Molecules and Cells

Molecular Basis of Hexanoic Acid Taste in *Drosophila melanogaster*

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Animals generally prefer nutrients and avoid toxic and harmful chemicals. Recent behavioral and physiological studies have identified that sweet-sensing gustatory receptor neurons (GRNs) in *Drosophila melanogaster* mediate appetitive behaviors toward fatty acids. Sweet-sensing GRN activation requires the function of the ionotropic receptors IR25a, IR56d, and IR76b, as well as the gustatory receptor GR64e. However, we reveal that hexanoic acid (HA) is toxic rather than nutritious to *D. melanogaster*. HA is one of the major components of the fruit *Morinda citrifolia* (noni). Thus, we analyzed the gustatory responses to one of major noni fatty acids, HA, via electrophysiology and proboscis extension response (PER) assay. Electrophysiological tests show this is reminiscent of arginine-mediated neuronal responses. Here, we determined that a low concentration of HA induced attraction, which was mediated by sweet-sensing GRNs, and a high concentration of HA induced aversion, which was mediated by bitter-sensing GRNs. We also demonstrated that a low concentration of HA elicits attraction mainly mediated by GR64d and IR56d expressed by sweet-sensing GRNs, but a high concentration of HA activates three gustatory receptors (GR32a, GR33a, and GR66a) expressed by bitter-sensing GRNs. The mechanism of sensing HA is biphasic in a dose dependent manner. Furthermore, HA inhibit sugar-mediated activation like other bitter compounds. Taken together, we discovered a binary HA-sensing mechanism that may be evolutionarily meaningful in the foraging niche of insects.

Keywords: attraction, aversion, gustatory receptor, hexanoic acid, ionotropic receptor, noni

INTRODUCTION

Taste perception plays an essential role in feeding behavior. Likewise, the aversion to harmful and toxic chemicals is critical for animals' survival. Hence, animals have evolved chemoreceptors to sense nutritious and non-nutritious chemicals, depending on their niche. In mice, various chemoreceptors are expressed in distinct populations of taste bud cells on the tongue. For example, two different groups of cells respond to carbohydrates and amino acids, respectively. A separate group of cells responds to bitter chemicals. The responsive taste receptors, T1Rs and T2Rs, mainly activate pertussis toxin-insensitive G-proteins (Gq), phospholipase C (PLC), and TRPM5 (involved in the sensing of semiochemicals) to potentiate the taste bud cells. In addition, two functionally distinct types of taste cells detect sour and salt (Puri and Lee, 2021). The intrinsic quality of tastants is initially sensed by the taste organ. This information is then transferred to the gustatory cortex in the brain. The connection between the periphery and the central nervous system is referred to as the labeled line model of taste coding.

Drosophila melanogaster is an excellent genetic model organism for studying the cellular and molecular mechanisms of each taste category (Shrestha and Lee, 2023). Similar to

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mammals, flies can detect sweet, amino acid, bitter, salty, and sour tastants. Taste chemoreceptors are distributed on the labellum (fly tongue), legs, wings, and internal pharynx (esophagus). The labellum contains 31 taste sensilla in each half. Taste sensilla are classified as long, intermediate, and short (L, I, and S), depending on the length. L- and S-type sensilla have four gustatory receptor neurons (GRNs), whereas I-type sensilla have only two GRNs. Gustatory receptors (GRs) and ionotropic receptors (IRs) are major chemoreceptors that detect sweet, amino acid, bitter, salty, nitrogenous waste, fermented histamine, vitamin C, and sour tastants in flies (Aryal et al., 2022a; Aryal and Lee, 2021; 2022; Dahanukar et al., 2007; Dhakal et al., 2021; Ganguly et al., 2017; Lee et al., 2010; McDowell et al., 2022; Rimal et al., 2019; 2020; Sang et al., 2019; 2021; Shrestha and Lee, 2021a; 2021b; Shrestha et al., 2022; 2023; Stanley et al., 2021; Thorne et al., 2004; Zhang et al., 2013a). Furthermore, transient receptor potential (TRPA1, TRPL, and painless) channels are involved in detecting pungent and aversive chemicals: aristolochic acid, wasabi, and camphor (Al-Anzi et al., 2006; Kang et al., 2010; Kim et al., 2010; Zhang et al., 2013b). Pickpocket (PPK23 and PPK28) channels are required for sensing water and contact pheromone (Cameron et al., 2010; Thistle et al., 2012). Rhodopsin G-protein coupled receptors (Rh1, Rh4, and Rh7) are required for detecting aristolochic acids (Leung et al., 2020). Otopetrin (OTOP1) is a well-conserved proton sensor in mammals and flies (Ganguly et al., 2021; Mi et al., 2021; Tu et al., 2018).

Naturally occurring fatty acids are carboxylic acids with an aliphatic chain containing an even number of saturated or unsaturated carbon atoms, from 4 to 28 (Chauhan and Varma, 2009). Among them, glycolic acid, citric acid, and lactic acid taste attractions for flies are mediated by GRs (GR5a, GR61a, and GR64a-f) and IRs (IR25a and IR76b) expressed in sweet-sensing GRNs (Shrestha and Lee, 2021a; Stanley et al., 2021). The aliphatic chain can be saturated or unsaturated. Viscosity increases with the longer chain length of saturated fatty acids, so long-chain saturated fatty acids have a greasier mouthfeel than those of less viscosity. Generally, we experience that marbling in steak can be tasty. Likewise, multiple studies show that flies like fatty acids, such as hexanoic acid (HA) and octanoic acid. The PLC pathway and the potential chemoreceptors (IR56d or GR64e) expressed in sweet-sensing GRNs mediate this attraction (Brown et al., 2021; Kim et al., 2018; Masek and Keene, 2013). Another study shows that fatty acids can activate sweet-sensing GRNs in sensilla on the legs, which require two broadly tuned IR25a and IR76b in addition to a specific IR, IR56d (Ahn et al., 2017).

