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THE ROLE OF CANONICAL AND NOVEL TASTE NEURONS IN AMINO ACID FEEDING IN DROSOPHILA MELANOGASTER

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ABSTRACT

Mammals and insects alike depend on foodstuffs as an essential source of proteins and their constitutive amino acids (AA), several of which cannot be synthesized by the organism. AA consumption by *Drosophila melanogaster* plays a crucial role in a variety of behaviors that impact the animal's survival and fitness, including feeding, mating, and egg laying. Underlying neural circuits that dictate such behaviors begin with the activation of distinct subsets of gustatory receptor neurons (GRNs) in the fly labellum, which subsequently transmit taste information to the brain for its integration. However, the mechanisms by which GRNs detect AAs to elicit behavioral responses in *Drosophila* are not fully characterized. The present study employs behavioral paradigms that evaluate the external and internal mechanisms of tryptone taste detection to describe the roles of three distinct GRN classes within the labellum in AA sensing. Through taste and feeding assays combined with chemogenetics, optogenetics, and chronic neuronal silencing, we describe the canonical roles of the *Gr64f*-expressing sweet GRNs and *Gr66a*-expressing bitter GRNs in AA feeding and contrast these circuits to an undescribed, novel set of *IR94e*-expressing GRNs. This work supports a combinatorial coding paradigm of taste coding in which one chemical ligand activates parallel sensory circuits to expand the range of the sensory experience in the fly.

Keywords: gustatory receptor neuron, amino acids, combinatorial coding, tastant, umami, tryptone

INTRODUCTION

Feeding is vital for the survival of all animals. This behavior varies tremendously between organisms when considering factors such as meal size, food preference, and manner of obtaining foodstuffs, but even between species its fundamental purpose is conserved; food provides the nutritional resources necessary for growth, repair, and survival. The requirement to consume beneficial, energy-dense nutrients through the diet has led to the evolution of chemosensory pathways that allow animals to contextualize their environment (Melcher et al., 2007).

Neurons expressing chemosensory receptors facilitate the convergence of internal metabolic cues and external signals from surroundings, enabling an organism to generate the specific and appropriate behavioral response to those stimuli. Chemical stimuli in food activate gustatory neurons in taste organs, which constitute the basis of the distinct taste modalities that promote the consumption of beneficial nutrients and avoidance of toxic substances (Scott, 2018). Taste neurons can be activated by a wide variety of chemical ligands, and their capacity to discriminate between diverse taste stimuli drives feeding behaviors (Thorne et al., 2004). There is evidence to suggest that chemosensory systems operate through the combinatorial coding of neurons, in which unique combinations of receptors are activated upon the binding of different chemical stimuli to increase the complexity of the sensory experience (Chen et al., 2019; Jaeger et al., 2018; Faure, 2009; Malnic et al., 1999). Still, the mechanisms by which the nervous system combinatorially encodes this sensory information to confer these behaviors are not fully understood.

The unique features of the model organism *Drosophila melanogaster*, including the capacity for its genetic manipulation, well-characterized neural circuitry, and definitive

behavioral paradigms, establish it as a prime candidate for research of the gustatory system and its combinatorial coding properties (Scott, 2018). There are 5 classes of gustatory receptor neurons (GRNs) housed in hair-like sensilla on the *Drosophila* labellum, which can be considered a functional homologue of the mammalian tongue (Amrein & Thorne, 2005). GRNs in *Drosophila* are characterized by their constituent taste receptors, which are divided into two classes: gustatory receptors (GRs) and ionotropic receptors (IRs). The majority of receptors in both classes are believed to be ligand-gated ion channels embedded in the neuronal membrane (Ma et al., 2024; Benton, 2008). GRNs in the fly labellum, at the tip of the proboscis, transmit taste information to the subesophageal zone (SEZ) of the central brain, where it can be integrated to inform feeding behavior (Fig. 1A, B) (Koh et al., 2014, Jaeger et al., 2018).

Activation of distinct classes of GRNs elicits distinct behavioral responses. Neurons corresponding to "sweet" taste express the sugar receptor *Gr64f* and invoke attractive behaviors upon activation, whereas "bitter" neurons express the *Gr66a* receptor for the activation of noxious chemicals to drive feeding aversion in the fly (Marella et al., 2006; Jiao et al., 2008; Shim et al., 2015). Conversely, the GRN population expressing *Ionotropic Receptor 94e* (IR94e) has yet to be fully characterized. Gr64f, IR94e, and Gr66a GRNs comprise 3 of 5 distinct GRN populations within the labellum, and the combined activation of these receptors by discrete chemical tastants contributes to the rich diversity of perceptive information in the fly (Fig. 1C). Thus, a comprehensive description of IR94e GRN function is required to fully understand the neural substrates of feeding behavior in *Drosophila*.

IR94e-expressing neurons were first described as a novel GRN population that does not overlap with other labellar GRN populations corresponding to sweet, bitter, high salt, or water perception (Jaeger et al., 2018). Complete characterization of the combinatorial coding

mechanisms of labellar GRNs in the fly necessitates identification of the taste stimuli that IR94e GRNs are activated by, thus prompting initial investigations to quantify neuronal activation. Significant IR94e GRN activation has been observed following stimulation with an amino acid (AA) mixture known as tryptone, derived from a digest of the casein protein (Guillemin et al., 2024). AAs have also been shown to activate both Gr64f and Gr66a GRNs (Aryal et al., 2022).

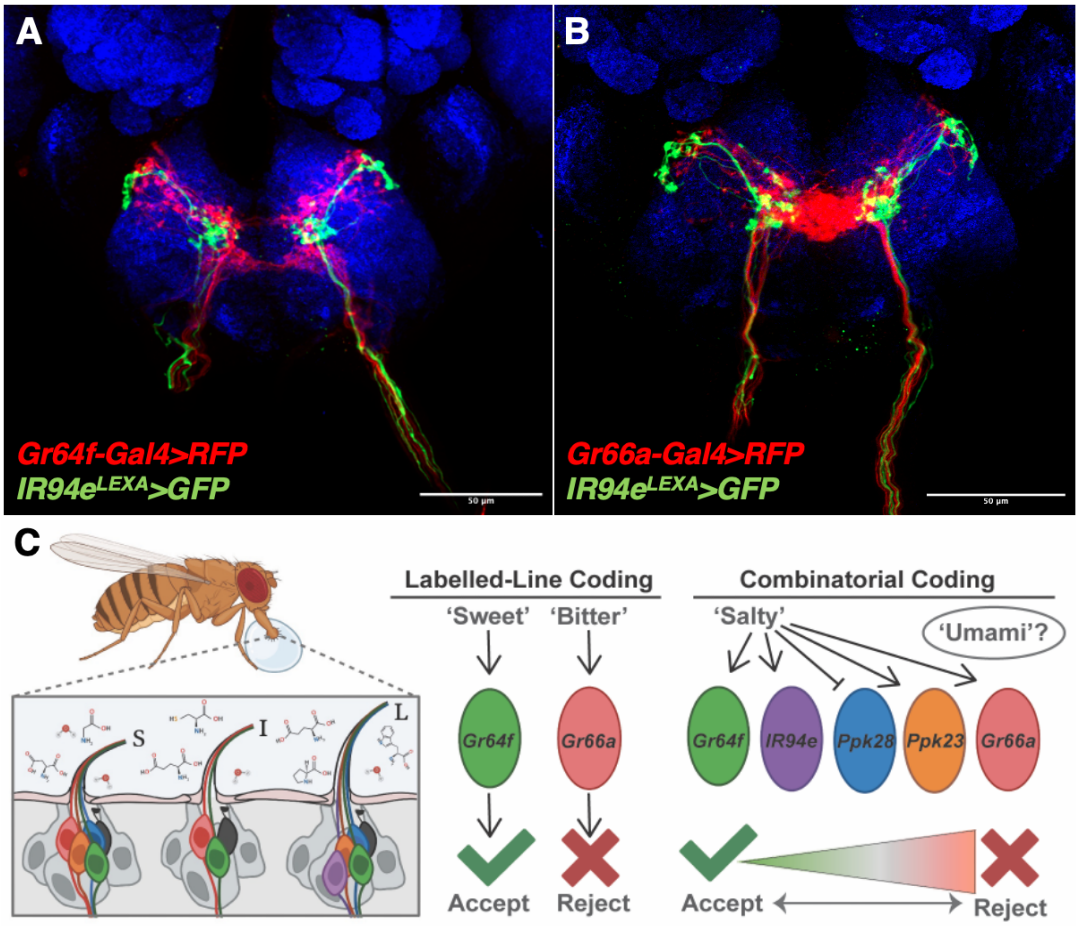

Figure 1: Populations of GRNs that project to the SEZ combinatorially encode taste information to enhance the sensory experience of the fly
(**A-B**) The axon terminals of IR94e GRNs expressing GFP co-labeled with canonical sweet Gr64f (**A**) and bitter Gr66a (**B**) GRNs expressing RFP in the SEZ of the brain. Scale bars = 50 µm. Adapted from Guillemin et al. (2024). (**C**) Model demonstrating the labelled-line and combinatorial coding paradigms of GRN activation. Labellar GRNs express ligand-gated receptors which are activated by chemical ligands in food. The combined activation of multiple GRN circuits expands the range of feeding behaviors that fall between the attraction and aversion spectrum.

