

Mechanisms of Carboxylic Acid Attraction in *Drosophila melanogaster*

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Sour is one of the fundamental taste modalities that enable taste perception in animals. Chemoreceptors embedded in taste organs are pivotal to discriminate between different chemicals to ensure survival. Animals generally prefer slightly acidic food and avoid highly acidic alternatives. We recently proposed that all acids are aversive at high concentrations, a response that is mediated by low pH as well as specific anions in *Drosophila melanogaster*. Particularly, some carboxylic acids such as glycolic acid, citric acid, and lactic acid are highly attractive to *Drosophila* compared with acetic acid. The present study determined that attractive carboxylic acids were mediated by broadly expressed *Ir25a* and *Ir76b*, as demonstrated by a candidate mutant library screen. The mutant deficits were completely recovered via wild-type cDNA expression in sweet-sensing gustatory receptor neurons. Furthermore, sweet gustatory receptors such as *Gr5a*, *Gr61a*, and *Gr64a-f* modulate attractive responses. These genetic defects were confirmed using binary food choice assays as well as electrophysiology in the labellum. Taken together, our findings demonstrate that at least two different kinds of receptors are required to discriminate attractive carboxylic acids from other acids.

Keywords: citric acid, glycolic acid, gustatory receptor, ionotropic receptor, lactic acid

INTRODUCTION

The sense of taste is crucial for the identification of nutritious foods and avoiding potentially toxic foods. Taste perception is mediated by receptors housed in the taste organs and enables animals to localize and discriminate between potential food items in the environment. Chemical signals from foods are distinguished by specialized receptors, which possess a discrete ligand response profile (Rimal and Lee, 2018). Sour is one of the five basic taste modalities of the gustatory system. Unlike sweet, bitter, and umami, salty and sour perception is bidirectional. In other words, animals prefer low concentrations of salt and sour compounds but also actively avoid high concentrations (Chen and Dahanukar, 2020).

In the fruit fly (*Drosophila melanogaster*), taste is primarily detected through ionotropic receptors (ion permeable receptors), including gustatory receptors (GRs), ionotropic receptors (IRs), and transient receptor potential (TRP) channels (Rimal and Lee, 2018). Fly GRs are generally required for sensing sweet and bitter compounds (Dahanukar et al., 2007; Weiss et al., 2011). Further, except for nine sweet-sensing GRs, most GRs are considered bitter-sensing GRs (Shrestha and Lee, 2021). These nine sweet-sensing GRs are categorized by the expression of reporters and recently verified functions. *Gr5a* is expressed in a large number of sweet-sensing gustatory receptor neurons (GRNs) (Marella et al., 2006) and is required for sensing trehalose (Dahanukar et al., 2001). *Gr43a* has a crucial role in internal fructose

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sensing in the brain (Miyamoto and Amrein, 2014). In fact, the *Gr64a* cluster (*Gr64a-f*) is the most important cluster for the perception of most sugars such as sucrose, maltose, trehalose, and glucose given the role of *Gr64f* as a sugar co-receptor (Jiao et al., 2008). Furthermore, *Gr64e* is required for sensing glycerol (Wisotsky et al., 2011) and fatty acids (Kim et al., 2018). However, *Gr61a* is only a putative sweet-sensing GR based on the reporter expression in sweet-sensing GRNs (Dahanukar et al., 2007; Miyamoto et al., 2013). Fly IRs are required for sensing saltiness and sourness in foods (Chen and Amrein, 2017; Zhang et al., 2013). Additionally, flies use IRs for tasting Ca^{2+} (Lee et al., 2018). Fly TRPs taste aversive and pungent chemicals such as wasabi and aristolochic acid (Al-Anzi et al., 2006; Kim et al., 2010).

The labellum, the major taste organ in *Drosophila*, harbors many taste sensilla that are classified based on length as short (S-type), intermediate (I-type), and long (L-type) (Hiroi et al., 2002; Shanbhag et al., 2001). Each taste sensillum comprises an apical pore system, which facilitates the recording of neural activity (Wieczorek and Wolff, 1989). The taste sensillum encompasses 2-4 GRNs. Generally, each L- and S-type sensillum harbors 4 GRNs, whereas I-type sensillum has two GRNs (Dethier, 1976; Falk et al., 1976; Fujishiro et al., 1984; Hiroi et al., 2004; Meunier et al., 2003; Nayak and Singh, 1983). Individual neurons for each taste modality are embodied by a discrete repertoire of receptors, which drive the transduction of stimuli elicited by a particular sensor to a higher brain center. After decoding these messages, the brain determines whether or not the food is safe to eat (Roper and Chaudhari, 2017).

Acid has a sour taste and a pungent smell. Odor sensation related to acids has been identified in *Drosophila melanogaster* and this process is known to be mediated by IRs. Electrophysiological analyses and calcium imaging have demonstrated that IR31a, IR64a, IR84a, IR75a, IR75b, and IR75c are specifically tuned to acidic molecules, in addition to the broadly tuned co-receptor IR8a (Abuin et al., 2011; Prieto-Godino et al., 2016; 2017; Silbering et al., 2011). However, acid perception in *Drosophila* remains largely uncharacterized. We recently proposed that IR7a mediates repulsive responses to acetic acid in *D. melanogaster* (Rimal et al., 2019). Furthermore, many carboxylic acids such as glycolic acid (GA), citric acid (CA), and lactic acid (LA) are very attractive even at extremely low pH (~2.5) (Rimal et al., 2019). In another study, starved flies exhibited appetitive responses to acetic acid (Devineni et al., 2019). Additionally, two broadly expressed IRs, IR25a and IR76b, detect acids during oviposition (Chen and Amrein, 2017).