Here, we identified controversial results using the same concentration of HA, although we agree with the attractive effect of HA at a 10-fold lower concentration than 1% HA the other researchers used. First, we newly identified that GR64d and IR56d are essential chemoreceptors of the sweet-sensing GRNs in L-type sensilla for detecting an attractive HA concentration (0.1%). Second, at least three GRs (GR32a, GR33a, and GR66a) are fundamental in eliciting the aversion to 1% HA (mostly used by other research groups), which is mediated by bitter-sensing GRNs in S-type sensilla. Although IR25a, IR56d, and IR76b may function in the legs (Ahn et al., 2017), IR25a and IR76b have no role in the labellum because those mutants have statistical non-significance in electrophysiology.

MATERIALS AND METHODS

Drosophila strains

All flies were grown at 25°C under 12-h light/12-h dark cycles. Both males and females were mixed randomly for the experiments. Wild-type (w^{1118}) was used as a control strain. We described the following lines previously (Aryal et al., 2022a): $Ir7a^1$, $Ir47a^1$, $Ir52a^1$, $Ir56a^1$, $Ir60b^3$, $Ir94a^1$, $Ir94c^1$, and $Ir94h^1$. We received $Ir7g^1$ (BL42420), $Ir8a^1$ (BL41744), $Ir10a^1$ (BL23842), $Ir21a^1$ (BL10975), $Ir48a^1$ (BL26453), $Ir48b^1$ (BL23473), $Ir51b^1$ (BL10046), $Ir52b^1$ (BL25212), $Ir52c^1$ (BL24580), $Ir56b^1$ (BL27818), $Ir56d^1$ (BL81249), $Ir62a^1$ (BL32713), $Ir67a^1$ (BL56583), $Ir75d^1$ (BL24205), $Ir85a^1$ (BL24590), $Ir92a^1$ (BL23638), $Ir94b^1$ (BL23424), $Ir94d^1$ (BL33132), $Ir94f^1$ (BL33095), $Ir94g^1$ (BL25551), $Ir100a^1$ (BL31853), *UAS-Kir2.1* (BL6596), $Gr2a^1$ (BL18415), $Gr10a^1$ (BL29947), $Gr22f^1$ (BL43859), $Gr23a^1$ (BL19287), $Gr28b^{Mi}$ (BL24190), $Gr36b^1$ (BL24608), $Gr36c^1$ (BL26496), $Gr58b^1$ (BL29065), $Gr59a^1$ (BL26125), $Gr77a^1$ (BL26374), $Gr93d^1$ (BL27800), $Gr94a^1$ (BL17550), and $Gr97a^1$ (BL18949) strains from the Bloomington Drosophila Stock Center. Dr. Craig Montell and Dr. R. Benton kindly provided strains *UAS-Gr64d* and *UAS-Ir56d* (Sánchez-Alcañiz et al., 2018), respectively. In addition, $Gr33a^1$, *Gr33a-GAL4* (Moon et al., 2009), $Gr8a^1$ (Lee et al., 2012), $Gr93a^3$ (Lee et al., 2009), $Gr98b^1$ (Shim et al., 2015), $Gr47a^1$ (Lee et al., 2015), and $Gr66a^{ex83}$ (Moon et al., 2006) fly strains were described in our previous studies (Shrestha et al., 2023). Previously, we used the following lines (Aryal and Lee, 2021; Aryal et al., 2022a; Shrestha and Lee, 2021a): *ppk23-GAL4*, *ppk28-GAL4*, $Gr22e^1$, $Ir25a^2$, *Ir76b1*, $Gr5a^{\Delta 5}$, $Gr61a^1$, *Gr66a-GAL4*, $Gr64a^{GAL4}$, $Gr64b^{LEXA}$, $Gr64c^{LEXA}$, $Gr64d^1$, $Gr64e^{LEXA}$, $Gr64f^{LEXA}$, *Gr64f-GAL4*, $Gr28a^1$, *Δ Gr32a*, $Gr36a^1$, $Gr39b^1$, $Gr59c^1$, and $Gr89a^1$. The *Ir56d-GAL4* line was obtained from the Korea *Drosophila* Resource Center (GIST, Korea). $Gr43a^1$ was generated in our other study.

Chemical reagents

HA (Cat. No. W255912), tricholine citrate (CAS No. 546-63-4), and sucrose (CAS No. 57-50-1, Cat. No. S9378) were purchased from Sigma-Aldrich (USA).

Proboscis extension response (PER) assay

The PER assay was carried out as previously described with some modifications (Poudel and Lee, 2016). First, the flies were starved for 18-20 h. Flies were then anesthetized on ice. Fly bodies and tarsi were confined inside a cut 200 µl pipette tip while the flies' heads and proboscis were exposed. The flies were kept in a humidified box for 1 h. Flies were given water to sip freely until satisfied to exclude the water-associated response. Kimwipe paper wicks served as the medium to deliver tastant stimuli to the flies. For low HA concentration, the water response represented the control, and then 0.1% HA was given. However, for high HA concentration, 2% sucrose concentration was given as an initial stimulus, and 1% HA was then delivered along with the 2% sucrose. The

proboscis was gently touched with moist wicks. Flies that did not show complete proboscis extension toward sucrose were discarded. The test solution was then administered, consisting of 1% HA with 2% sucrose stimulus, and the extension of proboscis was scored as the positive PER. Over 10 flies per trial were used as n = 1. Therefore, we calculated the rate of PER.

