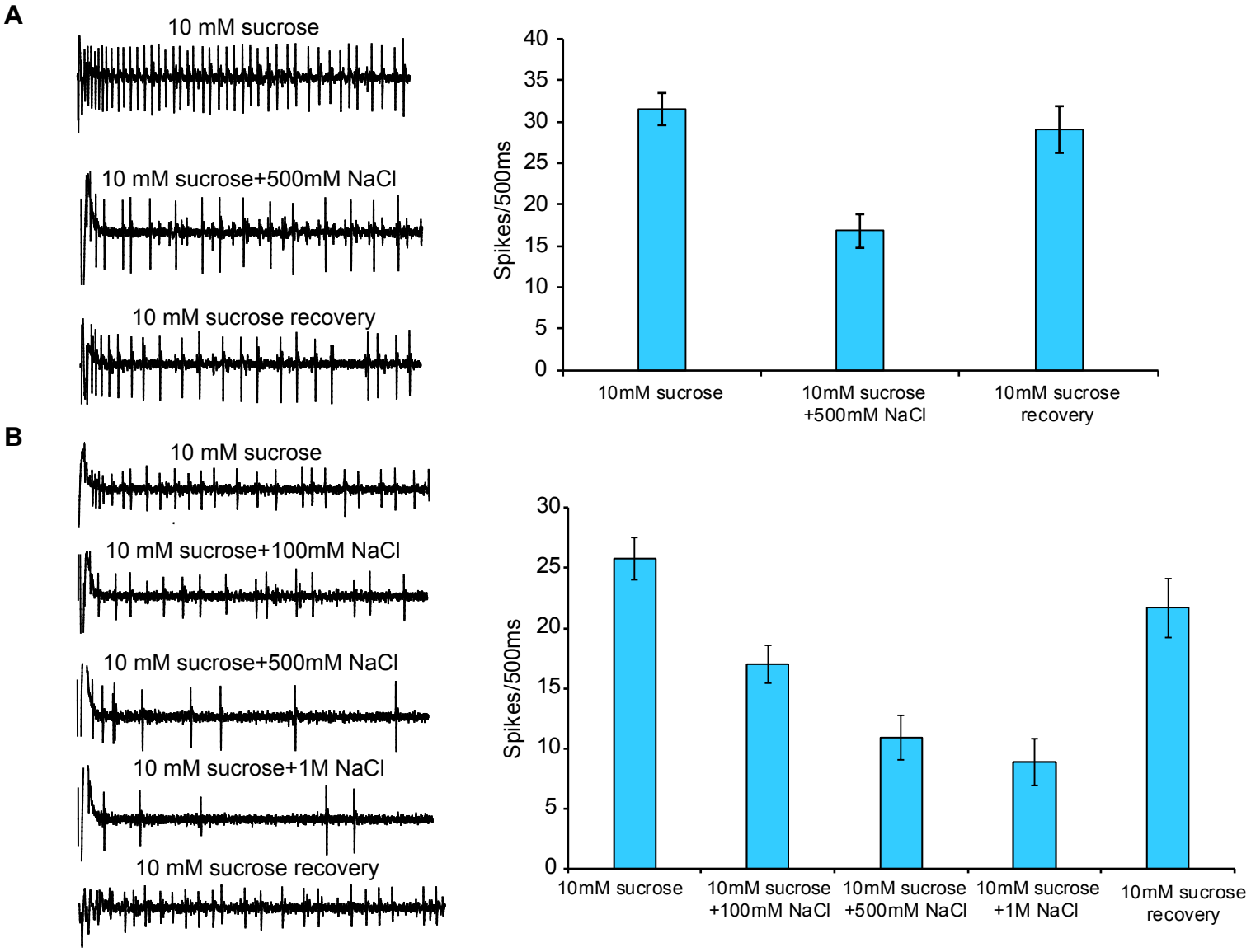

A
10 mM sucrose
10 mM sucrose+500mM NaCl
10 mM sucrose recovery
Spikes/500ms
40
35
30
25
20
15
10
5
0
10mM sucrose
10mM sucrose +500mM NaCl
10mM sucrose recovery
B
10 mM sucrose
10 mM sucrose+100mM NaCl
10 mM sucrose+500mM NaCl
10 mM sucrose+1M NaCl
10 mM sucrose recovery
Spikes/500ms
30
25
20
15
10
5
0
10mM sucrose
10mM sucrose +100mM NaCl
10mM sucrose +500mM NaCl
10mM sucrose +1M NaCl
10mM sucrose recovery

Figure 4.2: High salt concentrations inhibit sweet taste neuron activity.

A) Traces (left) and mean neuronal responses (right) for the first 500ms from labellar L sensilla of female *Ir76b*^{1} flies upon stimulation with the indicated stimulants. All recordings were obtained from 5-7 days old flies. n=13 sensilla from 3 flies. Error bars indicate S.E.M.