L-amino acids are the building blocks of proteins and are thus required for the proper function of virtually all cellular processes (Wu, 2021). Ten of the twenty common AAs cannot be synthesized by *Drosophila* and mammalian organisms alike and must be supplemented through dietary proteins (Park & Carlson, 2018). The taste of AAs is termed umami, meaning "delicious savory taste" in Japanese. Umami taste is characterized by the high prevalence of glutamate, found in many foodstuffs including seafoods, meats, and vegetables (Ghirri & Bignetti, 2012).

In a low-protein state relative to other macronutrients such as carbohydrates and fats, animals will consume available food so as to prioritize regaining proportionate protein levels. This phenomenon, termed the protein leverage hypothesis, precedes the overconsumption of concomitant sugars and fats which are implicated in metabolic disease (Gosby et al., 2014; Ahrentløv et al., 2024). Interestingly, diets composed of higher protein and lower carbohydrates and fats yield less energy for the organism than that of the reverse, a factor which raises inquiry about the metabolic factors that drive protein consumption in animals (Simpson & Raubenheimer, 2005). The aforementioned mix of AAs can be used as a chemical stimulus to investigate how IR94e, Gr64f, and Gr66a GRNs encode the behavioral responses to the umami taste.

In the present study, we provide evidence for the distinct contributions of individual GRN populations that are integrated in the brain to inform feeding behaviors and demonstrate the dose-dependent role of these sensory neurons in AA detection and consumption. Through genetic methods of neuronal activation and inhibition, we found that the combinatorial activation of Gr64f, Gr66a, and IR94e GRNs predicts flies' preference for foods with moderate concentrations of AAs. The canonical sweet and bitter GRNs were involved in attraction and

aversion to AAs, respectively, as expected. Interestingly, the novel IR94e GRNs contributed to AA avoidance at low-to-moderate concentrations, but this flipped with the consumption of high concentrations. These findings demonstrate a unique evolutionary adaptation in which multiple neuronal circuits can be activated by ligands in foodstuffs, expanding the breadth of sensory information that is then able to dictate feeding behaviors in the fly to promote survival.

MATERIALS AND METHODS

Flies

Experimental flies were kept on regular cornmeal food at 25°C in 60% relative humidity. Mated female flies between 2-10 days old were used in all experiments. The following fly stocks were used for experiments with final genotypes indicated in the figures and legends: *W[1118], Gr64f-Gal4, Gr66a-Gal4, IR94e(VT)-Gal4, UAS-VR1, UAS-*Gt*ACR1, UAS-Kir2.1.* A depiction of the binary expression systems used to generate flies in experiments for this study is depicted in Fig. 2A.

Feeding assays

Dye-based binary choice: flies were collected and subjected to the assay in groups of 10. Binary choice was performed as previously described (Fig. 2B) (Guillemin et al., 2024, Jaeger et al., 2018). Flies were transferred from food-deprivation vials containing 1% agar (if in food-deprived group) or from cornmeal food (if in fed group) to vials containing 10 µL drops of a dyed 1% agar solution. The solutions contained an experimental or control tastant mixed with either blue (0.125 mg/mL Erioglaucine, FD and C Blue#1) or red (0.5 mg/mL Amaranth, FD and C Red#2) dye. Colors were balanced between experimental solutions. Flies fed for 2 hours in the dark at 29°C before freezing at –20°C. Abdomen color was scored as red, blue, purple, or no

color. Preference index was calculated as $\frac{(\#\text{ of flies labeled with food 1 color}) - (\#\text{ of flies labeled with food 2 color})}{\text{total \# of flies that fed}}$. The number of flies eating either option was calculated as a percent: $\frac{\#\text{ of flies labeled blue,red,or purple}}{\text{total \# of flies in vial}} \times 100$.

Optogenetic PER: three days prior to the assay, flies were collected and placed on all-*trans*-retinal (ATR) or ethanol mixed into normal food for two days. One day prior to the assay, flies were transferred to starvation vials containing ATR or ethanol mixed into 1% agar for 24h. Vials were covered with aluminum foil to minimize exposure to light and kept at 25°C. Flies were mounted for labellar PER assay with a mouth pipette into 200 µL pipette tips cut so only the heads were exposed (Fig. 2C). Flies were mounted in a dark room with minimal light under a dissection microscope, permitted to recover in a humidity chamber for ~1 hour, and water satiated. Water was presented as the first stimulus as a negative control (to ensure flies did not exhibit PER to water). The experimental solutions were presented second in combination with a green LED powered by a 9V battery (~425 µWatts), held directly over the fly labellum. The final stimulus was 1 M sucrose, a positive control to ensure that flies were able to exhibit PER. The efficacy of optogenetic inhibition was validated by confirming the suppression of PER to 1 M sucrose in *Gr64f*>Gt*ACR1*, ATR+ flies in the presence of the green LED. Flies that responded to H_2O stimulation prior to stimulation with the experimental solutions were excluded. Flies that did not respond to 1 M sucrose after stimulation with the experimental solutions were excluded.

Statistical analysis

All statistical tests were performed in GraphPad Prism 10 software. Specific tests are indicated in the figure legends along with the number of replicates, which were generally chosen