Electrophysiology

Tip-recording tests were undertaken, as previously described (Shrestha et al., 2022). We collected 4- to 7-day-old flies and tranquilized them on ice. A reference glass electrode filled with Ringer's solution (3 mM $CaCl_2 \cdot 2H_2O$, 182 mM KCl, 10 mM NaCl, 10 mM Tris base, 1 N HCl; pH 7.2) was inserted into the thorax and reached their proboscis. The 5 to 6 live insects were prepared for each setting, and the identical procedure was repeated several times. Tricholine citrate (30 mM) solution was used as electrolytes in recording pipettes with tip diameters ranging from 10 to 20 μm to excite the sensillum for 5 s during recordings. The recording electrode was connected to a preamplifier (Taste Probe; Syntech, Netherlands), and the signals were collected and amplified by 10× using a signal connection interface box (Syntech) and a 100-3,000 Hz band-pass filter. Data for action potential (AP) of 12 kHz were recorded and analyzed using Autospike 3.1 software (Syntech). Each following recording had a stimulation interval of around 1 min. Only spikes evoked between 50 and 550 ms were counted. The response's average AP frequencies (spikes/s) are shown.

Survival assay

Survival tests were performed according to the guidelines of a previous study (Shrestha et al., 2022). Different food sources were prepared, including 1% sucrose and 1% sucrose added with 0.1%, 0.5%, 1%, and 2% HA. Ten male and 10 female flies of each sex, aged 3 to 4 days, were given each of these food sources. The viability of the fly was then measured every 12 h. The flies were then transferred to fresh vials with the same food supply.

Statistical analysis

The studies were conducted over a period of days. Data were analyzed using Prism 8.0 (GraphPad Software, USA) (RRID:SCR 002798). The raw data were presented in graphs. The sample size of each experiment is mentioned in the figure legend. Each error bar shows an SEM. A single-factor ANOVA and Scheffe's post hoc analysis were performed for multiple comparisons. The Origin program (Origin Lab Corporation; RRID:SCR 002815) was used to determine the statistical significance ($*P < 0.05$, $**P < 0.01$).

RESULTS

HA is toxic to *D. melanogaster*

HA is one of the representative fatty acids attractive to flies. Therefore, we tested the nutritional status of the flies to measure how long they could survive by feeding on HA only. As positive and negative controls, we fed 1% sucrose and complete starvation with 1% agar (Fig. 1A). Interestingly we found that the range of 0.5%-2.0% HA was toxic in the survival assay, although a low concentration of 0.1% HA increased starvation resistance. The lethality among 50% of the flies (LT_{50}) fed 1% sucrose was 250.50±10.87 h, whereas the LT_{50} was 146.00±8.24 h for 0.1% HA. However, the toxic range of HA reduced the life span even more than the starved condition while 0.1% HA enhanced the survivability. This demonstrates that HA may exert nutritious and harmful effects in flies.

HA activates a dose-dependent biphasic activity

Labellar taste sensilla have three categories (L-, I-, and S-type) according to the length. The dose-response curve by electrophysiology represented that L6 sensilla were most responsive to 0.1% HA (Fig. 1B). Higher concentrations of HA induced much lower neuronal responses on the L6 sensilla. However, we found that S6 sensilla responded to HA in a dose-dependent manner, while the responses of I8 sensilla to any concentration of HA were negligible. This was reminiscent of the arginine (an amino acid)-induced neuronal responses, which is a biphasic activation (Aryal et al., 2022a). In other words, a low concentration of arginine activates sweet-sensing GRNs, but a high concentration of arginine activates bitter-sensing GRNs. To test whether this applies to HA, we decided to ablate each specific GRN. Generally, GRNs can be classified into two attractive groups (sweet- and water-sensing GRNs) and two aversive groups (bitter- and calcium-sensing GRNs) (Lee et al., 2018). Using an inwardly rectifying potassium channel (Kir2.1), we inactivated each GRN (Figs. 1C and 1D). The responses to 0.1% HA by L6 sensilla were significantly dependent on the sweet-sensing GRNs because only *Gr64f-GAL4/UAS-Kir2.1* flies presented reduced AP, but others and control flies had similar neuronal activation (Fig. 1C). Next, we tested 1% HA. Surprisingly, it was found to activate S6 sensilla only, which harbor bitter-sensing GRNs (Fig. 1D). However, the other ablated flies and control flies showed normal responses in electrophysiology. Here, we demonstrated that a low concentration of HA induced attraction, which was mediated by sweet-sensing GRNs, and a high concentration of HA might induce aversion, which is mediated by bitter-sensing GRNs (Fig. 1E).

To further test this hypothesis, we performed behavioral assays with the same concentrations of HA and the same flies. The binary food choice assay is the most popular method to evaluate the gustatory function (Aryal et al., 2022b). However, the flies did not eat HA in sufficient amounts, which caused difficulty in performing the assay. Therefore, we tested behaviors using the PER (Poudel and Lee, 2016; Rimal and Lee, 2019). First, 10 to 15 flies per round were starved, immobilized, and sated with water (see detail in Materials and Methods section). Only flies showing PER to water stimuli were selected for testing HA. Again, we found that only the sweet-sensing GRNs-ablated flies showed decreased PER to 0.1% HA (Fig. 1F). The reduced PERs to 0.1% HA were comparable to the reduced PERs to sucrose of sweet-sensing GRNs-ablated flies. However, the other ablated flies presented normal attractive responses to 0.1% HA compared with control flies. This indicates the role of sweet-sensing GRNs in the perception of low HA. Next, we measured the PER to 1%