Here, we conducted screening experiments using loss of function mutants to elucidate the transduction mechanism of attractive acid perception. Further, feeding behavior assays and electrophysiological analyses were conducted to identify candidate taste receptors for carboxylic acids. Our findings demonstrated that two broadly required IRs, IR25a and IR76b, and most sweet-sensing GRs are essential to discriminate attractive sour tastes.

MATERIALS AND METHODS

Fly stocks

Strain *w¹¹¹⁸* was used as a control strain in this study. All flies were maintained at 25°C under a 12-h light/12-h dark cycle. Both male and female flies were used randomly for the experiments. The following lines were obtained from the Bloomington Drosophila Stock Center (<https://bdsc.indiana.edu/>): *Ir7g¹* (BL42420), *Ir8a¹* (BL41744), *Ir10a¹* (BL23842), *Ir21a¹* (BL10975), *Ir48a¹* (BL26453), *Ir48b¹* (BL23473), *Ir51b¹* (BL10046), *Ir52b¹* (BL25212), *Ir56b¹* (BL27818), *Ir62a¹* (BL32713), *Ir67a¹* (BL56583), *Ir75d¹* (BL24205), *Ir85a¹* (BL24590), *Ir92a¹* (BL23638), *Ir94b¹* (BL23424), *Ir94d¹* (BL33132), *Ir94f¹* (BL33095), *Ir94g¹* (BL25551), *Ir100a¹* (BL31853), and *UAS-Kir2.1* (BL6596). We previously described the source of the following lines in our previous study: *Ir7a¹*, *Ir47a¹*, *Ir52a¹*, *Ir56a¹*, *Ir60b³*, *Ir94a¹*, *Ir94c¹*, *Ir94h¹* (Rimal et al., 2019), *UAS-Ir25a* (Lee et al., 2018). C. Montell kindly provided strains *Ir76b¹*, *Ir76b-GAL4*, and *UAS-Ir76b*. Additionally, J.R. Carlson generously provided strains *Gr5a^{Δ5}*, *Gr5a^{Δ5}/FM7;UAS-Gr5a/CyO*, *Gr5a^{Δ5}/FM7;Gr5a-GAL4/CyO*, *Gr61a¹*, *Gr61a-GAL4*, and *Gr61a¹;UAS-Gr61a*. K. Scott provided strains *ppk23-GAL4* and *ppk28-GAL4*. *Gr43a^{GAL4}*, *Gr66a-GAL4*, *Gr5a-GAL4*, and Δ *Gr64a-f* were provided by H. Amrein. *Ir20a¹* and *Gr64f-GAL4* were obtained from A. Dahanukar and L.B. Vosshall provided strains *Ir25a-GAL4* and *Ir25a²*.

Chemical sources

GA (CAS No. 79-14-1), CA (CAS No. 77-92-9), LA (CAS No. 50-21-5), sucrose (CAS No. 57-50-1), tricholine citrate (TCC) (CAS No. 546-63-4), and sulforhodamine B (CAS No. 3520-42-1) were purchased from Sigma-Aldrich (USA). Brilliant blue FCF (CAS No. 3844-45-9, Cat No. 027-12842) was purchased from Wako Pure Chemical Industry (Japan).

Electrophysiology

Electrophysiology (i.e., tip recording assay) was performed as described in a previous study (Lee et al., 2009). First, 4- to 7-day old flies were anesthetized on ice. A reference glass electrode filled with Ringer's solution was inserted into the thorax of the flies. The electrode was then slowly extended towards the proboscis of the fly. To avoid experimental biases, 5 to 6 live insects were prepared per set-up and the same procedure was repeated for several rounds on different days. For the recordings, the sensilla were stimulated for 5 s with a mixture of chemical stimulant in a 30 mM TCC solution (i.e., electrolyte solution) in recording pipettes (10-20 μm tip diameter) connected to a preamplifier. The recorded signals were collected and amplified 10 \times using a signal connection interface box (Syntech, Netherlands) in conjunction with a 100-3,000 Hz band-pass filter. Recordings of action potentials were acquired using a 12-kHz sampling rate and analyzed using the Autospike 3.1 software (Syntech). To obtain proper signals, all recordings were conducted at 1 min intervals. The number of action potentials was counted from 50-550 ms after application of the stimulus. Therefore, the numbers of spikes/s in Figures mean that the numbers of spikes during 50-550 ms are divided by 1/2. The dots in each figure indicate the number of insects tested.