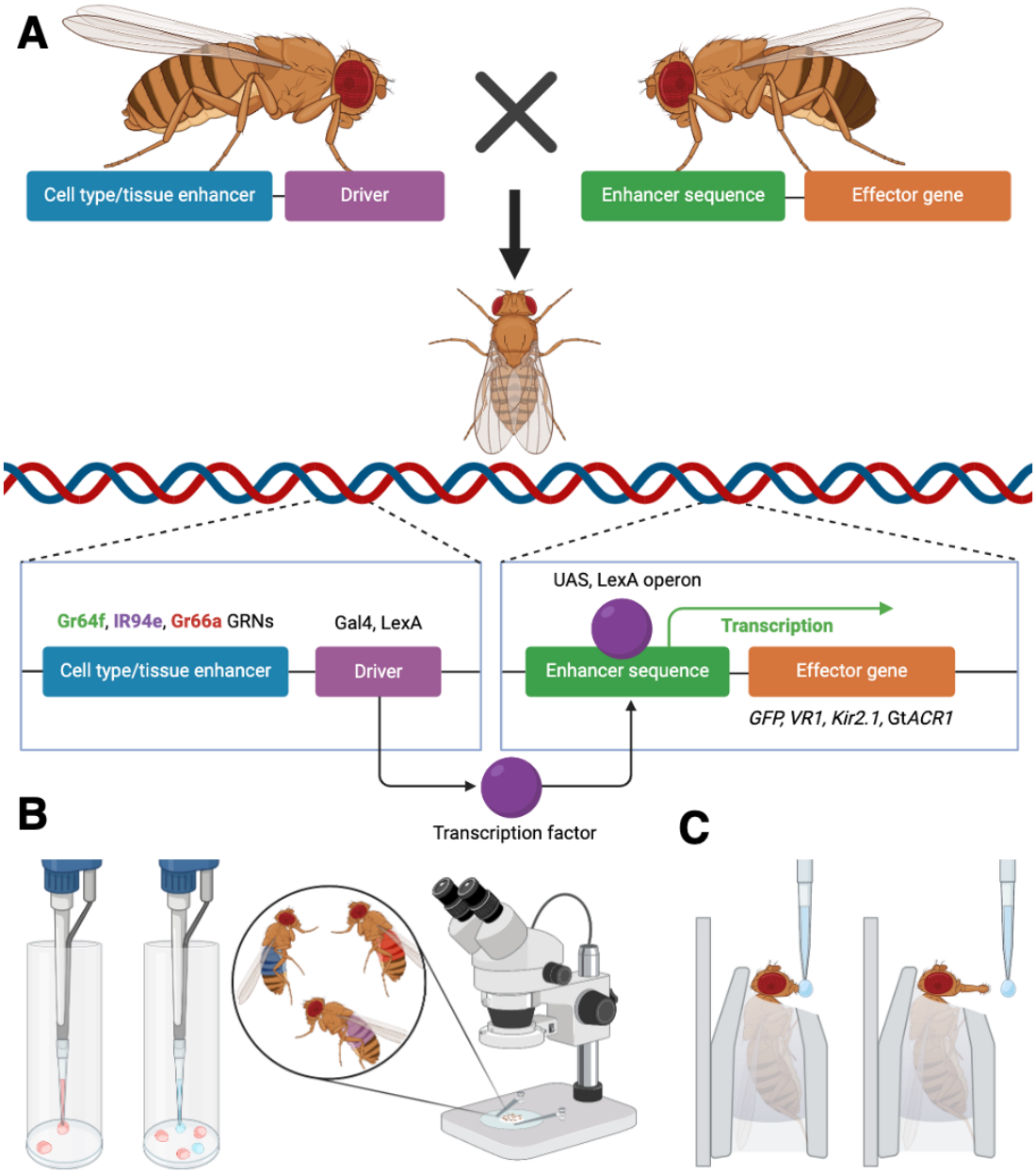

Figure 2: Genetic and behavioral methods for investigation of the neuronal circuits that inform feeding behavior in *Drosophila*

(**A**) Depiction of the manipulation of binary expression systems within the fly genome. Genetic manipulations for all behavioral assays used in this study were achieved through the use of the *Gal4/UAS* system. (**B**) Depiction of the dye-based binary choice experiment. The abdomen color was used to measure flies' preference for ingestion of the experimental tastants. (**C**) Depiction of the PER assay. Proboscis extension, or lack thereof, was used to measure flies' preference for foodstuffs following external detection of the experimental tastant.

based on variance and effect sizes in accordance with previously-established literature using the same assays. Asterisks indicate *p<0.05, p**<0.005, p***<0.0005, p****<0.0001.

RESULTS

The range of attraction and aversion in *Drosophila* feeding is determined by activation of different GRN populations

To investigate the combined roles of Gr64f, IR94e, and Gr66a GRNs in amino acid feeding, it was first necessary to determine the behavioral changes that follow the activation of each cell type. We expressed the mammalian-derived vanilloid receptor (VR1), a ligand-gated cation channel that is activated by capsaicin, the natural chemical found in capsicum peppers which produces the perception of spice (Caterina et al., 1997). GRNs that express VR1 become artificially activated when the fly comes into contact with capsaicin, thereby enabling a way to examine the behavioral response to direct chemogenetic activation of distinct neuronal populations (Marella et al., 2006). We crossed *Gal4* drivers for Gr64f, IR94e, and Gr66a GRNs with *UAS-VR1*, resulting in the expression of VR1 in defined sets of GRNs (Fig. 2A). To determine behavioral valence, we employed dye-based binary preference assays to examine preference for capsaicin or vehicle. As expected, *Gr64f>VR1* flies demonstrated a significantly increased preference for capsaicin and a higher percentage eating compared to genetic controls, a phenotype consistent with sugar attraction (Fig. 3A). *IR94e>VR1* flies demonstrated a weakly negative preference toward the vehicle, with a significantly higher number of flies consuming the vehicle compared to that of genetic controls, indicating an interest in the non-capsaicin option. Interestingly, the percentage of *IR94e>VR1* flies consuming any option was slightly increased (Fig. 3B). Predictably, *Gr66a>VR1* flies strongly preferred the vehicle, consistent with bitter

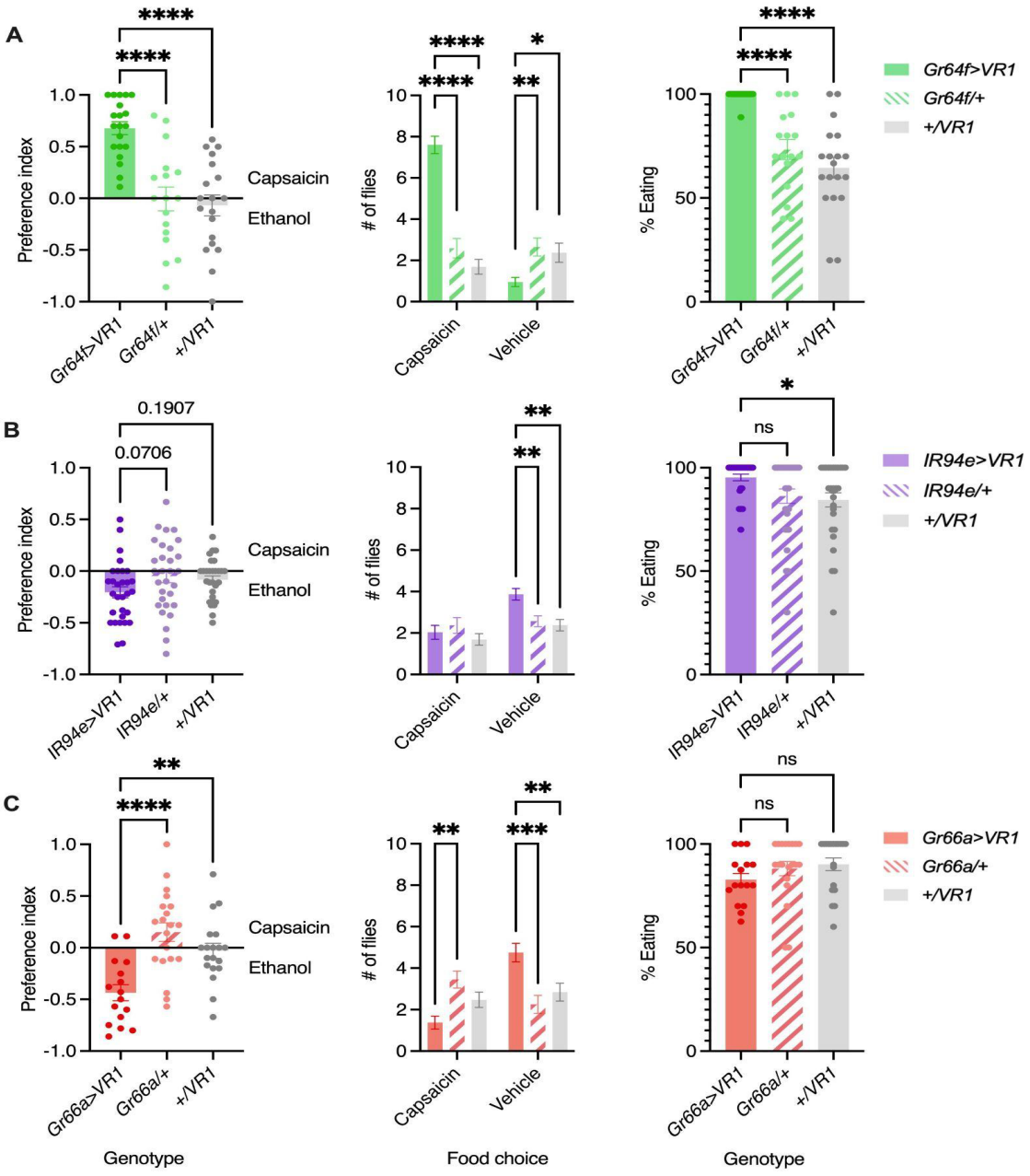

Figure 3: Chemogenetic activation of labellar GRNs demonstrates characteristic behavioral outputs

(**A-C**) Chemogenetic activation of Gr64f GRNs (**A**), IR94e GRNs (**B**), and Gr66a GRNs (**C**) using VR1 and 100 µM capsaicin versus vehicle (0.07% ethanol) in a dye-based binary choice assay. Preference index (left), number of flies consuming capsaicin vs. vehicle (middle), and percentage of flies eating (right) are shown. All mated females, n=20-30 groups of 10 flies per genotype. ns = p>0.20, trending p values indicated, p*<0.05, p**<0.01, p***<0.001, p****<0.0001 by way of one-way ANOVA with Dunnett's posttest (preference index, % eating) or two-way ANOVA with Tukey's posttest (number of flies eating). Each graph depicts the mean ± SEM.

aversion (Fig. 3C). The number of Gr66a-activated flies consuming the vehicle was markedly higher than that of the genetic controls, and the percentage of flies consuming any option trended downward, although not statistically significant (Fig. 3C). Taken together, these results suggest that feeding behaviors in *Drosophila* are mediated through the activation of individual GRN circuits, and their characteristic behavioral responses can be used to determine the magnitude of their roles in amino acid feeding.