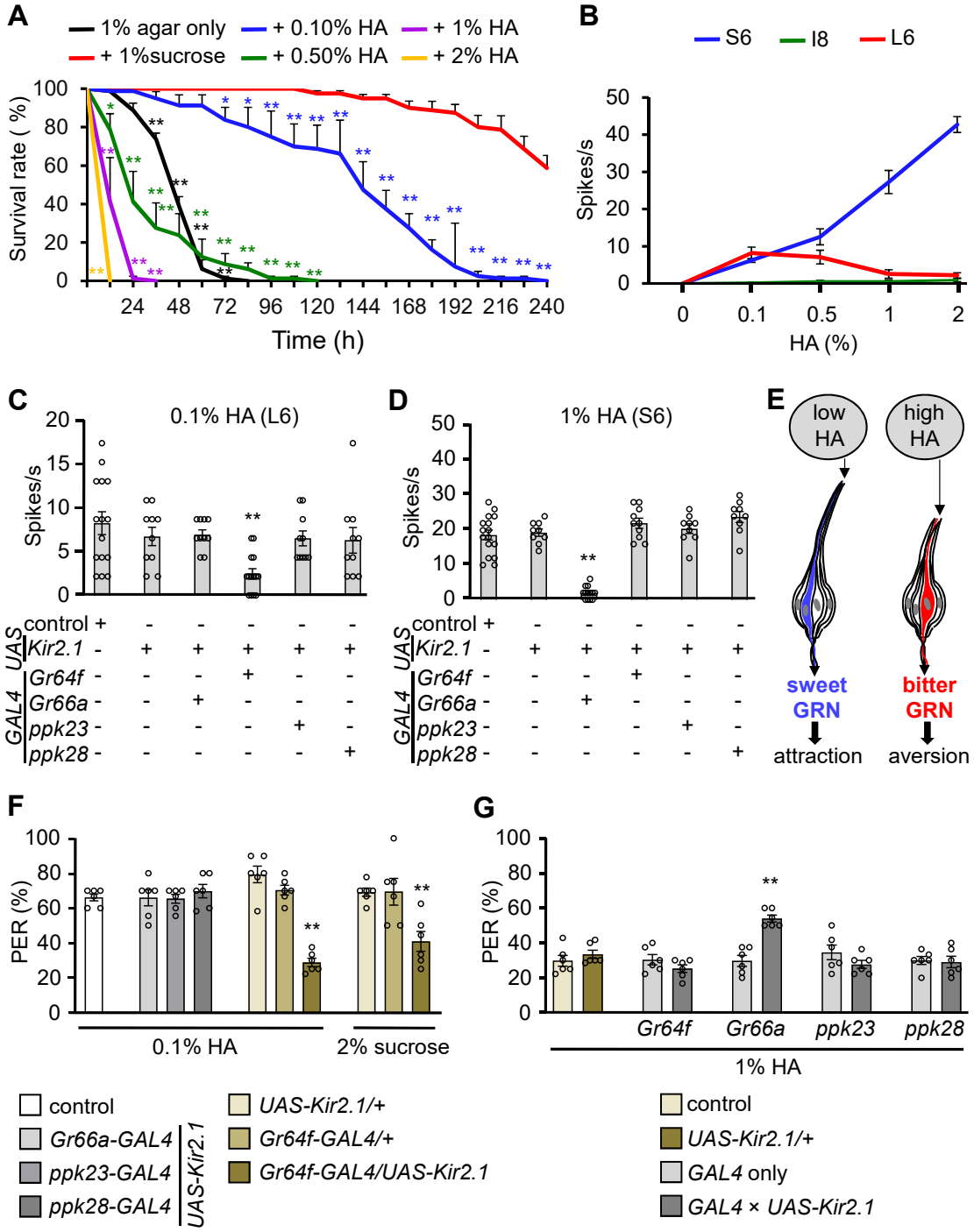

Fig. 1. Toxicity and biphasic activations of hexanoic acid (HA) in a dose-dependent manner. (A) Survival rate of control flies fed with 1% sucrose alone, 1% sucrose with the indicated amounts of HA (0.1%, 0.5%, 1%, and 2%), and 1% agar only (n = 4). (B) Dose-response curve of HA tip-recordings from S6, I8, and L6 sensilla (n = 10-12). (C) Tip recording in the presence of 0.1% HA after inhibiting different GRNs (*Gr64f-GAL4* [sweet-sensing], *Gr66a-GAL4* [bitter-sensing], *ppk23-GAL4* [calcium-sensing], and *ppk28-GAL4* [water-sensing]) by expressing *UAS-kir2.1* under the control of the indicated *GAL4*s on L6 sensilla (n = 10-15). (D) Tip recording in the presence of 1% HA after inhibiting above-mentioned GRNs by expressing *UAS-kir2.1* under the control of the indicated *GAL4*s on S6 sensilla (n = 10-16). (E) Diagrammatic representation showing the dual mechanism of HA sensation on sweet gustatory receptor neuron (GRN) and bitter GRN. (F) Proboscis extension response (PER) analysis of indicated neuron-ablated flies using above-mentioned *GAL4*s to 0.1% HA and *Gr64f-GAL4*-ablated flies to 2% sucrose (n = 6). (G) PER response of neuron-ablated flies of above-mentioned *GAL4*s to 1% HA (n = 6). All error bars represent SEMs. Single-factor ANOVA coupled with Scheffe's post hoc analysis was performed to compare multiple sets of data. Asterisks indicate statistical significance compared with the control (*$P < 0.05$, **$P < 0.01$).