Binary food choice assay

Binary food choice assays were conducted as described in a previous study (Lee et al., 2010). Approximately 50-70 flies (3-6 days old; mixed sexes) were starved for 18 h in a humidified chamber. Two different food sources containing 1% agarose were then prepared. One food source contained 2 mM sucrose, whereas the other contained 2 mM sucrose mixed with different concentrations of acid. These food sources were mixed with either blue food coloring (brilliant blue FCF, 0.125 mg/ml) or red food coloring (sulforhodamine B, 0.1 mg/ml). The two mixtures were distributed in alternating wells of a 72-well microtiter dish (Cat. No. 438733; Thermo Fisher Scientific, USA). Approximately 50-70 starved flies were then transferred to the dish within 30 min of food preparation. The dishes were incubated in a dark, humidified chamber, and the flies were allowed to feed for 90 min at room temperature. After the feeding process, the flies were sacrificed at -20°C . The color of their abdomens was analyzed under a stereomicroscope. Upon visual inspection, blue (N_B), red (N_R), or purple (N_P) flies were tabulated. The preference index (PI) was calculated according to the following equation: $(N_B - N_R)/(N_R + N_B + N_P)$ or $(N_R - N_B)/(N_R + N_B + N_P)$, based on the dye/tastant combinations. PI values of 1.0 or -1.0 indicated that the flies exhibited a complete preference for one food choice or the other, whereas a PI of 0.0 indicated that the flies had no preference between the two food choices.

Quantification and statistical analyses

All the experiments were repeated on different days and the data were analyzed using GraphPad Prism 8 (GraphPad Software, USA) (RRID: SCR_002798). The dots in the graph represent the number of trials for the experiment. All error bars represent SEM. Multiple sets of data were compared using single-factor ANOVA coupled with Scheffe's post hoc analysis. All statistical analyses were conducted using the origin program (OriginLab [USA]) (RRID: SCR_002815). The asterisks in the figures indicate statistical significance ($*P < 0.05$, $**P < 0.01$).

RESULTS

Low concentrations of carboxylic acids are attractive to *Drosophila*

Many organic acids such as GA, LA, and CA are edible and can be used as energy sources (Fig. 1A). GA is the smallest α -hydroxyl acid, which is produced during photorespiration in sugar-crop plants. LA is produced from simple carbohydrates by LA bacteria. Furthermore, CA is abundant in most fruits and vegetables. These carboxylic acids elicit appetitive responses in animals at environmentally relevant concentrations (Rimal et al., 2019). Binary food choice assays were conducted to characterize sour feeding behavior in flies (see the Materials and Methods section for more details). Control flies exhibited almost no bias to 0.1% carboxylic acids (pH ~ 3) (Fig. 1B). According to our observations, the flies preferred a 0.5%-1% carboxylic acid range (pH 2.4-2.7) with 2 mM of sucrose (Fig. 1B) or without sucrose (Fig. 1C). However, this attraction was reversed by increasing the concentrations

of carboxylic acids (Figs. 1B and 1C). Sucrose did not affect the discrimination of these acids except in the 5%-10% CA treatment. Specifically, the preference for relatively high concentrations of CA was affected by the presence of sucrose (Figs. 1B and 1C). Therefore, our findings demonstrated that sour taste is biphasic and that the flies preferred ecologically relevant concentrations of GA, LA, and CA.

Sweet-sensing GRNs are involved in the attractive response to carboxylic acids

Next, surgical dissections were conducted to test whether other chemosensory organs besides the labellum were required for carboxylic acid attraction. Flies have two main olfactory organs: the antennae and maxillary palp. When we removed each organ, flies generally preferred ingesting 1% GA, LA, and CA (Fig. 1D). In addition to the labellum, the legs and wing margin also contain taste sensilla. Dissecting the wings or forelegs did not affect carboxylic acid preference (Fig. 1D). However, these observations cannot completely rule out the possible contribution of these chemosensory organs to carboxylic acid perception, as removing the remaining midlegs and hindlegs was impossible without affecting the behavioral assays. Furthermore, many taste receptors in flies are also expressed in the legs. Next, we sought to determine which GRNs were required to detect 1% GA, LA, and CA (Fig. 1E). Flies have four types of GRNs. Specifically, sweet-sensing and water-sensing GRNs are required for attraction, whereas bitter-sensing and calcium-sensing GRNs are necessary for aversion. When the sweet-sensing GRNs were inactivated by expressing inwardly rectifying Kir2.1 (Paradis et al., 2001) under the control of a *Gr64f-GAL4* driver (Dahanukar et al., 2007), GA-, LA-, and CA-mediated attraction was attenuated (Fig. 1E). Conversely, inactivating the water-sensing GRNs using the *ppk28-GAL4* driver line (Cameron et al., 2010) had no effect on carboxylic acid feeding preference (Fig. 1E). Furthermore, attraction remained unchanged in flies with inactivated bitter-sensing (*Gr66a-GAL4*) and calcium-sensing (*ppk23-GAL4*) GRNs (Dunipace et al., 2001; Lee et al., 2018; Moon et al., 2006) (Fig. 1E). These results suggest that sweet-sensing GRNs mediate the preference for appetitive concentrations of carboxylic acids.