Amino acid feeding in *Drosophila* is driven by internal and external factors

To achieve a comprehensive understanding of amino acid feeding in *Drosophila*, we first performed an assay to determine the preference for low (1%), moderate (5%), and high (10%) concentrations of the AA mixture tryptone (w/v) depending on their hunger state. We conducted a dye-based preference assay in which *W[1118]* flies which have the same genetic background as the genetically modified flies used in our experiments. Flies were kept on the standard diet (fed group) or deprived of regular food and only given water for 24h prior to the experiment (food-deprived group). Fed flies demonstrated a significant decrease in preference for tryptone compared to food-deprived flies at all concentrations (Fig. 4A). The difference between groups was minimized at 5% tryptone, the concentration believed to be the most attractive (Guillemin et al., 2024). 10% tryptone yielded the lowest preference for both groups (Fig. 4A). Unsurprisingly, the percentage of fed flies consuming any option was significantly lower than food-deprived flies for all tryptone concentrations (Fig. 4B). Again, this difference was minimal at 5% tryptone, but most extensive at 1% tryptone, potentially because this concentration may not exceed far past the threshold of AA detection in the fly. Overall, these data support the notion that AA consumption in flies is dose-dependent and mediated by internal state. Furthermore, these results provide a

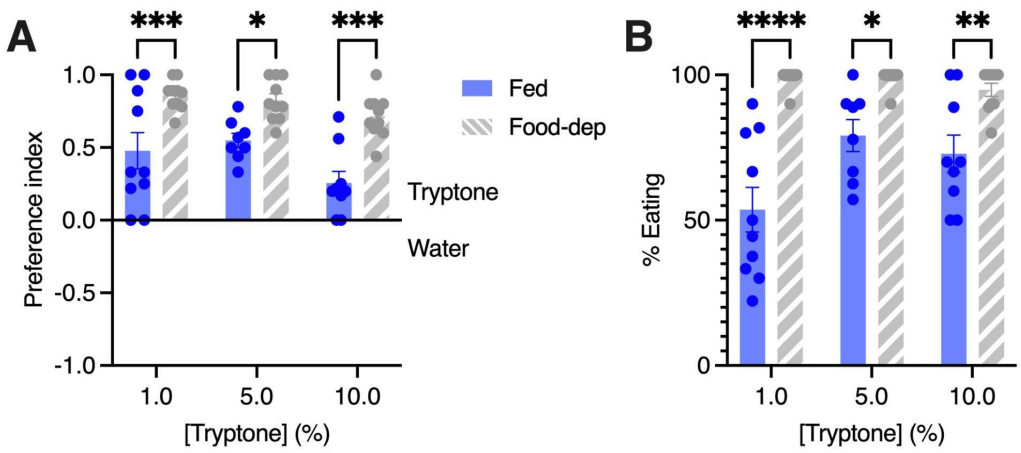

Figure 4: Amino acid consumption is dependent on concentration and internal hunger state
(**A**) Dye-based binary preference assay of W1118 flies that were food-deprived for 24h or kept on regular food. Food-deprived flies were kept on 1% agar for thirst. (**B**) Preference index for 1%, 5%, or 10% (w/v) tryptone vs. water. n=8-10 groups of 10 mated female flies per group. p*<0.05, p**<0.01, p***<0.001, p****<0.0001 by way of two-way ANOVA with Sidak's posttest (**A, B**). Each graph depicts the mean ± SEM.

basis for the continuation of this investigation with the use of flies in the fed state to avoid the ceiling effect of maximal preference, as evidenced by food-deprived flies.

Next, we sought to determine each of the GRNs' individual contributions to the preference for AA at the three different tryptone concentrations. We expressed *Kir2.1*, an inward-rectifying potassium channel embedded in the cell membrane that permits the free movement of K^+ ions out of the cell, leading to neuronal hyperpolarization and inhibition of their activity (Johns et al., 1999). Crossing Gr64f-, IR94e-, and Gr66a-Gal4 drivers with UAS-Kir2.1 allowed us to genetically silence each of these populations for examination of flies' amino acid preference in the absence of their activity. *Gr64f>Kir2.1* flies demonstrated a slight decrease in 1% tryptone preference; the strongest difference was observed between *Gr64f>Kir2.1* and *+/Kir2.1* flies, while trending differences between *Gr64f>Kir2.1* and *Gr64f/+* flies for 1%

tryptone preference did not achieve statistical significance (Fig. 5A). However, silencing of Gr64f GRNs significantly increased the percent eating compared to *Gr64f/+* controls. Expression of Kir2.1 in IR94e GRNs did not alter preference for 1% tryptone, but percent eating was higher than that of *IR94e/+* flies (Fig. 5B). Silencing Gr66a activity significantly increased 1% tryptone preference compared to both genetic controls (Fig. 5C). This increased preference in the absence of Gr66a GRN activity is further highlighted by the increased number of *Gr66a>Kir2.1* flies that consumed 1% tryptone compared to both genetic controls. The results of this assay demonstrate the role of the canonical sweet and bitter cells in the consumption of amino acids at low concentrations. Expectedly, low-tryptone preference was decreased in the absence of Gr64f GRNs, whereas preference increased in the absence of Gr66a GRNs. These data suggest the roles of the canonical sweet and bitter GRNs in promoting attraction and aversion to amino acids, respectively, even at low concentrations that are close to the detection threshold. Conversely, the minimal change in preference to tryptone in the absence of IR94e GRNs at this concentration suggests that their activation is not integral for the consumption of low amino acid concentrations in food.

We next utilized the same genetic models and dye-based preference assay to examine flies' behavioral response to 5% tryptone. Expression of *Kir2.1* in Gr64f neurons considerably decreased flies' preference to 5% tryptone (Fig. 6A). This difference was prominent not only in the preference index, but also in the number of flies choosing either 5% tryptone or water. The percentage of *Gr64f>Kir2.1* flies eating any option did not differ from either genetic control (Fig. 6A). In line with results from 1% tryptone in Fig. 5, genetic silencing of IR94e GRNs had, at most, a modest effect on amino acid feeding at 5% tryptone (Fig. 6B). The difference in preference between *IR94e>Kir2.1* and *IR94e/+* flies did not achieve statistical significance,