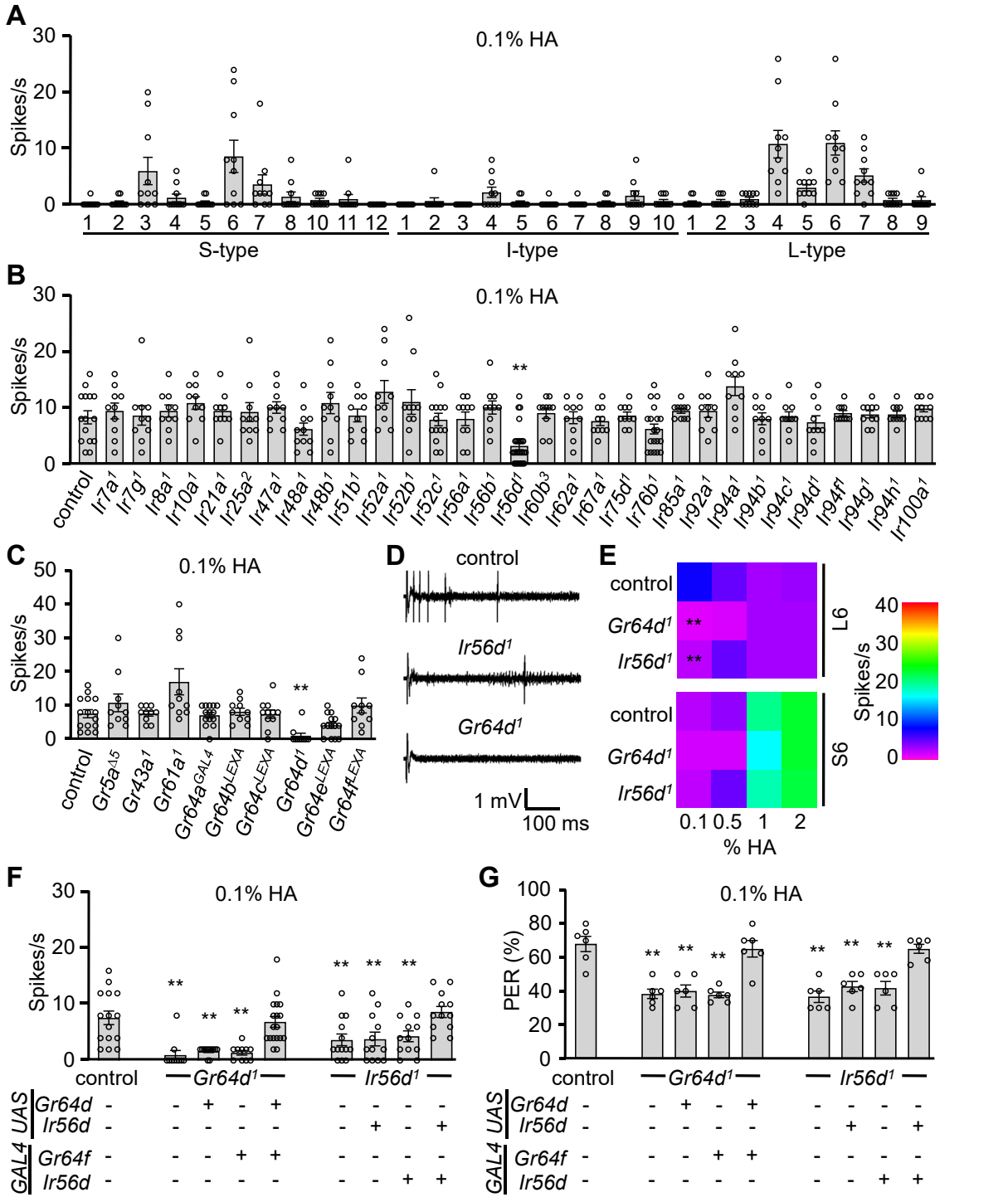

Fig. 2. Genetic screens using electrophysiology with 0.1% hexanoic acid (HA) and the behavioral assay. (A) Tip recordings from all labellar sensilla of control flies (n = 10) by stimulation with 0.1% HA. (B) Tip-recording analyses from L6 sensilla to 0.1% HA for control and 31 *Ir* mutants (n = 10-15). (C) Tip-recording analyses from L6 sensilla to 0.1% HA for control and nine sweet *Gr* mutants (n = 10-16). (D) Representative sample traces of control and candidate mutants (*Gr64d*^{1} and *Ir56d*^{1}) from (B) and (C). (E) Tip recordings with dose responses from L6 and S6 sensilla to 0% to 2% HA for control, *Gr64d*^{1}, and *Ir56d*^{1} (n = 10-20). (F) Recovery experiments using tip-recording assays from L6 sensilla for *Gr64d*^{1} and *Ir56d*^{1} defects. Genetically recovered flies were driven by crossing each wild-type gene with *Gr64f-GAL4* and *Ir56d-GAL4*, respectively (n = 10-18). (G) Proboscis extension response (PER) analyses showing the defect and rescue response from labellum for *Gr64d*^{1} and *Ir56d*^{1} defects (n = 6). All error bars represent SEMs. Single-factor ANOVA coupled with Scheffe's post hoc analysis was performed to compare multiple sets of data. Asterisks indicate statistical significance compared with the control (**$P < 0.01$).

HA (Fig. 1G). The PERs were relatively low compared with 0.1% HA in control flies (w^{1118} as well as *UAS-Kir2.1*/+). We interpreted that it was caused by the activation of bitter-sensing GRNs. These reduced PERs were comparable in all the tested *GAL4* only or each ablated fly, except *Gr66a-GAL4*/*UAS-Kir2.1* flies (Fig. 1G). The finding specifies the function of bitter-sensing GRNs in detecting high dose of HA. Overall, we conclude that HA induces a biphasic response, depending on the concentration.