B) Traces (left) and mean neuronal responses (right) for the first 500ms from labellar L sensilla of female *Ir76b*^{1} flies upon stimulation with the indicated stimulants. All recordings were obtained from 5-7 days old flies. n=9 sensilla from 3 flies. Error bars indicate S.E.M.

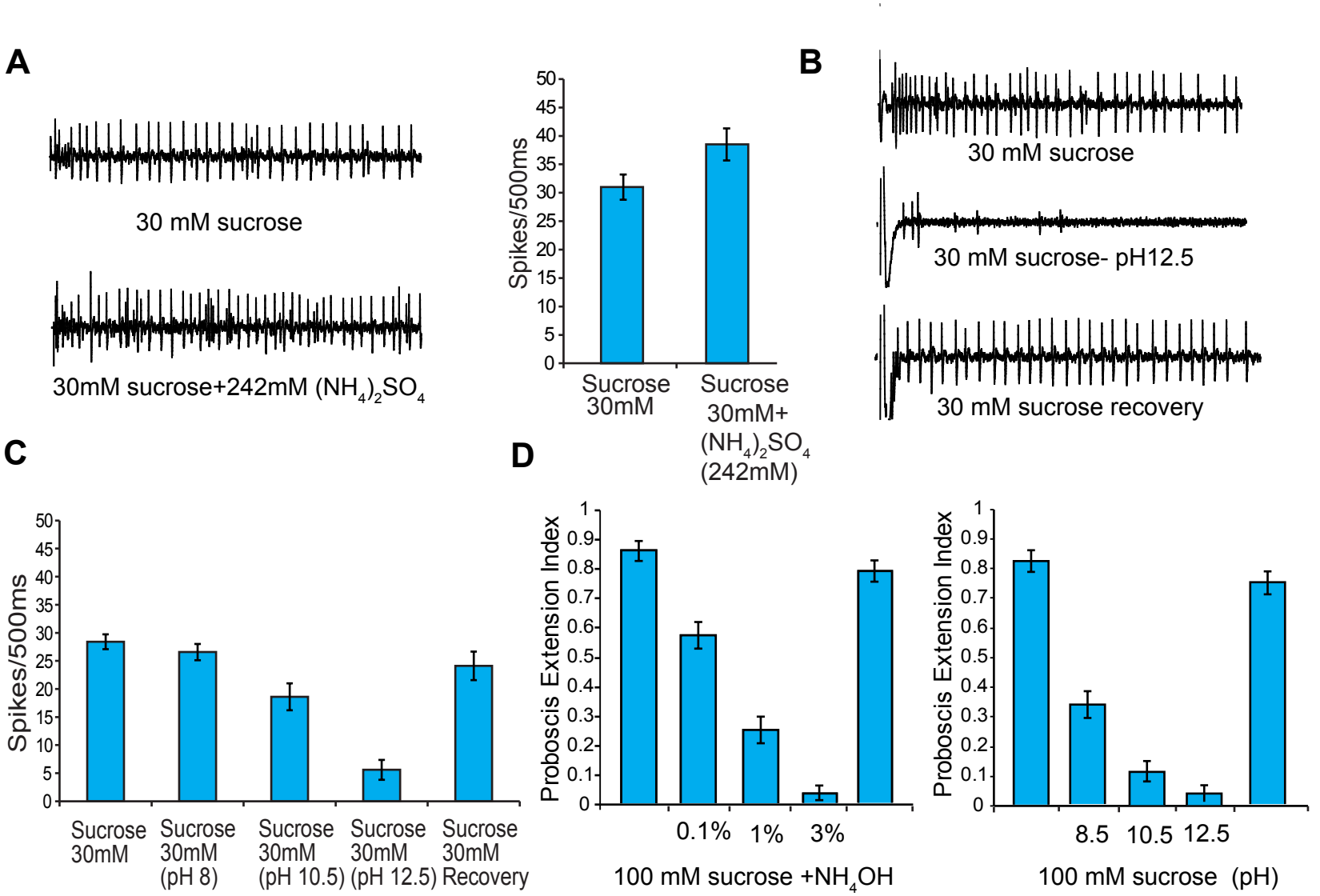

A
30 mM sucrose
30mM sucrose+242mM $(NH_4)_2SO_4$
Spikes/500ms
Sucrose 30mM
Sucrose 30mM+ $(NH_4)_2SO_4$ (242mM)
B
30 mM sucrose
30 mM sucrose- pH12.5
30 mM sucrose recovery
C
Spikes/500ms
Sucrose 30mM
Sucrose 30mM (pH 8)
Sucrose 30mM (pH 10.5)
Sucrose 30mM (pH 12.5)
Sucrose 30mM Recovery
D
Proboscis Extension Index
0.1%
1%
3%
100 mM sucrose +NH_4OH
Proboscis Extension Index
8.5
10.5
12.5
100 mM sucrose (pH)

Figure 4.3: Sweet neuron inhibition by ammonia is caused by high pH of the solution.