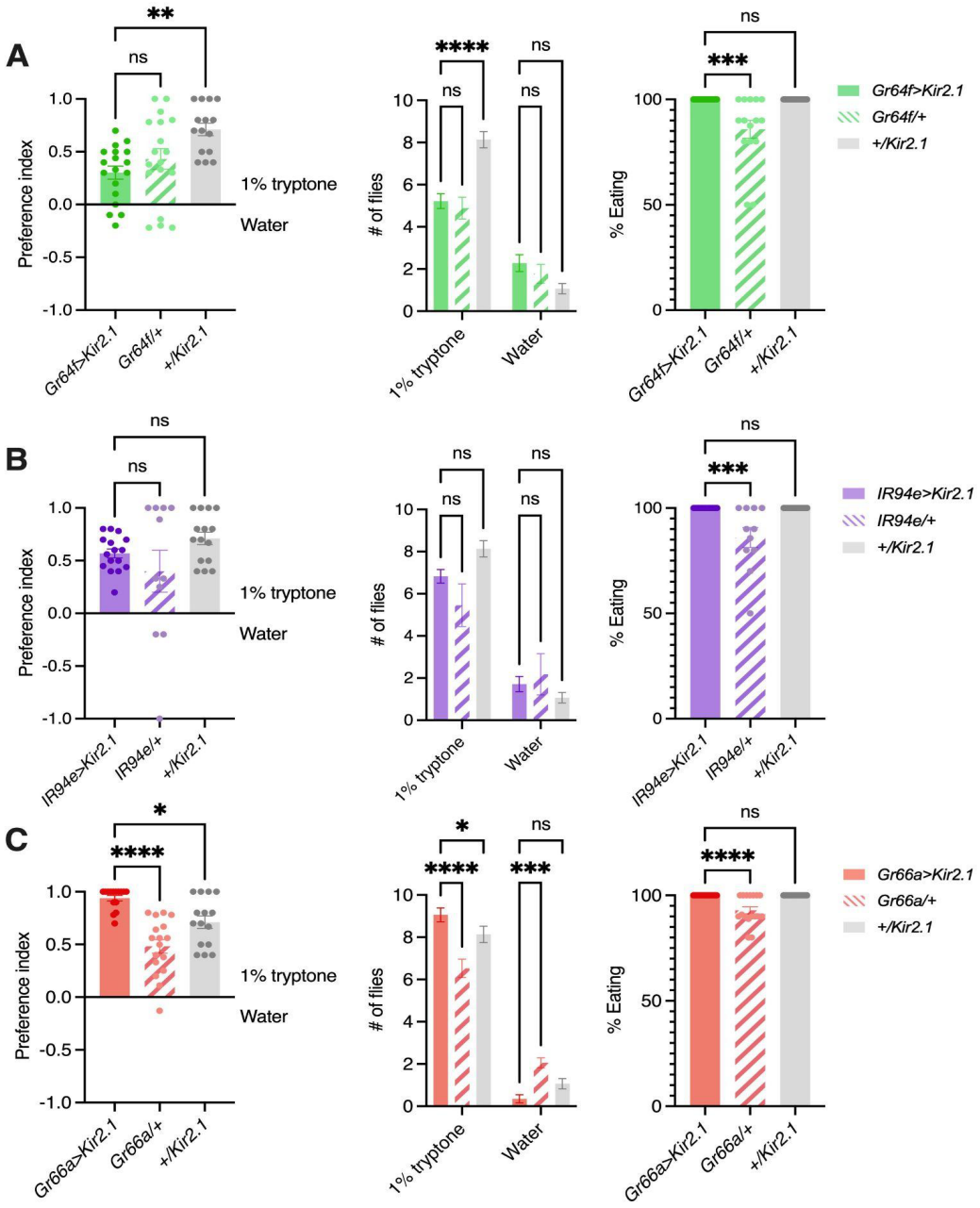

Figure 5: Sweet and bitter GRNs drive feeding of low AA concentrations, with minimal influence of IR94e GRNs

(**A-C**) Dye-based preference assay of flies with Gr64f (**A**), IR94e (**B**), and Gr66a (**C**) GRNs that have been electrically silenced through expression of the inward-rectifying K^+ channel *Kir2.1*. Preference index (left), number of flies eating 1% tryptone or water (middle), and total % of flies eating either or both options (right). N = 16-20 groups of 10 mated female flies per genotype, ns = p>0.05, p*<0.05, p**<0.01, p***<0.001, p****<0.0001 by way of one-way ANOVA with Sidak's posttest (preference index, % eating) or two-way ANOVA with Dunnett's posttest (# of flies). Each graph depicts the mean ± SEM.

while *IR94e>Kir2.1* flies displayed a slight decrease in preference compared to *+/Kir2.1* controls. There was no difference between percent eating at this concentration (Fig. 6B). Furthermore, silencing Gr66a GRN activation yielded no observable differences in 5% tryptone preference or percent eating compared to genetic controls (Fig. 6C). Based on findings presented in Fig. 4, we suspect that 5% tryptone is the most preferable amino acid concentration of the three investigated here; this is in line with the apparent ceiling effect that accompanies the absence of aversive Gr66a GRN activity (Fig. 6C).

After we had confirmed the roles of Gr64f, IR94e, and Gr66a GRNs in feeding preference for low and medium AA concentrations, we performed the same assay at 10% tryptone to investigate the roles of these circuits in high AA concentrations. In agreement with previous data, Gr64f-silenced flies demonstrated a significant reduction in preference for 10% tryptone. The number of *Gr64f>Kir2.1* flies consuming water was greater than those consuming tryptone, indicating a clear preference for the non-tryptone option (Fig. 7A). Notably, no differences were observed in percent eating, which was maximal for the Gr64f-silenced group and controls. 10% tryptone elicited a strongly negative preference in IR94e-silenced flies that was significantly lower than both genetic controls, a surprising result supported by the vast majority of flies consuming water instead of 10% tryptone (Fig. 7B). *IR94e>Kir2.1* flies also demonstrated mildly lower percent eating compared to *+/Kir2.1* flies. These results were unanticipated following previous assays demonstrating the mild effects of IR94e inhibition on amino acid feeding at lower concentrations (Fig. 5B, Fig. 6B). Electrical silencing of Gr66a GRNs prompted an increase in preference for 10% tryptone (Fig. 7C). In regard to food choice, a majority of *Gr66a>Kir2.1* flies chose to consume tryptone instead of the water option. Moreover, silencing of Gr66a GRNs increased the percent eating compared to *Gr66a/+* controls,

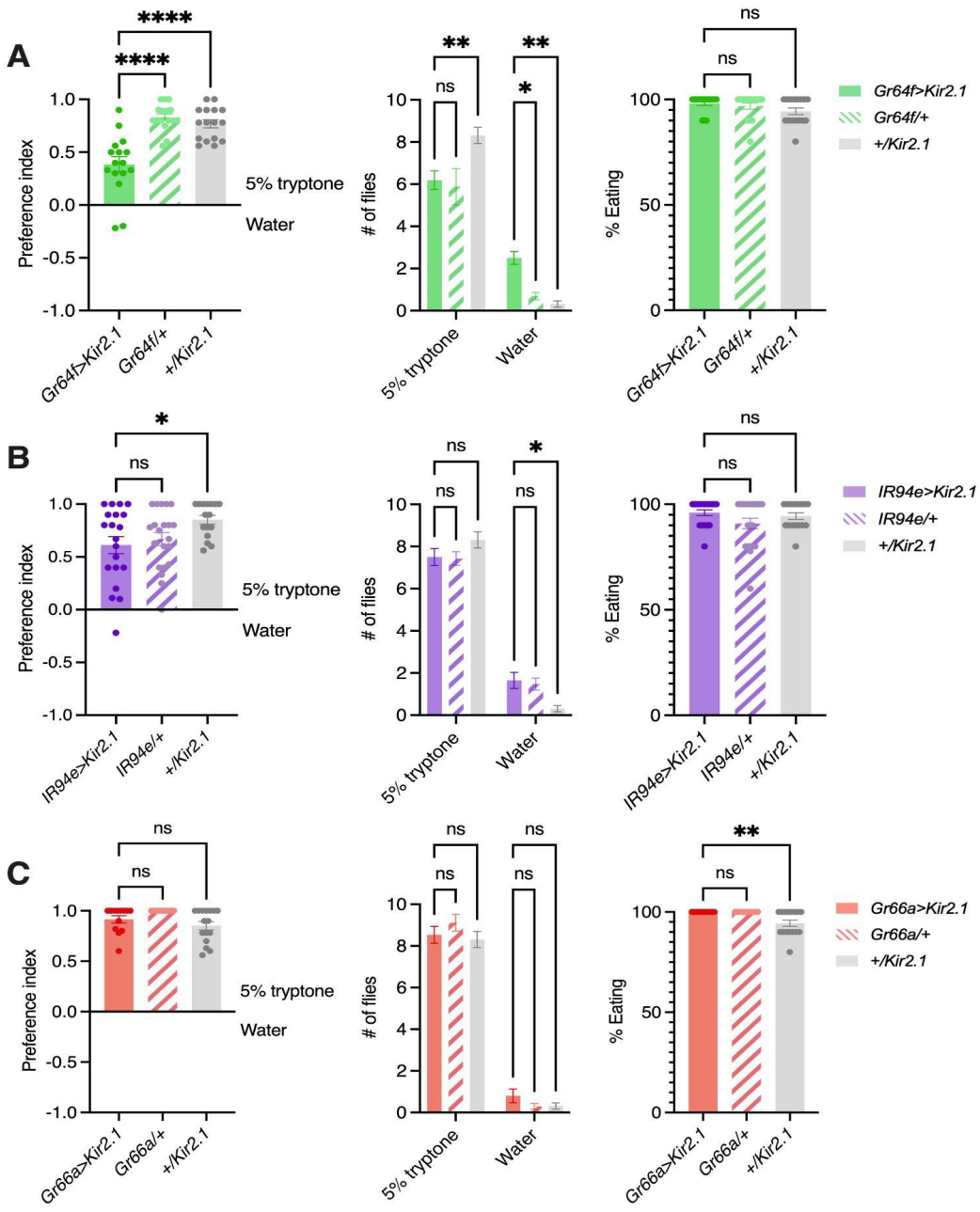

Figure 6: Canonical sweet and bitter GRNs drive feeding of moderate AA concentrations, with minimal influence of IR94e GRNs

(**A-C**) Dye-based preference assay of flies with Gr64f (**A**), IR94e (**B**), and Gr66a (**C**) GRNs that have been electrically silenced through expression of the inward-rectifying K^+ channel *Kir2.1*. Preference index (left), number of flies eating 5% tryptone or water (middle), and total % of flies eating either or both options (right). N = 15-21 groups of 10 mated female flies per genotype, ns = p>0.05, p*<0.05, p**<0.01, p****<0.0001 by way of one-way ANOVA with Sidak's posttest (preference index, % eating) or two-way ANOVA with Dunnett's posttest (# of flies). Each graph depicts the mean ± SEM.

although no difference from *+/Kir2.1* was observed (Fig. 7C). Altogether, these data support the notion that variability in foodstuffs, such as concentration of a chemical ligand, may promote the differential activation of individual GRN circuits. The combination of parallel GRN circuits in the fly may then encode taste information with increased specificity for that food, enabling a more nuanced behavioral response.