Some L-type sensilla mediate robust responses to physiologically relevant concentrations of carboxylic acids

Each sensillum possesses one sweet-sensing GRN, which was labeled using the *Gr64f-GAL4* driver. To identify relevant sensilla in the labellum that were sensitive to 1% GA, LA, and CA, we investigated the peripheral nerve responses to each carboxylic acid by performing tip recordings with control flies. The L4, L6, and L8 sensilla robustly responded to all three acids (Fig. 2A). Four additional L-type sensilla (L3, L5, L7, and L9) showed moderate responses to the same stimuli (Fig. 2A). However, none of the S-type and I-type sensilla or the L1 and L2 sensilla were significantly activated by the acids (Fig. 2A). Next, the behavioral abnormalities of the flies with inactivated sweet-sensing GRNs (*Gr64f-GAL4/UAS-Kir2.1*) were confirmed by conducting electrophysiology tests from the robust L4, L6, and L8 sensilla (Figs. 2B and 2C). As expected, we found that high-frequency action potentials in the L4,

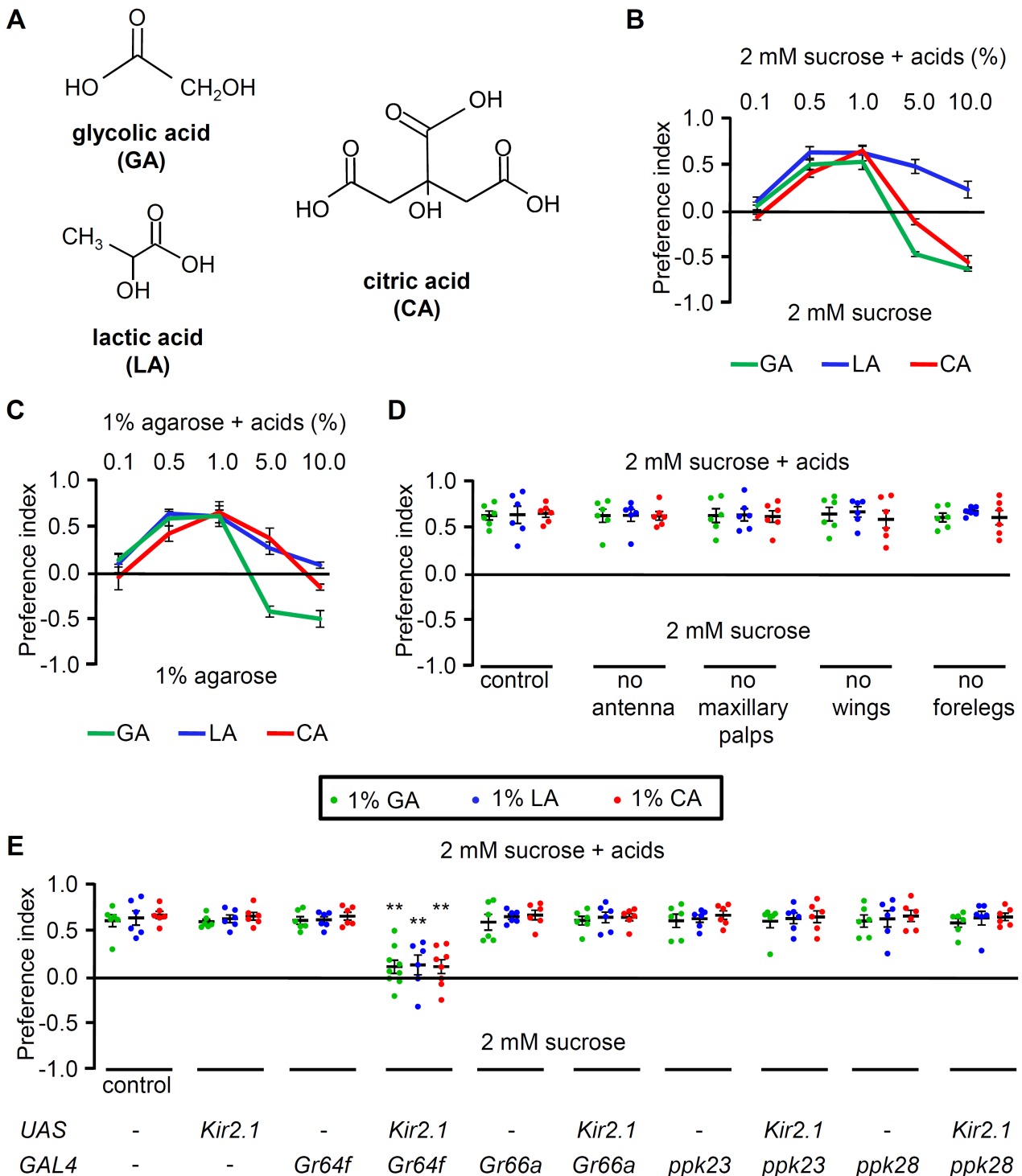


Fig. 1. Low concentrations of carboxylic acids are attractive to *Drosophila*. (A) Chemical structures of GA, LA, and CA. (B) Binary food choice assays using control flies (w^{1118}). The food choices were prepared with either 2 mM sucrose only or 2 mM sucrose mixed with different concentrations of GA, LA, and CA ($n = 6$). (C) Binary food choice assays using control flies (w^{1118}) in the absence of sucrose with different concentrations of GA, LA, and CA ($n = 6$). (D) Binary food choice assays with organ-ablated control flies (2 mM sucrose or 2 mM sucrose with 1% GA, 1% LA, or 1% CA) ($n = 6$). (E) Binary food choice assays after inactivating different GRNs using *UAS-Kir2.1* under the control of the indicated *GAL4*s. 1% GA, 1% LA, and 1% CA were tested ($n = 6$). Multiple sets of data were compared using single-factor ANOVA coupled with Scheffe's post hoc test. Asterisks indicate statistical significance compared with the control (** $P < 0.01$). All error bars represent the SEM.