A) Representative traces (right) and mean responses for the first 500msecs recorded when labellar L-type sensilla are stimulated with the indicated tastants. All recordings were obtained from 5-7day old wild type (CS) flies. n= 6 sensilla from 2 flies. Error bars indicate S.E.M.

B) Representative traces for the first 500msecs obtained from L-type labellar sensilla upon stimulation with the indicated tastants. Recordings are from wild type(CS) female flies, 5-7days of age.

C) Mean responses for the first 500msecs obtained from the labellar L-sensilla when stimulated with the indicated tastants. All recordings are from 5-7 days old female CS flies. n= 7-17 sensilla from 2-5 flies. Error bars indicate S.E.M.

D) Proboscis extension responses of female CS flies to 30mM sucrose alone as well as mixed with indicated percentage of NH4OH (left) and NaOH to reach the indicated pH. n= 14 flies in both cases. Error bars indicate S.E.M.

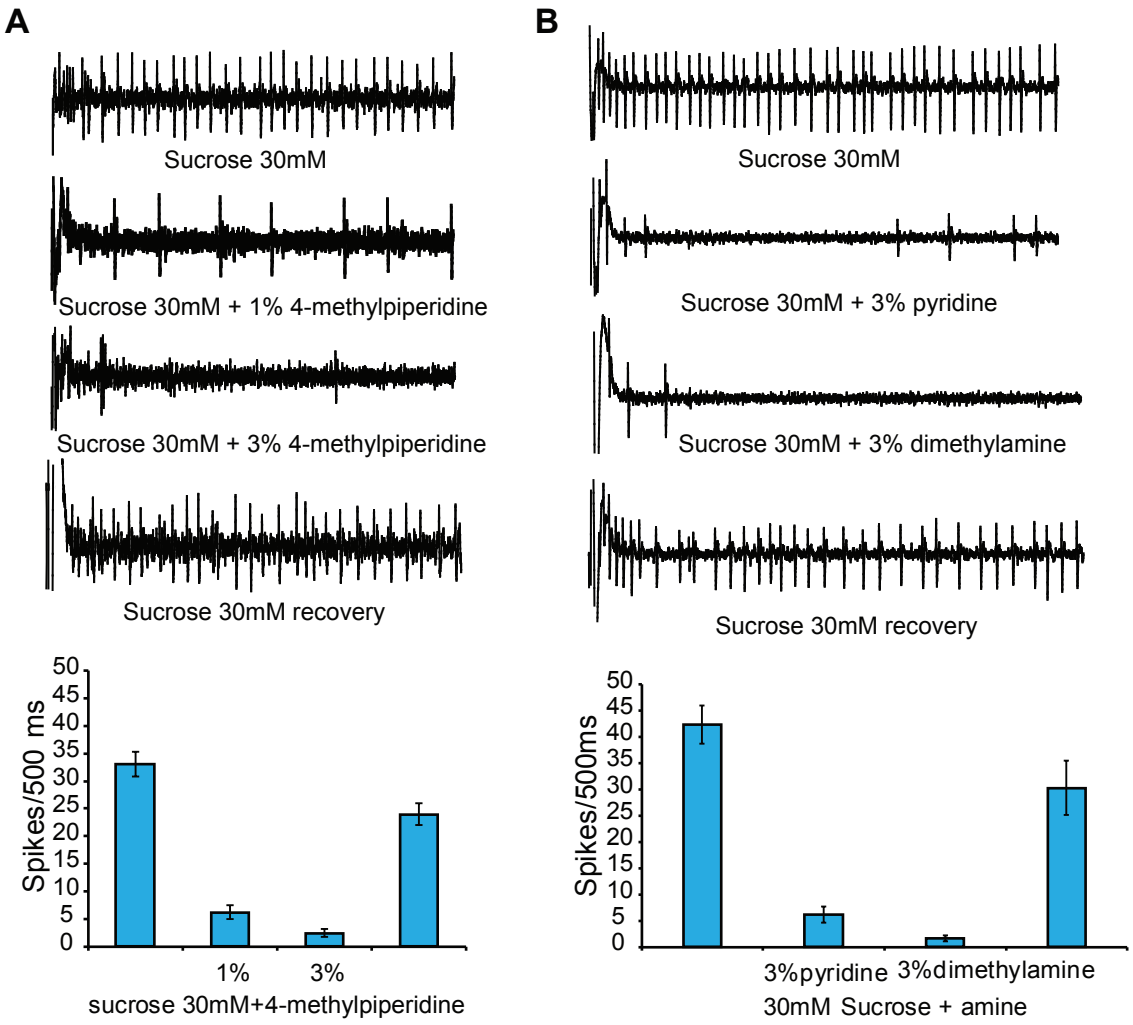

A
Sucrose 30mM
Sucrose 30mM + 1% 4-methylpiperidine
Sucrose 30mM + 3% 4-methylpiperidine
Sucrose 30mM recovery
Spikes/500 ms
50
45
40
35
30
25
20
15
10
5
0
1%
3%
sucrose 30mM+4-methylpiperidine
B
Sucrose 30mM
Sucrose 30mM + 3% pyridine
Sucrose 30mM + 3% dimethylamine
Sucrose 30mM recovery
Spikes/500ms
50
45
40
35
30
25
20
15
10
5
0
3%pyridine
3%dimethylamine
30mM Sucrose + amine

Figure 4.4: Basic amines can also inhibit sweet taste neuron activity.

A) Representative traces (top) and mean responses(bottom) for the first 500msecs recorded when labellar L-type sensilla are stimulated with the indicated tastants. All recordings were obtained from 5-7day old wild type (CS) flies. n= 13-22 sensilla from 3-6 flies. Error bars indicate S.E.M.

B) Representative traces (top) and mean responses(bottom) for the first 500msecs recorded when labellar L-type sensilla are stimulated with the indicated tastants. All recordings were obtained from 5-7day old wild type (CS) flies. n= 10 sensilla from 3 flies. Error bars indicate S.E.M.