The results using the dye-based consumption assay indicate roles for all 3 sensory cells; however, this assay is subject to metabolic factors of internal state and the preference may be driven more than purely by the external sensory component. Therefore, we next wanted to identify the individual roles of these GRNs in the *detection* of AAs, rather than overall consumption, thereby necessitating the use of the proboscis extension response (PER) assay. We previously found that AAs are sufficient to stimulate taste and initiate feeding through the PER (data not shown), and here we tested the role of each GRN population in this response by expressing the optogenetic channel Gt*ACR1* using the Gal4/UAS system. Gt*ACR1* is a light-gated anion channel that hyperpolarizes neurons, and its expression as a transgene in the *Drosophila* genome rapidly inhibits neuronal circuits (Gorunova et al., 2015; Deere et al., 2023). Activation of the *Gt*ACR1 channel requires all-*trans*-retinal (ATR) supplementation and green light at an approximate wavelength of 515 nm (Mohammad et al., 2017). Previous unpublished data from our lab has used Gt*ACR1* in combination with a PER assay. This optogenetic approach enables a model of acute neuronal inhibition that can be used to study the impact of Gr64f, IR94e, and Gr66a GRNs in the detection of AAs at varying concentrations.

A model for the optogenetic inhibition of neuronal activity during stimulation in the PER assay is depicted (Fig. 8A). Following the acute silencing of Gr64f GRNs (experimental:

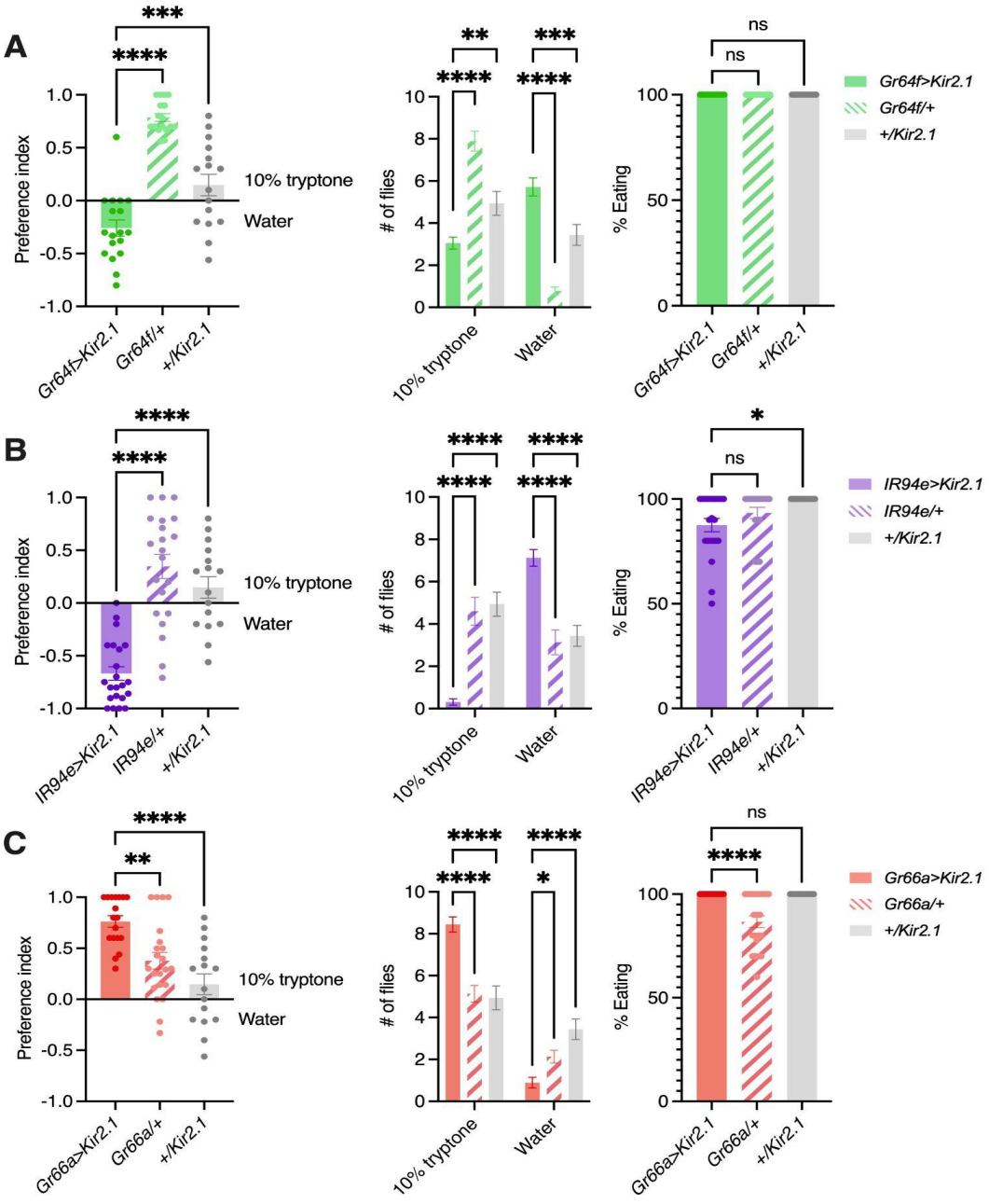

Figure 7: The inhibition of IR94e GRN activity elicits a robust decrease in preference for 10% tryptone, while Gr64f and Gr66a GRNs show canonical behavioral outputs
(**A-C**) Dye-based preference assay of flies with Gr64f (**A**), IR94e (**B**), and Gr66a (**C**) GRNs that have been electrically silenced through expression of the inward-rectifying K^+ channel *Kir2.1*. Preference index (left), number of flies eating 10% tryptone or water (middle), and total % of flies eating either or both options (right). N = 16-22 groups of 10 mated female flies per genotype, ns = p>0.05, p*<0.05, p**<0.01, p***<0.001, p****<0.0001 by way of one-way ANOVA with Sidak's posttest (preference index, % eating) or two-way ANOVA with Dunnett's posttest (# of flies). Each graph depicts the mean ± SEM.

Gr64f>Gt*ACR1* ATR+ group), PER to 1%, 5%, and 10% tryptone was decreased relative to ATR- controls (Fig. 8B), in line with consumption assays. Inhibition of IR94e GRNs significantly increased PER to all concentrations of tryptone, despite showing a robust decrease in 10% tryptone preference in the two-choice experiment (Fig. 8C). Interestingly, expression of Gt*ACR1* in Gr66a GRNs plus ATR supplementation resulted in a dose-dependent increase in PER; a negligible difference between groups at 1% tryptone became statistically significant at higher concentrations, which was most prominent at 10% tryptone (Fig. 8D). Overall, the results from this experiment demonstrate the magnitude by which the GRN populations discussed here are involved in the external sensing of AAs, leading to the initiation of feeding behaviors (i.e., extending the proboscis toward AA-containing food). Furthermore, these data highlight the multivariate nature of AA feeding. The taste-induced activation of labellar GRNs is not the sole mediator of AA intake, as this is one factor in a system which relies on the convergence of signals transmitted by multiple neuronal circuits to produce complex behavioral outputs (Steck et al., 2018).