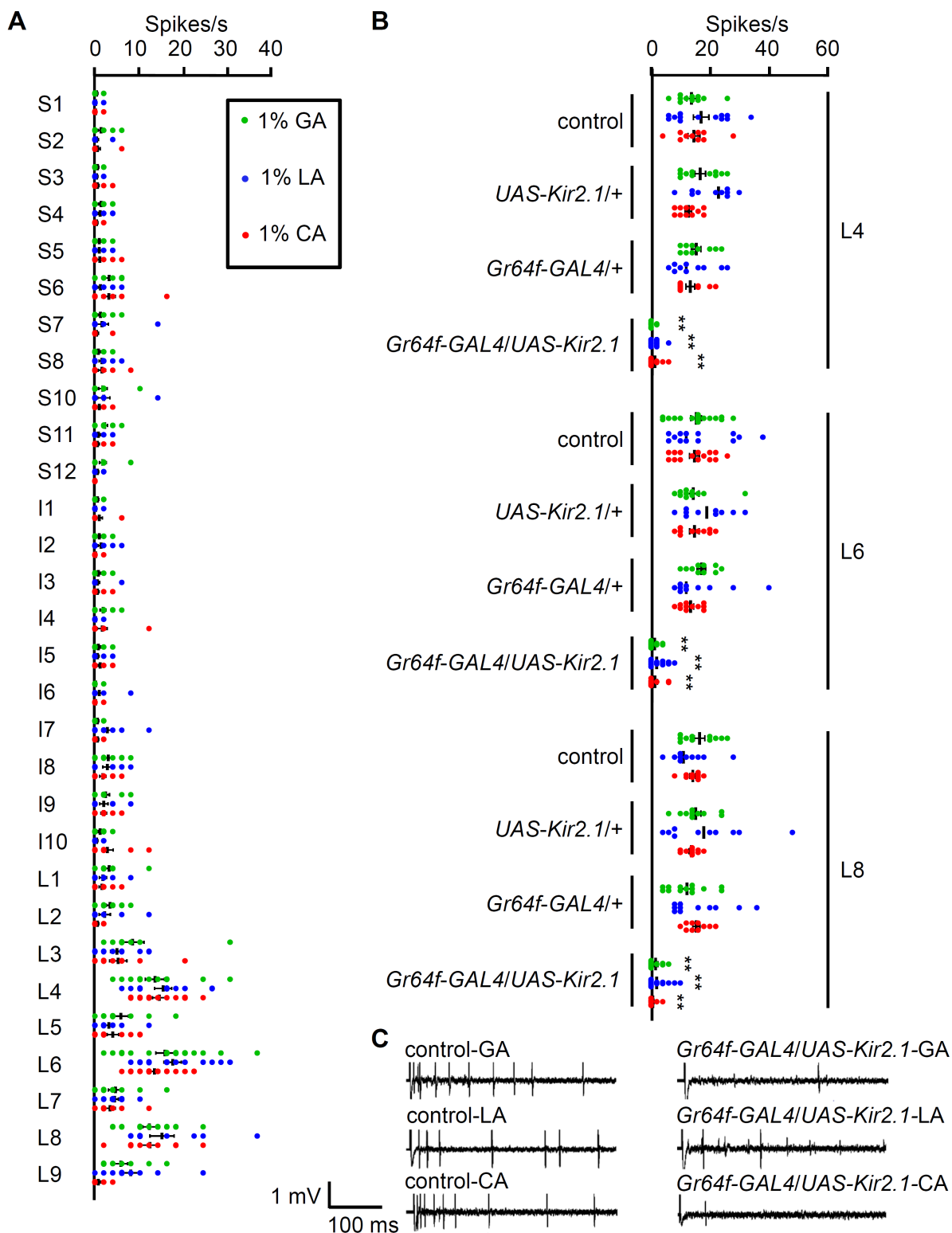


Fig. 2. 1% carboxylic acid-induced neuronal responses in the labellum. (A) 1% GA, 1% LA, and 1% CA were tested from all the labellar sensilla for electrophysiology mapping ($n = 10-18$). All error bars represent the SEM. (B) Tip recordings from L4, L6, and L8 sensilla after inactivating sweet-sensing GRNs using *UAS-Kir2.1* under the control of *Gr64f-GAL4* ($n = 10-15$). All error bars represent the SEM. Multiple sets of data were compared using single-factor ANOVA coupled with Scheffe's post hoc test. Asterisks indicate statistical significance compared with the controls (** $P < 0.01$). (C) Representative sample traces of (B).

L6, and L8 sensilla of the control flies were significantly suppressed, although the nerve activity of both parent strains was similar to that of the control (Fig. 2B). These data suggest that molecular sensors for carboxylic acids are present in L-type sensilla.