IR56d and GR64d are required for the neuronal responses of 0.1% HA

We identified that 0.1% HA was attractive and activated sweet-sensing GRNs. Therefore, we systematically analyzed all 31 sensilla using 0.1% HA to find responsive sensilla (Fig. 2A). As a result, we found that S3, S6, L4, L6, and L7 were significantly stimulated by 0.1% HA. Next, we screened available mutant libraries of IRs and sugar GRs from the most responsive sensilla, L6 (Figs. 2B-2D). First, we identified IR56d and GR64d from the screening. Second, dose-response profiles of L6 and S6 sensilla were characterized for control, $Gr64d^{1}$, and $Ir56d^{1}$ flies (Fig. 2E). Again, the two mutants were significantly different from control flies only for the response from L6 sensilla to 0.1% HA and not from S6 sensilla to 0.1% HA. This indicated that the S3 and S6 sensilla responses to 0.1% HA were mainly mediated by bitter-sensing GRNs or combined responses rather than solely mediated by sweet-sensing GRNs. Third, we also confirmed the deficit responses from the L4 sensilla of $Gr64d^{1}$ and $Ir56d^{1}$ (Supplementary Fig. S1). However, the responses of S3 and L7 were not significant. Fourth, we recovered the reduced neuronal responses and behavioral deficits by the wild-type cDNA expression driven by *Gr64f-GAL4* or its own *GAL4* (Figs. 2F and 2G). These data indicate that flies possess both GR and IR dependent mechanisms for gustatory attraction to low HA. Overall, we concluded that GR64d and IR56d were indispensable for the attractive responses to HA.

GR32a, GR33a, and GR66a are essential for the neuronal responses of 1% HA

To test the aversive effect of HA, we performed mapping analyses of the neuronal responses from all 31 sensilla to 1% HA (Fig. 3A). From the results, we identified that most S-type sensilla were responsive to 1% HA, although all the I- and L-types did not respond. S3, S5, S6, S7, and S10 sensilla produced the highest APs by the stimulation with 1% HA. Next, we screened IRs and GRs (Figs. 3B and 3C). We found that previous potential candidates (IR25a, IR56d, and IR76b) were normal in electrophysiology (Fig. 3B). However, we found that broadly required bitter GRs (GR32a, GR33a, and GR66a) presented significantly decreased neuronal responses (Figs. 3C and 3D). Furthermore, these deficits were completely recovered by its own gene driven by its own *GAL4* (Fig. 3E). These genetic experiments confirmed that bitter GRs are necessary for high HA-induced nerve responses. Finally, we tested the responses elicited by HA at dose ranges of 0% to 2% on S6 sensilla of control, $\Delta Gr32a$, $Gr33a^{1}$, and $Gr66a^{ex83}$ flies (Fig. 3F). We found that all three mutants had significant deficits in their responses to HA concentrations ranging from 0.5% to 2%, although $Gr33a^{1}$ had deficits even at 0.1% HA. However, the S6 sensilla of all nine sweet GR mutants responded normally to 1% HA (Fig. 3G).

To further confirm the deficits of three GR mutants in electrophysiology, we performed the PER assay using 1% HA (Fig. 4A). Again, the PERs of $\Delta Gr32a$, $Gr33a^{1}$, and $Gr66a^{ex83}$ flies were significantly increased compared with the control flies. Moreover, the defects were completely recovered by the rescued flies of the $\Delta Gr32a$, $Gr33a^{1}$, and $Gr66a^{ex83}$ flies expressing their own wild-type genes. The deficits of PER using three mutants were detectable at the stimulus of 0.5% HA but not 0.1% HA (Fig. 4B). This indicates that 0.1% HA is not an aversive concentration, although the PER is reduced compared with sucrose only in control flies.

HA inhibits sugar responses

The PER responses were reduced by increasing the concentration of HA (Fig. 4B). The reduced PER to 0.1% HA was investigated. However, *Gr32a* and *Gr66a* mutants had no defects activating S6 sensillum at 0.1% HA, although the *Gr33a* mutant had defects (Fig. 3F). This means that activation of S6 by 0.1% HA is marginal in aversion. There are at least two mechanisms in bitter chemical sensation; bitter chemicals directly activate bitter-sensing GRNs, and bitter chemicals can inhibit sugar activation, which is called sugar inhibition (Chu et al., 2014; French et al., 2015; Jeong et al., 2013). To test sugar inhibition by HA, we measured sugar responses of L6 sensilla (Fig. 5). Then, the sucrose responses were compared with neuronal responses to the mixture of sucrose and HA. Sugar inhibition was detected for HA concentrations ranging from 0.1% to 1%. Therefore, we conclude that HA simultaneously activates the bitter-sensing GRNs and suppresses the sweet-sensing GRNs.

DISCUSSION

In this study, we characterized the cellular and molecular basis of HA sensation. First, we found a novel function of HA in the bitter-sensing GRNs, which was mediated by at least three GRs: GR32a, GR33a, and GR66a. The full collection of bitter GRs requires at least three receptors. For example, the expression of GR8a, GR66a, and GR98b is required to fully recapitulate the L-canavanine receptor (Shim et al., 2015). Likewise, GR93a, Gr33a, GR39a, and GR66a are required to recapitulate the caffeine receptor (Dweck and Carlson, 2020). However, we only identified the broadly expressed GRs. Therefore, further studies are required to find specific GRs to recapitulate the HA receptor. Based on the results of the mapping, specific GRs should be expressed by neurons of S-type but not I-type sensilla. In addition, we also characterized the sugar inhibition effect of HA-like bitter chemicals in a dose-dependent manner. A high concentration of HA can directly activate bitter-sensing GRNs and inhibit attractive signals like sugar at the same time.