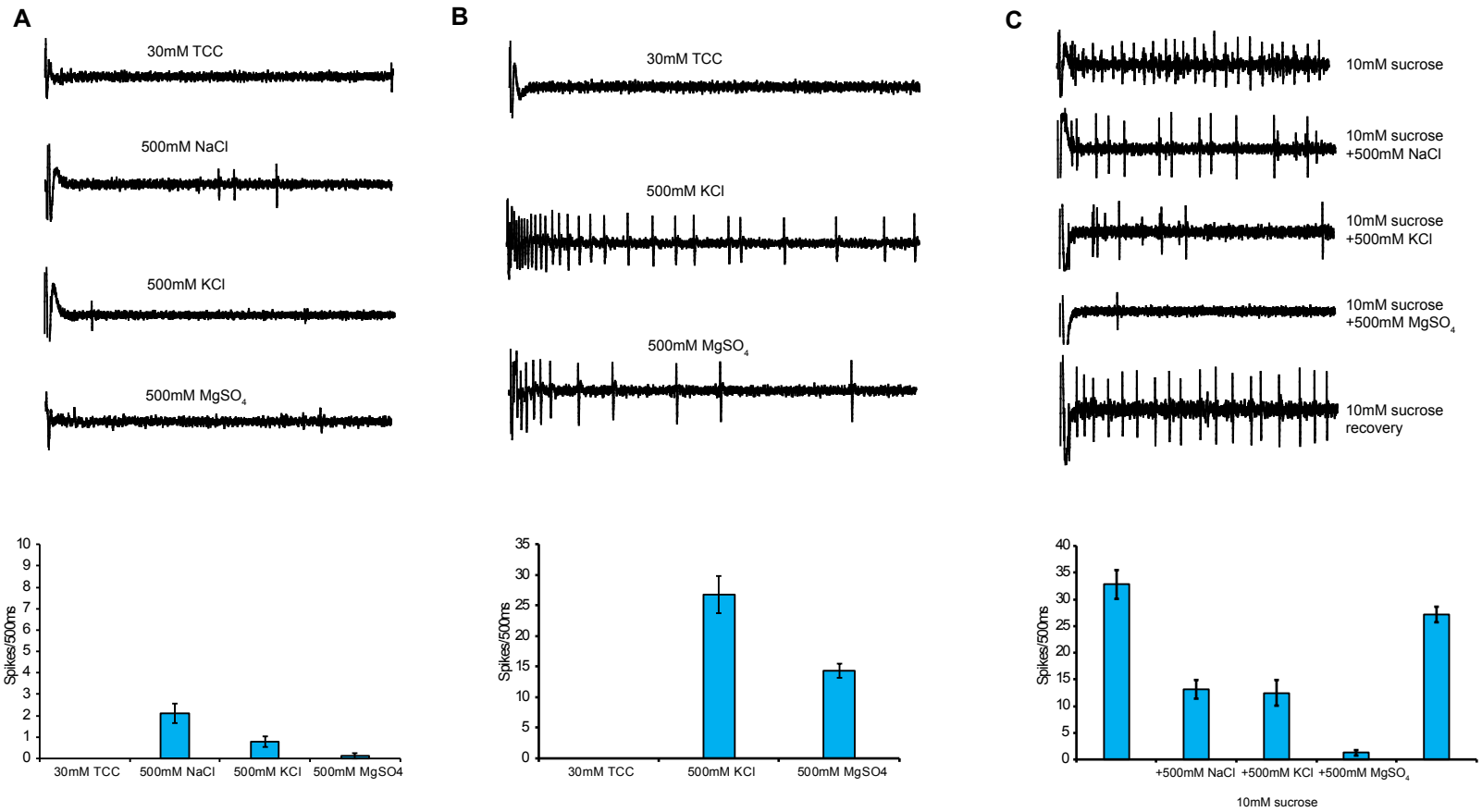

A
30mM TCC
500mM NaCl
500mM KCl
500mM MgSO4
Spikes/500ms
30mM TCC
500mM NaCl
500mM KCl
500mM MgSO4
B
30mM TCC
500mM KCl
500mM MgSO4
Spikes/500ms
30mM TCC
500mM KCl
500mM MgSO4
C
10mM sucrose
10mM sucrose +500mM NaCl
10mM sucrose +500mM KCl
10mM sucrose +500mM MgSO4
10mM sucrose recovery
Spikes/500ms
+500mM NaCl
+500mM KCl
+500mM MgSO4
10mM sucrose

Figure 5: High concentrations of several salts can inhibit sweet neuron activity.

A) Traces (top) and mean neuronal responses (bottom) for the first 500ms from labellar L sensilla of female *Ir76b*^{1} flies upon stimulation with the indicated stimulants. All recordings were obtained from 5-7 days old flies. n=9 sensilla from 2 flies. Error bars indicate S.E.M.

B) Traces (top) and mean neuronal responses (bottom) for the first 500ms from labellar L-sensilla of female *w*^{1118} flies upon stimulation with the indicated stimulants. All recordings were obtained from 5-7 days old flies. n=8-9 sensilla from 2 flies. Error bars indicate S.E.M.

C) Traces (top) and mean neuronal responses (bottom) for the first 500ms from labellar L sensilla of female *Ir76b*^{1} flies upon stimulation with the indicated tastants. All recordings were obtained from 5-7 days old flies. n=10-13 sensilla from 3-4 flies. Error bars indicate S.E.M.

Chapter 5: Conclusion.

SUMMARY OF RESULTS:

The purpose of this study was to ascertain how taste perception between multiple appetitive tastants is modulated by several external and internal factors. To that end, we set out to determine the molecular and cellular basis for both yeast and amino acid taste (**Chapter 2**). Our next step was to characterize changes in feeding preferences as well as behavioral and taste neuron sensitivity in response to macronutrient deprivation (**Chapter 3**). Lastly, we also investigated the mechanisms that mediates these changes. We characterized the inactivation of appetitive neurons by two different repulsive tastants viz. high salt and ammonia that leads to behavioral aversion (**Chapter 4**). We for the first time, characterized amino acid taste in