IR25a and IR76b are indispensable mediators of carboxylic acid-induced nerve activity in L-type sensilla

To identify which molecules are involved in carboxylic acid detection, 30 candidate *Ir* loss-of-function mutants were tested via electrophysiology assays (Benton et al., 2009; Lee et al., 2018; Rimal et al., 2019) (Fig. 3A). The library included the *Ir7a* and *Ir62a* mutants, which are essential for the repulsion of acetic acid and Ca^{2+} , respectively (Lee et al., 2018; Rimal et al., 2019). *Ir76b* is required for sensing Na^+ and Ca^{2+} minerals and amino acids (Ganguly et al., 2017; Lee et al., 2017; 2018; Zhang et al., 2013). *Ir25a* is also broadly expressed and required for sensing minerals (Chen and Amrein, 2017; Lee et al., 2018; Sánchez-Alcañiz et al., 2018). We thus focused on the L4, L6, and L8 sensilla, which exhibit relatively high action potential frequencies in response to carboxylic acids (Fig. 2A). Our screening results indicated that the *Ir25a*² and *Ir76b*¹ mutations significantly impaired LA-induced action potentials (Fig. 3A). In contrast, the other 28 mutations elicited normal LA responses (Fig. 3A). To address whether IR25a and IR76b are necessary for neuronal firing in response to other acids, we further evaluated the response of L4 and L6 sensilla to 1% GA and 1% CA (Figs. 3B and 3C). Our findings indicated that the strong responses to the carboxylic acids in the sweet-sensing GRNs were mediated by IR25a and IR76b.

To test whether *Ir25a* and *Ir76b* are required in these GRNs, recovery experiments were conducted using their specific enhancer *GAL4s*, which are very broadly expressed in the labellum (Lee et al., 2018). *Ir25a-GAL4* and *Ir76b-GAL4* are expressed not only in sweet-sensing GRNs but also in bitter- and calcium-sensing GRNs (Lee et al., 2018). Each wild-type cDNA was expressed in the mutant background under the control of its specific *GAL4* (Figs. 3D and 3E). We found that each *GAL4* in combination with its specific *UAS-Ir25a* or *UAS-Ir76b* fully rescued the GA-, CA-, and LA-induced action potentials in the neurons (Fig. 3D) and restored the attraction to 1% carboxylic acids (Fig. 3E). Furthermore, we also expressed *UAS-Ir25a* or *UAS-Ir76b* under the control of *Gr5a-GAL4*, which is exclusively expressed in the sweet-sensing GRNs (Thorne et al., 2004) for cell-type-specific rescue (Figs. 3D and 3E). Again, the expression of IR25a and IR76b in the sweet-sensing GRNs was enough to recover the neuronal activity as well as attractive behavior (Figs. 3D and 3E). However, *UAS*-only or *GAL4*-only parent strains in the mutant background had deficits that were similar to the mutants (Figs. 3D and 3E). These genetic experiments clearly confirmed that IR25a and IR76b are necessary for carboxylic acid-induced nerve activity.

Sweet *Gr*s are required for carboxylic acid-induced attraction and neuronal activity

Given that sweet-sensing GRNs are required for acid sensation (Figs. 1E and 2B), sweet *Gr* mutants were examined in more detail. Fruit flies possess nine sweet *Gr*s including *Gr5a*

(trehalose), *Gr43a* (fructose), *Gr61a* (unknown), and *Gr64a-f* cluster (sucrose, maltose, trehalose, glucose, glycerol, and fatty acids) (Dahanukar et al., 2001; Jiao et al., 2007; 2008; Kim et al., 2018; Miyamoto and Amrein, 2014; Miyamoto et al., 2012). Surprisingly, we found that all the sweet *Gr*s except for *Gr43a* had very suppressed neuronal responses to all the carboxylic acids (Fig. 4A). Furthermore, all mutants including the *Gr43a*^{GAL4} mutant exhibited severe deficits in the binary food choice assays (Fig. 4B), indicating that the physiology and behavior results for *Gr43a* were inconsistent. However, *Gr43a* is a known fructose sensor in the brain, suggesting that the ingestion of carboxylic acids as an energy source is controlled both by internal sensors as well as peripheral sensation. These results suggest that sweet-sensing GRs are involved in the signal transduction of acid sensation rather than direct sensors.

The *GAL4/UAS* system in the mutant background was used to genetically recover the deficits of *Gr5a* and *Gr61a*. Using its specific *GAL4* drivers, the mutant deficits of *Gr5a* and *Gr61a* were significantly recovered to levels similar to those of the wild-type control upon exposure to GA, LA, and CA, as demonstrated by our electrophysiology analyses (Fig. 4C) and binary food choice assays (Fig. 4D). However, we did not further verify six genes of the *Gr64* cluster. Nevertheless, these results strongly suggest that sweet GRs are involved in the tuning of acid responses in the labellum. Furthermore, our study is the first to demonstrate the role of *Gr61a* deficits in carboxylic acid attraction. *Gr61a* is known to be expressed in sweet-sensing GRNs and is phylogenetically close to sweet GRs (Dahanukar et al., 2007; Robertson et al., 2003). Therefore, *Gr61a* was functionally verified as a sweet *Gr* in this study.