We also identified GR64d and IR56d as sensors on the labellum that respond to a low concentration of HA. IR56d is known to be expressed by the sweet-sensing GRNs in the labellum as well as legs (Ahn et al., 2017; Brown et al., 2021). Therefore, we found consistent results in the electrophysiolo-

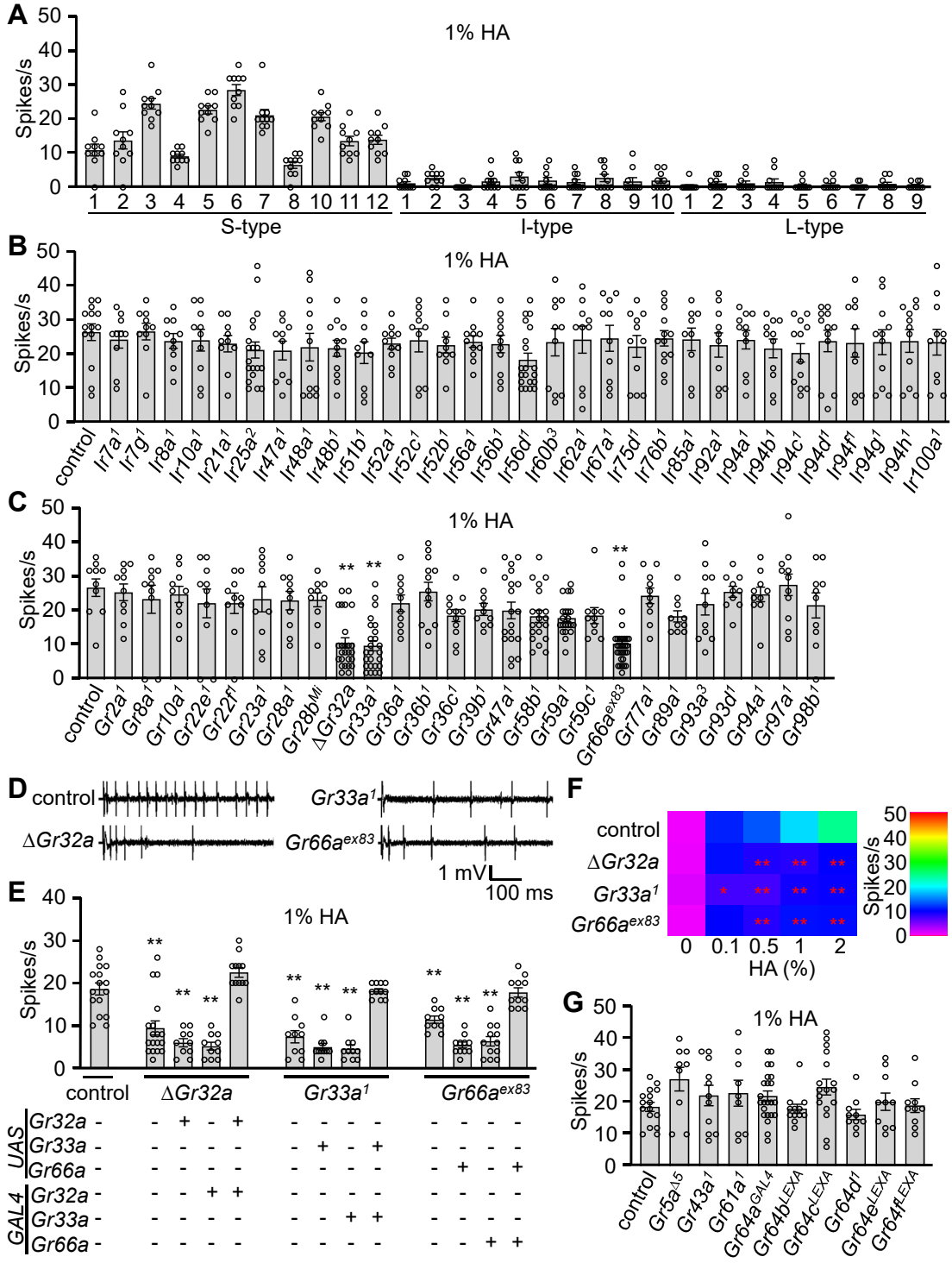

Fig. 3. Genetic screens using electrophysiology with 1% hexanoic acid (HA). (A) Mapping analyses using tip recordings from all 31 sensilla to 1% HA (n = 10). (B) Tip-recording assays from S6 sensilla of control and 31 *Ir* mutants (n = 10-23). (C) Screens with control and 26 *Gr* mutants to 1% HA using electrophysiology (n = 10-20). (D) Representative sample traces of control, Δ*Gr32a*, *Gr33a*^{1}, and *Gr66a*^{ex83} from (C). (E) Genetic rescues of Δ*Gr32a*, *Gr33a*^{1}, and *Gr66a*^{ex83} deficits in the neuronal responses to 1% HA aversion using its own *GAL4*/*UAS* systems (n = 10-16). (F) Heat map analyses representing dose-dependent responses of control, Δ*Gr32a*, *Gr33a*^{1}, and *Gr66a*^{ex83} using tip recordings from S6 sensilla to indicated concentration of HA (n = 10-20). (G) Tip-recording analyses from S6 sensilla to 1% HA for the control and nine sweet *Gr* mutants (n = 10). All error bars represent SEMs. Single-factor ANOVA coupled with Scheffe's post hoc analysis was performed to compare multiple sets of data. Asterisks indicate statistical significance compared with the control (**P* < 0.05; ***P* < 0.01).