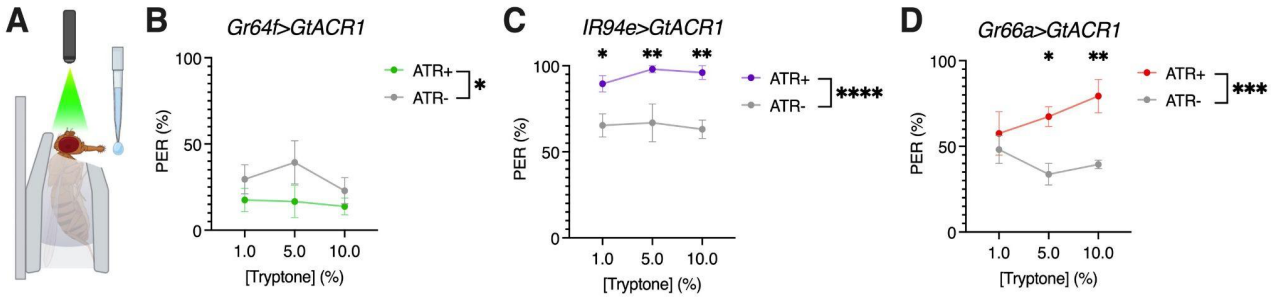

Figure 8: Individual GRN circuits on the labellum mediate AA detection
(**A**) Depiction of the PER assay in conjunction with optogenetic inhibition of labellar GRNs. (**B-D**) PER (%) of flies with Gr64f (**B**), IR94e (**C**), and Gr66a (**D**) GRNs that have been optogenetically silenced through the expression of the light-gated anion channel Gt*ACR1*. PER is reported as the percentage of flies that extended their proboscis during stimulation with the indicated tastant. PER to H_2O (negative control) and 1 M sucrose (positive control) was also measured. Each graph depicts the mean ± SEM.

DISCUSSION

The nervous system has evolved to receive, encode, and transmit a wide breadth of sensory information from the environment in the process of informing behavior that is advantageous for the organism. An extensive understanding of these mechanisms can lead to progress in a variety of domains, from the inhibition of pathogenic disease vectors to the treatment of metabolic disease. This study helps to characterize the neural circuitry that drives the intake of umami-rich food and provides evidence for the combinatorial coding properties of the *Drosophila* nervous system. The work presented here contributes to what is known about the neuronal detection of chemical ligands in *Drosophila* and, on a broader scale, provides insight into the factors that motivate organisms to consume protein over other macronutrients.

The behavioral significance of Gr64f, IR94e, and Gr66a neuronal activation

The nervous system's evaluation of nutritional content is a prominent factor underlying the intake of specific foods and thereby the proportion of macronutrients in the diet. *Drosophila melanogaster* is a generalist feeder who, likely accompanying its dispersal across the world, has adapted the ability to feed on a wide range of foodstuffs (Scott, 2018). Food selection in the fly is dictated by appetites for specific nutrients, suggesting the integration of the nervous system with metabolic processes (Malita et al., 2022). In the present study, AA detection and consumption was altered following the manipulation of three distinct neuronal circuits that are found in *Drosophila* labellar sensilla, suggesting that the encoding of AA taste information is modulated by each of the circuits studied. The canonical sweet and bitter circuits were consistent with the attraction and aversion to AAs, respectively, in both behavioral paradigms. These findings further establish the predominant roles of the Gr64f and Gr66a GRN circuits in reflexive feeding

behaviors which promptly inform the fly of the food's nutritious composition. Conversely, the activation of IR94e GRNs by AAs does not yield such predictable behavioral phenotypes. This absence of a characteristic behavioral output implies the existence of additional factors that modulate IR94e GRNs to enable behaviors beyond the scope of food acceptance or rejection. The possession of this tuning mechanism is evolutionarily advantageous for an organism, whose fitness would be improved with the ability to alter macronutrient feeding based on internal state or concentration of a ligand in food or prioritize other behaviors such as egg-laying.

The ratio of proteins to carbohydrates in the diet of mated female fruit flies has marked effects on lifespan, egg production, and egg-laying and leads females to prioritize higher protein proportions to maximize egg production (Rodrigues et al., 2015). Thus, the consumption of high-protein foods in females may be driven by the nutritional demands of reproduction rather than their own metabolic needs. Our lab has recently demonstrated the role of IR94e GRNs in the reciprocal regulation of AA feeding and egg-laying behaviors. This study identified a circuit in which female-specific IR94e GRNs synapse onto second-order projection neurons that synapse onto the descending interneurons responsible for oviposition (Guillemin et al., 2024; Wang et al., 2020). Behavioral responses to the activation of this circuit through AA stimulation showed that the *IR94e* receptor suppressed feeding but increased oviposition, suggesting that the IR94e circuit may function as a tuning mechanism by which flies can first evaluate the nutritional state of chemicals in food prior to egg laying to help increase the viability of their offspring. Gr66a activation in tarsal GRNs was also shown to suppress feeding behavior to increase oviposition (Joseph & Heberlein, 2012). However, further investigation is necessary to determine the reciprocal nature of these two behaviors, which are both fundamental to the species' success.

The combinatorial nature of labellar GRNs in *Drosophila*

The notion that foodstuffs containing a mixture of chemical ligands – a probable occurrence which is relevant for all animal species – can activate parallel GRN circuits, ultimately leading to an increased range and complexity of behaviors, is not thoroughly described in fruit flies; yet this phenomenon can be investigated through the neuronal inhibition of individual circuits and the behavioral phenotypes that arise. The results of the present study provide evidence to support a combinatorial coding paradigm in *Drosophila* GRNs, whereby the same set of chemicals activates multiple sensory cell types in parallel to produce a range of behavioral output (Fig. 9). This has previously been established for salt taste encoding in *Drosophila* (Jaeger et al., 2018), and here we establish this in AA feeding, expanding on the work of others (Aryal et al., 2022). The absence of Gr66a GRN activity promotes near-maximal consumption of tryptone at all concentrations in fed flies in a dye-based preference assay (Figs. 5C, 6C, 7C), despite fed *W[1118]* controls exhibiting only moderate preferences (Fig. 4A). This higher-intensity attraction that is typically counterbalanced by Gr66a activity is presumably from the contributions of Gr64f GRNs that are not inhibited. This result is further observed in the opposite direction following suppression of Gr64f GRN activity (Figs. 5A, 6A, 7A) and in alternative behavioral assays such as PER (Fig. 8). With Gr64f GRN silencing, the loss of attraction and switch to aversion, particularly with 10% tryptone (Fig. 7A), is presumably from the contributions of Gr66a GRNs that are not inhibited. How exactly the IR94e GRN activity may modulate the balance between these sweet and bitter GRN circuits is less clear, but our results suggest the contribution may vary depending on the state of the fly.

When exploring the combinatorial coding paradigm using this approach in *Drosophila* GRN circuits, the method of neuronal silencing may demonstrate specific properties which

cannot be directly compared. Experiments in the present study utilized flies with the expression of the K^+ channel *Kir2.1*, a model of chronic neuronal inhibition (Baines et al., 2001), in the binary choice assay to measure flies' post-ingestive AA preference. The constitutive silencing of a specific GRN circuit may elicit compensatory activity by other GRNs, such that their phenotypic role is altered in addition to the neuronal population of interest. In this study, we also employed the expression of the light-gated anion channel Gt*ACR1* as a measure of acute neuronal inhibition. These experiments yielded consistent characteristic behaviors following inhibition of the canonical sweet Gr64f and bitter Gr66a populations with either silencing method. However, the results from the investigation of IR94e GRNs prompt further inquiry as the two silencing methods produced opposing behavioral results at high tryptone concentrations (Fig. 7B, 8B). Additional behavioral paradigms can be combined with neuronal silencing methods to help rectify these differences.

Another aspect of combinatorial coding is the existence of multiple receptors, within and across cell types, that may be sensing similar chemicals. Inquiry into the potential existence of an AA receptor has emerged from the identification of the ionotropic receptor family in *Drosophila* sensory cells, particularly the role of the broadly-expressed coreceptor *IR76b* (Toshima & Schleyer, 2019). *IR76b*-expressing GRNs are activated by free amino acids and were shown to be necessary for the cellular and behavioral outputs of free amino acid taste (Ganguly et al., 2017). This study demonstrated that *IR76b* is expressed by multiple functionally distinct GRN types in both tarsi and labellar sensilla, and these neurons mediate distinct behavioral responses following stimulation with AAs. Recent work from our lab has shown that IR94e GRNs detect AAs through an IR complex composed of the *IR94e, IR25a,* and *IR76b* receptors (Guillemin et al., 2024). Thus, characterization of the mechanism behind the IR94e-mediated attraction to high

AA concentrations observed in our dye-based preference experiments necessitates further study into the role of *IR76b* in this circuit. Flies with mutations in the *IR94e* receptor or a genetic knockdown of *IR76b* specifically in the IR94e GRNs can be exposed to the same two-choice assay to determine whether the phenotype is consistent with that of IR94e GRN silencing.