flies and also discovered that *Ir76b* together with *Ir20a* and other factors act as amino acid taste receptors. While characterizing compensatory changes upon macronutrient deprivation we discovered that while deprivation of sugar/yeast enriched diet leads to compensatory changes through a *Dop2R* dependent mechanism, sugar enriched/yeast deprived diet employs a *dilp5* dependent pathway. Additionally, we also report that sugar and salt neuron can be inactivated by high pH while high concentrations of salt can inhibit sweet neuron activity.

Molecular and cellular basis of yeast and amino acid taste in flies:

Modulation of yeast feeding preference by mating is an intriguing phenomenon. Males and virgin females show little preference for yeast, but, on mating, female flies undergo a dietary switch in which they develop a strong preference for yeast (Ribeiro and Dickson, 2010; Vargas et al., 2010).

Here, we report that free amino acids present in yeast forms the basis of yeast preference. We identify a subset of 5 amino acids: serine, phenylalanine, alanine, threonine and glycine that elicit the maximum preference in female flies in binary choice assays. We further identify *Ir76b*+ neurons in the tarsi of the foreleg of females that respond to amino acids using calcium imaging experiments. Consistent with the behavior assays, tarsal neurons from male forelegs show little if any response to amino acids. We further demonstrate that *Ir76b* is necessary for amino acid taste responses. Both cellular response and behavioral preference to amino acids is lost in *Ir76b* mutant flies but are restored when *Ir76b* is rescued in the mutant background. Interestingly, *Ir76b* function is conserved in a mosquito species, *Anopheles gambiae*, suggesting Ir76b may play a role in amino acid detection in mosquitoes as well. Furthermore, we demonstrate that additional Irs beside *Ir76b* are also essential for amino acid taste. Evidences suggest that *Ir20a* combines with *Ir76b*, gating sodium channel activity of *Ir76b*, forming functional amino acid receptor complex together with other yet unknown factors.