DISCUSSION

Carboxylic acids such as glycolic acid, lactic acid, and citric acid are especially attractive to flies at ecologically relevant concentrations

A major unresolved question in the field of taste perception is whether animals distinguish different acid levels based only on relative acidity. Based on the outcomes of this study as well as our previous findings, flies clearly prefer GA, LA, and CA at a pH level of approximately 2.5. However, we previously demonstrated that wild-type flies clearly avoided acetic acid and propionic acid at pH 2.5 (Rimal et al., 2019). These observations made us speculate whether these preferences were driven by the carbon backbone of the carboxylic acid compounds. Specific anions and their concentrations are also important factors that determine food preference in flies, which is very similar to how other animals distinguish different carbohydrates. In mice, for example, the G-protein coupled receptors T1R2 and T1R3 are required for carbohydrate sensing (Puri and Lee, 2021; Zhao et al., 2003). GR64f is a co-receptor in flies (Jiao et al., 2008), but the mechanisms by which different sugars can be identified by different combinations of sweet GRs remain uncertain. Heterologous expression of a minimum number of sweet GRs should be conducted to address these questions. However, previous attempts to conduct these experiments based on many trials of

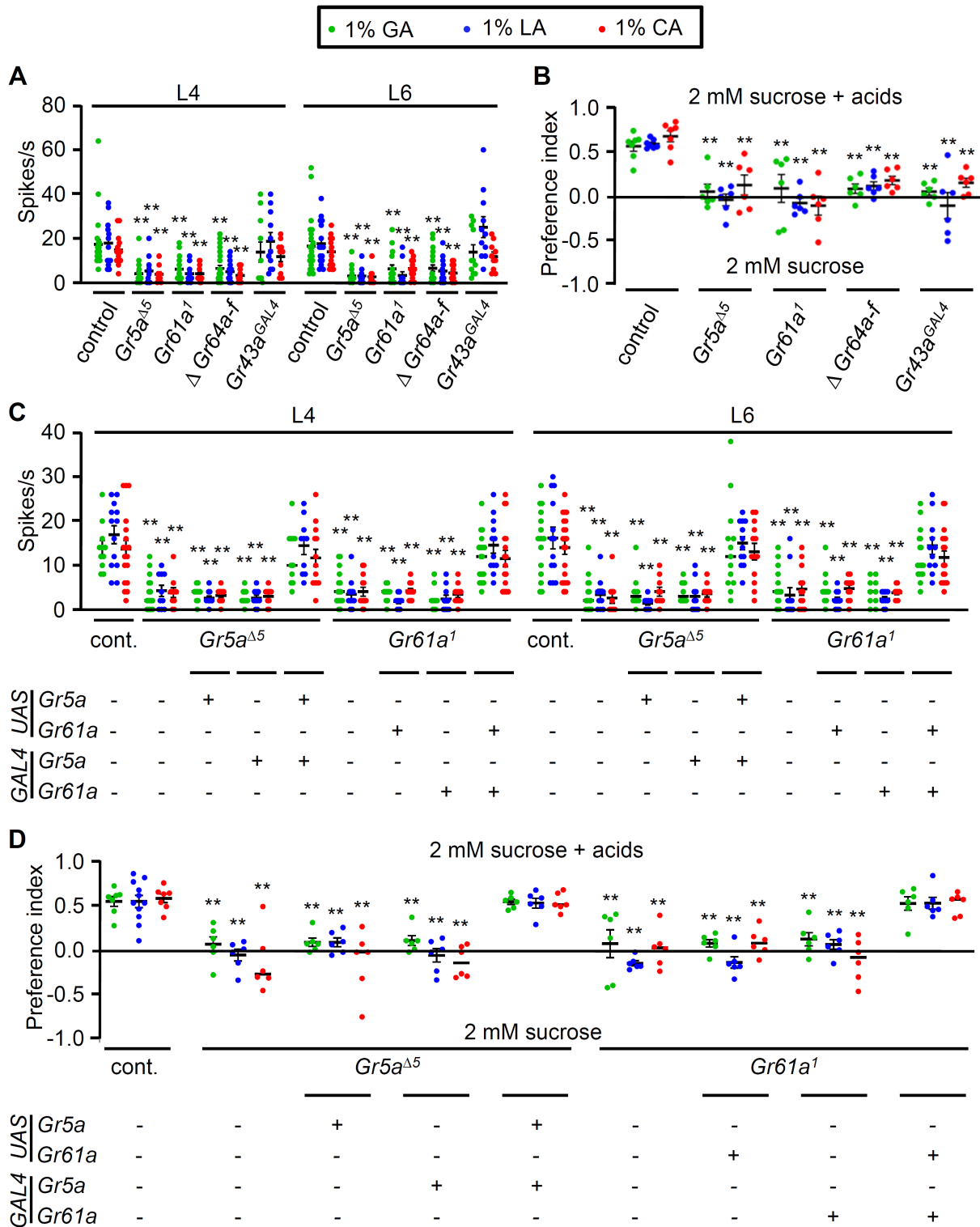


Fig. 4. Sweet GRs are required for carboxylic acid sensation. (A) Neuronal firing responses of the control, *Gr5a^{Δ5}*, *Gr61a¹*, Δ *Gr64a-f*, and *Gr43a^{GAL4}* mutants from L4 and L6 sensilla stimulated by the indicated acids ($n = 10-14$). (B) Binary food choice assays of control and sweet *Gr* mutants: *Gr5a^{Δ5}*, *Gr61a¹*, Δ *Gr64a-f*, and *Gr43a^{GAL4}* ($n = 6-8$). (C) Electrophysiological rescue of *Gr5a^{Δ5}* and *Gr61a¹* mutant defects on L4 and L6 sensilla elicited by the indicated acids by expressing *UAS-Gr5a* or *UAS-Gr61a* under the control of its specific *GAL4s* ($n = 10-16$). (D) Behavioral rescue of *Gr5a^{Δ5}* and *Gr61a¹* deficits in the indicated acid attraction by expressing *UAS-Gr5a* or *UAS-Gr61a* under the control of their specific *GAL4s* ($n = 6-8$). All error bars represent the SEM. Multiple sets of data were compared using single-factor ANOVA coupled with Scheffe's post hoc test. Asterisks indicate statistical significance compared with the control (** $P < 0.01$).

several groups have not been successful.

Acid sensing is thought to prevent the consumption of unripe foods or foods containing harmful bacteria. However, we found that ecologically relevant concentrations (range, 10–150 mM) of carboxylic acids may provide a good energy source to flies. This attractive behavior is mediated by the expression of two IRs (IR25a and IR76b) and sweet GRs (except GR43a) in the sweet-sensing GRNs.

Molecular sensors for sour taste

Recent studies (including this study) have proposed that sour sensation is quite complex in flies compared with sugar or bitter sensation (Ganguly et al., 2021; Mi et al., 2021; Rimal et al., 2019; Stanley et al., 2021). Wild-type females prefer acid-laced food to lay eggs, which is mediated by the expression of IR25a and IR76b in the GRNs of the tarsal segment (Chen and Amrein, 2017). Here, we demonstrated that flies with excised forelegs fed normally with acid-laced food, whereas the inactivation of sweet-sensing GRNs