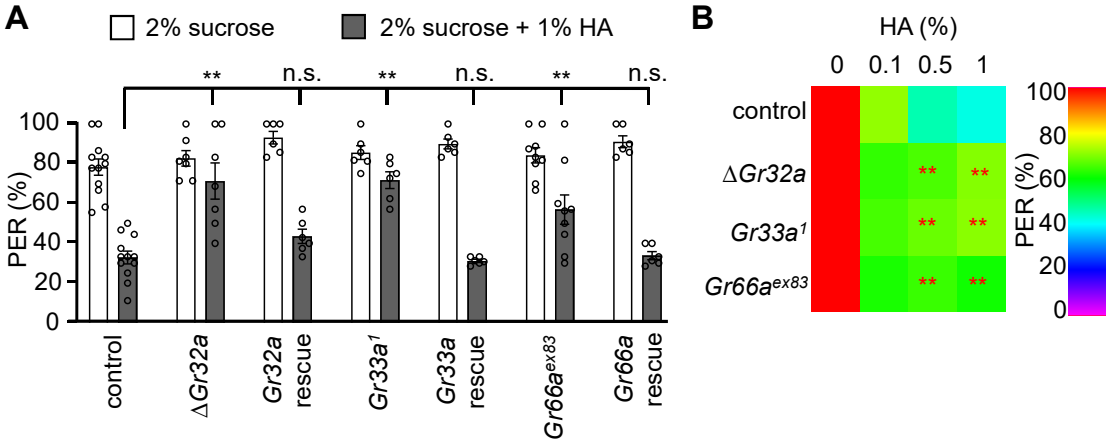

Fig. 4. Behavioral analysis by stimulation of labellum using 1% hexanoic acid (HA). (A) Behavioral rescues of Δ*Gr32a*, *Gr33a*^{1}, and *Gr66a*^{ex83} deficits to the 1% HA aversion using the expression of each *UAS* transgene driven by the respective *GAL4* (n = 6-11). (B) Concentration-dependent proboscis extension response (PER) responses by the stimulus to the labellum of control, Δ*Gr32a*, *Gr33a*^{1}, and *Gr66a*^{ex83}. The mixture of different concentrations of HA (0%, 0.1%, 0.5%, and 1%) and 2% sucrose was provided as experimental stimulus, and 2% sucrose only as control stimulus (n = 6-9). All error bars represent SEMs. Single-factor ANOVA coupled with Scheffe's post hoc analysis was performed to compare multiple sets of data. Asterisks indicate statistical significance compared with the control (**P < 0.01; n.s., indicates non-significance).

gy as well as PER assay by stimulating the labellum. Moreover, GR64d is a newly identified HA receptor because our electrophysiology and behavioral assay showed deficits in detecting 0.1% HA. However, previously characterized GR64e as a HA receptor was dispensable to detect HA in our experiments.

The taste perception in *Drosophila* involves the activation of specific GRNs via specific receptors in response to different chemicals. We identified two attractive and three aversive HA receptors, although we did not show recapitulation of these receptors in the GRNs that do not normally respond to HA. Each IR has its own kinetics to be activated or deactivated by chemicals. Recent study provides the model that the initiation of stimulus activates IR (IR25a) and removal of stimulus activate sweet GRs by lactic acid (Stanley et al., 2021). Likewise, it is possible that IR56d is involved in the onset response of HA and GR64d is activated by the offset response of HA. In the case of HA, only low concentrations between 0.1% and 0.5% can activate sweet-sensing GRNs. In contrast, concentrations over 1% HA did not induce any neuronal responses in the sweet-sensing GRNs. Once all HA receptors are identified, the activation threshold of the receptors can be tested by expressing these receptors in heterologous systems. The inhibition mechanism of over 1% HA in sweet-sensing GRNs is not known so far. However, the activations of bitter-sensing GRNs are highly dependent on the dose of HA.

The range from 0.1% to 0.5% HA induced similar levels of neuronal activation from L6 and S6, which may induce complex behavior. S-type sensilla have sweet-sensing and bitter-sensing GRNs, although L-type sensilla only have sweet-sensing GRNs. Therefore, the neuronal responses from S6 sensilla in this range can be expected from sweet-sensing GRNs. However, it should be tested with each GRN-ablated flies, GR and IR mutants. HA activates sweet-sensing GRNs to induce attraction and inhibits feeding behavior via direct activation of bitter-sensing GRNs and sugar inhibition. Moreover, different GRNs may be connected to different neural circuits that interpret the same chemical signal in various ways. Therefore, the perception of taste in *Drosophila* is a complex and dynamic process, influenced by both the sensitivity and specificity of GRNs and their neural circuits.

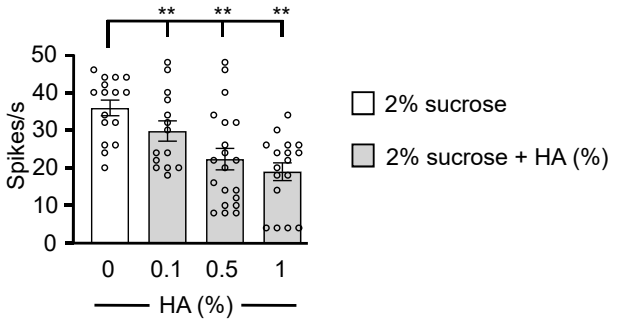

Fig. 5. Sugar inhibition by hexanoic acid (HA). Tip-recording analyses from L6 sensilla with 2% sucrose only or mixture of the indicated concentrations of HA (0%, 0.1%, 0.5%, and 1%) and 2% sucrose (n = 14-20). All error bars represent SEMs. Single-factor ANOVA coupled with Scheffe's post hoc analysis was performed to compare multiple sets of data. Asterisks indicate statistical significance compared with the control (**P < 0.01).

Fruit flies may evolve their chemoreceptors to survive in specific ecological niches. For example, HA is one of the fatty acids highly enriched in fruits like noni. Noni is toxic for all *Drosophila* except *D. sechellia* (Prieto-Godino et al., 2017). Therefore, *D. sechellia* has adapted to survive in environments of relatively high concentrations of HA. *D. sechellia* has a single amino acid change in IR75b, which allows the detection

of HA in olfaction (Prieto-Godino et al., 2017). Likewise, HA taste perception may act as a selective pressure on the evolution of *D. sechellia*, a sister species of *Drosophila*, allowing it to survive in the niche of noni. It will be fascinating to test *D. sechellia* by analyzing the related genes.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org)

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AUTHOR CONTRIBUTIONS

R.N.P. and B.S. conceived and performed experiments. Y.L. wrote the manuscript and supervised the project.

CONFLICT OF INTEREST

The authors have no potential conflicts of interest to disclose.

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