Compensatory changes upon macronutrient deprivation:

Taste preferences as well as sensitivity to specific tastants are affected when flies are deprived of macronutrients. Feeding the flies with sugar deprived/yeast enriched food increases their preference and sensitivity for sucrose and at the same time decreases their preference for amino acids. On the other hand, feeding the flies with sugar enriched yeast deprived food increases their preference for amino acids but decreases their preference and sensitivity for sucrose. *Dop2R,* a dopamine receptor, plays an important role in increasing sugar preference and sensitivity as well as decreasing amino acid preference upon feeding the flies with sugar deprived/yeast enriched diet.

Dop2R function is required in the GABAergic neurons for this modulation of sugar preference and sensitivity. A *Drosophila* insulin like peptide, *dilp5,* which is downregulated upon feeding with sugar enriched/yeast deprived diet is required for altered sugar preference and sensitivity but not for altered amino acid preference.

Interestingly, *Dop2R* is not required for altered preference upon feeding with sugar enriched/yeast deprived diet nor is *dilp5* required for altered preferences upon feeding with sugar deprived/yeast enriched diet. Thus, deprivation of different nutrients employs different neuromodulatory pathways that engender compensatory changes in fly taste behavior.

High pH and high salt concentration inhibits sweet neurons:

We discovered that sweet neurons are inhibited by high pH where increasing the pH of a sugar solution further decreases the sugar mediated response. This enables the fly to detect and avoid ammonia and various basic toxic amines that are present in nature. Interestingly, ammonia can also inhibit low salt neurons suggesting that all appetitive taste neuronal pathways are subject to modulation by toxic compounds.

Furthermore, we demonstrate that high concentrations of salt are also capable of inhibiting sugar neuron activity in a concentration dependent manner, indicating that multiple types of aversive stimuli can reduce sweet neuron activity.

DISCUSSION:

In Chapter 2, we discovered that flies can detect multiple amino acids and that preference for specific amino acids in adult flies does not depend on whether they are essential amino acids or not. While three out of five of the most attractive amino acids

were non-essential, some of the essential amino acids like leucine and lysine were among the least preferred. Another study on the amino taste preferences of larval *Drosophila* also finds that amino acid preference in the larvae is not biased towards essential amino acids. This is somewhat surprising since essential amino acids are the ones that cannot be synthesized within the body and needs to be replenished through diet. A previous study suggests that in tsetse flies, the amino acids that elicit the greatest response are the ones that occur in human sweat, which can drive feeding behaviors (DER et al., 1998). Hence, the preference for specific amino acids may be due to the composition of their natural food source. A detailed study of the chemical identity of the food sources of naturally occurring flies is required to validate if food composition drives the fly's amino acid preferences. Intriguingly, except for phenylalanine, the top five amino acids preferred by the larvae (aspartic acid, glutamic acid, cysteine, phenylalanine and asparagine) does not overlap with that of the adult flies (Croset et al., 2016). The differences in amino acid preferences could stem from the differences in chemical ecologies of the different niches occupied by the adult and the larval flies. Interestingly, most of the amino acids eliciting high calcium responses in larvae overlaps with that of the adults (Croset et al., 2016). It indicates that the amino acid receptor complexes might be similar in larvae and adults, but the larvae may ascribe different weightage to amino acid sensitive sensory neurons than the adults. From our studies, there could be more than one amino acid receptor complex and also, the different amino acid sensitive neurons are likely to have different tuning properties. It is likely, to adapt to their particular niches, different sets of amino acid sensitive neurons are given more weightage in the larval brain than in the adult flies.

Preliminary studies suggest that Irs other than *Ir76b* are involved in amino acid responses. However, the exact nature of the amino acid receptor complexes could not be deciphered. Ectopically expressing *Ir20a* and *Ir76b* confers amino acid sensitivity to sweet neurons but *Ir20a* cannot confer amino acid sensitivity when expressed in the salt neurons. This suggests that additional components may be required to form the functional amino acid receptor complex. Several of our candidate Irs viz. *Ir47a* and *Ir56d* were found to be expressed in the sweet neurons via transgenic reporter analysis (Koh et al., 2014). There is a possibility that they form functional amino acid receptor together with *Ir20a* and *Ir76b*.Detailed studies by heterologous expression of the different receptors in different combinations in cell culture will be helpful to identify the different functional amino acid receptor complexes.

A large fraction of amino acid sensitive neurons in the tarsi overlapped with sweet neurons. Hence, it is likely that the amino acid receptor requires participation of sweet Grs. If this is true, it would be the first instance of a functional receptor formed by members off different receptor families. Since Ir76b and Ir20a together are sufficient to confer amino acid sensitivity to sweet neurons, by expressing them in sweet GRNs in different sweet Gr mutant background it is possible to investigate if sweet Grs play a role in amino acid taste. If certain sweet Grs are required for amino acid taste, then if it is mutated, Ir76b and Ir20a will not be able to confer amino acid taste sensitivity.