External and internal modulation of IR94e GRNs remains unclear

Whereas the present study provides evidence for the encoding of AA taste information by the activation of labellar GRN circuits, there is an additional level of regulation by internal state factors which form a negative feedback loop to promote homeostasis. Gustatory sensitivity to specific macronutrients is altered depending on the concentration of the nutrient in the diet, a phenomenon that is observed across species, including in humans (Ganguly et al., 2021; Strilbytska et al., 2022; Abeywickrema et al., 2023). This is consistent with our findings of AA preference in the fed versus food-deprived state, with food-deprived flies demonstrating a significantly increased preference for tryptone at all concentrations compared to fed flies (Fig. 4). According to the protein leverage hypothesis, the drive to maintain sufficient protein levels in the diet is greater than that of other macronutrients (Gosby et al., 2014). An interesting experimental follow-up to examine these factors in greater detail involves investigating tryptone preference in flies following manipulation of the proportions of various dietary AAs.

Of the macronutrients, proteins are shown to have the greatest effect on satiety, despite yielding the least amount of energy following metabolic breakdown (Westerterp-Plantenga et al., 1999; Flatt, 2001). Metabolism of high-protein foods is mediated by internal factors in *Drosophila*, several of which are known to impact feeding behavior, including diuretic hormone

44, the gut hormone tachykinin, and adipokinetic hormone, the *Drosophila* homologue of glucagon (Yang et al., 2018; Ahrentløv et al., 2024). The extent to which metabolic hormones and other state-dependent factors affect labellar GRN circuits to alter AA feeding in flies is an intriguing topic which has not yet been described.

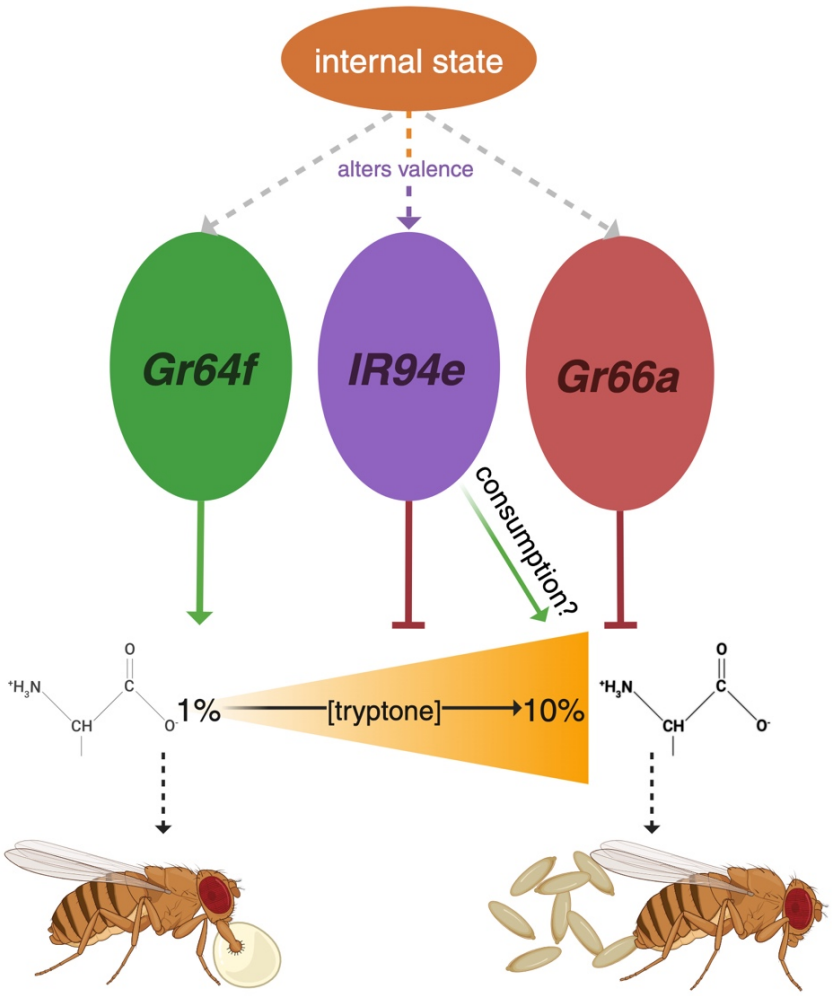

Figure 9: A model for the neural circuitry underlying the detection and consumption of AAs in tryptone
AAs activate Gr64f GRNs to promote feeding attraction and drive the consumption of protein-containing foods, whereas Gr66a GRN activation inhibits feeding and prioritizes other behaviors such as egg-laying. IR94e GRNs detect AAs to prompt aversive responses that inhibit feeding. However, unlike what was observed from the canonical sweet and bitter cells, the behavioral valence of IR94e GRNs is altered through the suspected modulation of metabolic signaling to promote the consumption of high AA concentrations in foodstuff.

An additional but important consideration of this study is the biological relevance of the tryptone solution utilized in experiments investigating AA preference. The AAs in tryptone are derived from a digest of the casein protein found in milk, which contains all essential dietary AAs (Wretland, 1947). However, the AAs in food are more likely to be consumed along with additional chemical ligands; thus, one food is activating more than one GRN circuit at a time, and/or is repeatedly activating one circuit through different ligands, potentially affecting a fly's subsequent behavioral outputs. The most common AA in food is glutamate, which was shown to significantly activate IR94e GRNs, as was 5% tryptone (Dai et al., 2022; Guillemin et al., 2024). Thus, while the AA solutions used in these experiments may not be entirely representative of foodstuffs in the natural environment, they are capable of activating the neuronal populations of interest while providing a baseline index for representative AA preference in *Drosophila*. Importantly, tryptone contains AAs that can be directly sensed by enteroendocrine cells of the *Drosophila* female midgut, insulin-producing cells in larvae, and widely-expressed peripheral receptors (Ahrentløv et al., 2024; Nässel & Vanden Broeck, 2016; Aryal et al., 2022). The behavioral drive to consume AAs through these internal sensing mechanisms cannot be ruled out in this investigation of the intake and preference of tryptone. However, the PER experiments were measurements of external AA sensing only. It is possible that the IR94e-mediated reversal in the preference for 10% tryptone is prompted by one or several of these factors, which would require the ingestion of AAs for 1) sensing by these downstream metabolic pathways and 2) the transmission of afferent signals which project to the SEZ of the brain to modulate the role of the IR94e circuit in feeding behavior.

The IR94e-mediated drive to consume foods high in protein may require additional modulation by other taste circuits or sensory modalities. Animals use a combination of sensory

modalities to contextualize their environment; flies have been shown to integrate taste, odor, and mechanosensory cues when evaluating foodstuffs (Oh et al., 2021). This may explain the increase in preference to tryptone following the acute loss of IR94e GRN activity in the PER assay, which specifically examines the activation of labellar GRNs whilst physically prohibiting the activation of GRNs located elsewhere on the fly, such as the legs, wings, and ovipositor. The strong IR94e-mediated preference for the consumption of 10% tryptone may require coactivation by other external modulatory pathways that could impact the behavioral outputs of AA detection. When activation of these non-labellar populations is inaccessible (i.e., in the labellar PER assay), IR94e GRNs may exert an inhibitory role in AA feeding initiation.

In conclusion, we demonstrate the behavioral outputs of distinct GRN populations in the labellum of *Drosophila melanogaster* following the consumption of low, moderate, and high concentrations of AAs. We provide support for the notion that individual circuits are integrated in the brain to inform the fly's feeding behavior, and that whilst the canonical Gr64f and Gr66a neurons promote attraction and aversion to AAs, respectively, the unique behavioral phenotype presented by IR94e GRNs implicate its function as an AA-sensing neuron in the labellum. Future studies must examine the external and internal modulatory factors that influence the detection and consumption of AAs in *Drosophila*. This work, along with future studies, improves our understanding of the motivational drive that underlies protein feeding and the mechanisms by which the nervous system encodes taste information to drive behavior.

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