suppressed the attractive behavior. IR25a and IR76b are very broadly expressed in the labellum as well as the tarsal segments (Chen and Amrein, 2017; Lee et al., 2018). These observations suggest that IR25a and IR76b in the labellum and legs are important for the detection of acid-laced foods. Furthermore, the role of IR25a in LA sensation was also evaluated (Stanley et al., 2021). However, the authors found that IR76b did not contribute to LA attraction, which was controversial among different groups. Our study provides not only feeding behavior data, but also single sensillum recordings in mutant flies and genetic recovery with specific *GAL4* drivers, as well as with the sweet-sensing GRN marker *Gr5a-GAL4*. Furthermore, our study evaluated the response of flies to three different carboxylic acids. Therefore, we believe that our findings provide solid evidence for the involvement of IR76b and IR25a in acid attraction.

Our study also demonstrated that most sweet GRs are required for acid attraction and neuronal firing in sweet-sensing GRNs. While we were preparing for the present study, the involvement of sweet GRs in LA attraction was also evaluated by another group (Stanley et al., 2021). The authors confirmed that the $\Delta Gr64a-f$ mutant could not normally sense LA using calcium imaging (Stanley et al., 2021). Together with our present and previous findings, the aforementioned study demonstrated that sweet GRs mediate acid-induced neuronal activation in sweet-sensing GRNs. Further, as demonstrated by our behavioral experiments and tip recordings, we genetically restored the ability of the *Gr5a* and *Gr61a* mutants to exhibit attractive responses to three carboxylic acids. We finally sought to identify the anatomical structures where *Gr43a* is required for the sensing of carboxylic acids. Our findings indicated that GR43a did not mediate acid sensation in the labellum, as normal neuronal activities to the acids were identified in the *Gr43a* mutant. However, the *Gr43a* mutant exhibited defects that were similar to those of other sweet *Gr* mutants.

Previous studies on IRs and GRs have not been able to conclusively demonstrate their role as molecular sensors for sour taste because none of these studies have demonstrated the direct activation of the IRs by acid. Recently, however, sev-

eral studies have proposed otopetrin (i.e., a proton-selective channel protein) as a molecular sensor of sour taste in mammals and flies (Ganguly et al., 2021; Mi et al., 2021; Tu et al., 2018). These studies have demonstrated the direct activation of the otopetrin channel coupled with deficits in acid-mediated behavior, suggesting that this evolutionarily conserved channel protein has a role in proton sensing. Again, sour taste appears to be mediated by not only protons but also anions and their concentrations. Moreover, the integration of peripheral acid-sensing information in the brain appears to be much more complex than we anticipated. Nevertheless, we propose that IRs and GRs are equally important to discriminate acid attraction.

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AUTHOR CONTRIBUTIONS

B.S. and Y.L. conceived and designed the experiments. B.S. performed the experiments. B.S. and Y.L. wrote the manuscript. Y.L. supervised the study.

CONFLICT OF INTEREST

The authors have no potential conflicts of interest to disclose.

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