Also, the number of neurons where *Ir20a* is coexpressed with *Ir76b* is fewer than the number of neurons that respond to amino acids in calcium imaging experiments, implying that there are could be more than one functional amino acid receptors. More experiments, as explained above, are necessary to determine the identity of the different receptors and their tuning properties.

Some earlier studies on post-mating behavior suggest that sex peptide, deposited by the males on the female reproductive tract during mating, directly acts on sex peptide receptors (SPR) present in sensory neurons, thereby modulating their sensitivities (Kubli, 2003). When we compare yeast sensitivity in mated female flies where *SPR* was present or silenced in *Ir76b+* neurons, we did not observe a change in yeast sensitivity. Some recent studies have shown that SPR-SAG (sex peptide abdominal ganglion) axis and octopamine plays an important role in post mating dietary switch of yeast and amino acid preference (Feng et al., 2014; Rezával et al., 2014) . However, a large gap exists in our understanding of how these neuronal circuits eventually alter sensitivity. Given that the male tarsal neurons do not respond to amino acids, a possible explanation could be that those pathways eventually modulate peripheral sensitivity to amino acids. But since virgin flies do not have a highly different amino acid response than the mated females (Ganguly et al., 2017), changes in the relative weightage ascribed to the amino acid neurons in higher order brain centers can be another possibility. The molecular basis of the differences in amino acid peripheral sensitivity between male and female flies is another intriguing question. An answer could lie in the RNAi screen that we conducted to detect the amino acid receptors. Silencing some Irs increases yeast sensitivity in females. A similar analysis in males is required to identify Irs which when silenced increase the yeast sensitivity of male flies. It is probable that these Irs can bind with and further gate the functional amino acid receptors and render them incapable of binding with amino acids.

Although our study does not provide an insight into the mechanism responsible for post mating dietary switch of yeast and amino acid preference it does provide an understanding of the cellular and molecular basis of amino acid taste.

Investigating how these neurons and receptors are modulated by mating, can eventually lead to a thorough understanding of the mechanism governing post-mating dietary switch. Interestingly, preferences for low salt and polyamines, two other tastes requiring *Ir76b* function, also changes upon mating (Hussain et al., 2016; Walker et al., 2015). This indicates the possibility that *Ir76b* neurons are somehow modulated by mating and subsequent physiological changes.

We further find that feeding sugar deprived/yeast enriched food decreased amino acid preference in flies but increased their preference and sensitivity for sucrose. Also, feeding the flies with yeast deprived/ sugar enriched diet increased their preference for amino acid but decreased their preference and sensitivity for sucrose. Several studies have showed increase in yeast preference upon yeast deprivation (Liu et al., 2017; Ribeiro and Dickson, 2010; Vargas et al., 2010). One particularly interesting study by Liu et al., 2017, showed increased yeast feeding and also decreased sucrose feeding in flies upon yeast deprivation. They identified a subset of dopaminergic neurons, the DA-WED neurons, to be responsible for these changes. Downstream *Dop1R*+ neurons mediate decreased sugar preference while downstream *Dop2R*+ neurons are required for increased preference. Thus, it is possible that to keep total caloric intake constant, increased appetite for one nutrient is accompanied with decreased appetite for other nutrient types. Mating increases preference and appetite for amino acids and yeast in flies but whether it leads to decreased preference for sugar as well has not been investigated. Comparing the neuronal activity of sweet taste neurons in age-matched virgin and mated females will provide an answer to this question.

A decrease in sugar sensitivity of the mated females would indicate that sugar and amino acid preferences are antagonistic in dietary switch as well suggesting that this is a more general phenomenon. While, if there are no changes it the antagonistic nature of sugar and amino acid preferences is typical of nutrient imbalance paradigms, and dietary switch on mating does not engender same effects.

We find that two different pathways account for altered taste responses following macronutrient deprivation. While modulation of taste responses upon feeding with sugar deprived/ yeast enriched food require *Dop2R* function; feeding with sugar enriched/yeast deprived diet employs *dilp5* a Drosophila insulin like peptide for the same purpose. In both cases we observe changes in peripheral sensitivity which is a novel finding. Most previous studies reported changes in presynaptic activity of sweet neurons upon starvation but denied any changes in spike frequency obtained from tip recordings (Inagaki et al., 2012). However, one particular study also reported increased spike frequency of the sweet neurons in response to sucrose when the flies were fed with sucralose (Wang et al., 2016). Sucralose being a non-nutritive sweet compound, the group conducting this study suggested that these flies were subjected to sweet/energy imbalance (Wang et al., 2016) while other groups have refuted the claim, providing evidences that the flies were essentially starved (Park et al., 2017). Irrespective of whether the flies were sweet/energy deficient or starved, changes in spike frequency of the neurons support our observation that excitability of the sensory neurons can be modulated by nutritional status. Earlier studies investigating changes in neuronal responses upon starvation recorded from the sugar neurons with only one sugar concentration i.e. 100mM of sucrose, which is quite high and the response of the flies might have plateaued at that concentration (Inagaki et al., 2012).

A more careful recording with a range of sugar concentration, like what we have done in Chapter 3, might have been more informative in understanding the changes.

How peripheral responses are modulated upon nutrient deprivation is still not well understood. We found that *Dop2R* function in GABAergic neurons is required for increased sucrose sensitivity upon feeding. We hypothesize that sugar deprivation leads to increased dopamine secretion which through Dop2R mediated signaling pathway decreases GABA secretion by GABAergic neurons onto the sweet neurons. Indeed previous studies have demonstrated that sweet neuron activity could be controlled by GABA signaling acting through GABA-B-R2 receptor (Jeong et al., 2016; Pool et al., 2014). However, we did not find any role of *GABA-B-R2* in increased sugar sensitivity upon sugar deprivation. The preliminary results indicated that *GABA-B-R3* might be important in this phenotype, but more experiments are required to validate a role of *GABA-B-R3*. An obvious approach will be to compare the performances of *GABA-B-R3* mutant flies and flies where *GABA-B-R3* is rescued only in sweet taste neurons in the mutant background, in behavior assays and electrophysiological recordings. If *GABA-B-R3* is required for compensatory changes following feeding with sugar deprived/ yeast enriched diet, no changes will be observed in the mutant upon feeding with sugar deprived diet but the changes will be restored in the rescue. Another possibility is that multiple sub-types of GABA receptors expressed in the sweet neurons modulates sweet neuron sensitivity upon sugar deprivation and silencing one subtype is not sufficient to alter the phenotype.

dilp5 is required for compensatory changes following feeding with sugar enriched/yeast deprived diet. *dilp5* presumably exerts its action via insulin receptor (InR).

However, the cellular subtype where *InR* function is required for modulation of taste is yet to be studied.

Interestingly, although mating and nutritional imbalance both changes fly's taste responses they appear to employ different pathways. While dietary switch following mating recruits a SPR and octopamine dependent pathway no such effect is observed in nutrient deprivation paradigms. Furthermore, deprivation of different nutrition engenders compensatory changes through different pathways. This probably serves to maintain a stricter control on diet and to ensure intake of appropriate diet upon different physiological conditions.

Intriguingly, peripheral sweet neurons are modulated also by presence of harmful contaminants in the food. We show that both high pH and high salt can inhibit sweet neuron activity. Additionally, high pH also inhibits appetitive salt neuron activity. Together with previous reports of inhibition of sweet neurons by bitter and acids it appears that sweet neurons are inhibited by any known aversive compound. Thus, interestingly, sweet neurons play a very important role in fly taste. They can detect and differentiate between various sweet compounds, a subset of them can detect amino acids and they are also inhibited by various toxic or noxious chemicals. Thus, even in the absence of the aversive neurons they are able to guide the flies to an appropriate feeding behavior i.e. ingesting nutritious food and avoiding food sources contaminated with toxic chemicals. This raises the possibility that more ancient groups of arthropods had only sugar neurons and other taste neuron types emerged latter as adaptive measures to survive in more specialized environments. However, more investigation is required to validate this hypothesis.

Also, whether the neuronal inhibition occurs through inactivation of the sweet Grs themselves or through any other means is not understood. An approach identical to the one reported by Freeman et al., 2014, will be useful in investigating this. Antennal ab1c neurons expressing single sweet Grs can be stimulated with sugar-aversive compound mixtures. A decrease in activity compared to that elicited by the sugar alone would indicate a role of the receptor itself on the inhibition.

IMPORTANCE OF THE STUDY:

We have identified the cellular and molecular basis of a novel taste category in flies. Blood feeding behavior in mosquitoes, like yeast feeding of flies, is an outcome of post-mating dietary switch. Amino acid taste receptor is expected to play an important role in the blood feeding and disruption of the amino acid receptor can hinder blood feeding. Since we discovered that amino acid co-receptor property of Ir76b is functionally conserved in mosquitoes, it can be used as a molecular tool to impair blood feeding. Additionally, ligands binding to other Ir members of the receptor complex can be utilized to prevent mosquitoes from blood feeding. We have also discovered that macronutrient deprivation leads to compensatory changes via two different pathways. Incidentally dopamine and insulin are both present in human and they have been implicated in many metabolic pathways. Thus, the nutrient deprivation paradigm can be developed to serve as a model for studying nutritional imbalances in men. Last but not the least, we have identified novel categories of tastants that can inhibit sweet neurons. This study can be used to develop antifeedants that can prevent agricultural crops from being damaged by insect pests. Something like a salt or a non-toxic amine will provide a much better and safer option to use than toxic insecticides.

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