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Original Article

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An inhibitory mechanism for suppressing high salt intake in *Drosophila*

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High concentrations of dietary salt are harmful to health. Like most animals, *Drosophila melanogaster* are attracted to foods that have low concentrations of salt, but show strong taste avoidance of high salt foods. Salt in known on multiple classes of taste neurons, activating *Gr64f* sweet-sensing neurons that drive food acceptance and 2 others (*Gr66a* bitter and *Ppk23* high salt) that drive food rejection. Here we find that NaCl elicits a bimodal dose-dependent response in *Gr64f* taste neurons, which show high activity with low salt and depressed activity with high salt. High salt also inhibits the sugar response of *Gr64f* neurons, and this action is independent of the neuron's taste response to salt. Consistent with the electrophysiological analysis, feeding suppression in the presence of salt correlates with inhibition of *Gr64f* neuron activity, and remains if high salt taste neurons are genetically silenced. Other salts such as Na_2SO_4, KCl, $MgSO_4$, $CaCl_2$, and $FeCl_3$ act on sugar response and feeding behavior in the same way. A comparison of the effects of various salts suggests that inhibition is dictated by the cationic moiety rather than the anionic component of the salt. Notably, high salt-dependent inhibition is not observed in *Gr66a* neurons—response to a canonical bitter tastant, denatonium, is not altered by high salt. Overall, this study characterizes a mechanism in appetitive *Gr64f* neurons that can deter ingestion of potentially harmful salts.

Key words: taste, feeding, sweet taste inhibition, high salt, *Drosophila*

Introduction

The sense of taste enables animals to forage for nutritious food while also avoiding potentially hazardous compounds that they might encounter in the environment. Avoidance of toxic or harmful compounds is essential for survival and most animals have evolved highly sophisticated machineries to detect them. T2R taste receptors in mammals, for example, detect a wide range of naturally occurring toxins (Chandrashekar et al. 2000), and there is a much larger diversity of T2R taste receptors as compared to those that sense appetitive sweet and umami tastes (Behrens et al. 2007; Meyerhof et al. 2010).

A similar repertoire feature exists in *Drosophila melanogaster*, despite its distant evolutionary relationship with mammals. Within the Gustatory receptor (Gr) family in flies, many more Grs are dedicated to detecting aversive compounds as compared to tastants of other categories (Dweck and Carlson 2020; Ling et al. 2014; Robertson et al. 2003; Weiss et al. 2011). In addition, there are several other types of receptors that sense aversive compounds, including ionotropic receptors (Ir), opsins (Rh), transient receptor potential (Trp) channels, and *OtopLA* (Chen and Dahanukar 2020; Dhakal et al. 2021; Ganguly et al. 2021; Kang et al. 2010; Kim et al. 2010; Lee et al. 2017, 2018; Leung et al. 2020; Mi et al. 2021; Montell 2021).

Flies employ at least 2 different mechanisms for detecting and avoiding aversive chemicals. First, deterrent gustatory receptor neurons (GRNs), such as the *Gr66a* bitter GRNs, house receptor complexes required for sensing aversive compounds (Chen and Dahanukar 2020; Montell 2021). Activation of these GRNs leads to cessation of feeding (Thorne et al. 2004; Wang et al. 2004). A second mechanism involves direct inhibition of sugar-sensing GRNs by aversive tastants such as bitter compounds and high concentrations of organic acids (Charlu et al. 2013; French et al. 2015; Jeong et al. 2013). Previous research demonstrated that OBP49a, an olfactory binding protein (Obp) synthesized by the accessory cells and found in the sensillary endolymph, is necessary for bitter compound-mediated inhibition of sugar-sensing *Gr64f* GRNs (Jeong et al. 2013). How organic acids inhibit *Gr64f* GRN firing is not well understood, but it has been implied that it is the anionic moieties of the carboxylic acids rather than the protons that mediate inhibition (Ganguly et al. 2021). Additional mechanisms that act via cellular interactions between bitter GRNs and appetitive circuits involve GABAergic interneurons (Chu et al. 2014; Pool et al. 2014).

Salts are ionic compounds with anionic and cationic moieties. They provide essential micronutrients needed for proper bodily functions. However, consumption of too much salt can be harmful. In mammals, for example, ingestion of high quantities of salts has been linked to increased risk of hypertension, osteoporosis, gastrointestinal cancer, and autoimmune disease (Heaney 2006; Jones et al. 1997; Luft et al. 1979; Sharif et al. 2018; Strazzullo et al. 2009; Wu et al. 2021). Feeding on high concentrations of salt has been shown to be

Editorial Decision 8 May 2023.

detrimental in flies as well (Murashov et al. 2021; Naikkhwah and O'Donnell 2011, 2012; Stergiopoulos et al. 2009; Xie et al. 2019). As a result, though most animals prefer food with low concentrations of salt, higher concentrations are typically rejected.

A number of recent studies have investigated how salt is encoded in the taste system and found that NaCl is in fact sensed by all GRNs in the labellum (Dweck et al. 2022; Jaeger et al. 2018; Lee et al. 2017; McDowell et al. 2022; Zhang et al. 2013). Attraction to low salt is mediated by *Gr64f* GRNs (Dweck et al. 2022; Jaeger et al. 2018), which sense various other categories of appetitive tastants including sugars and fatty acids (Chen and Dahanukar 2020; Dahanukar et al. 2007; Marella et al. 2006; Masek and Keene 2013; Montell 2021; Tauber et al. 2017). On the other hand, high salt avoidance is mediated by its action on *Gr66a* bitter-sensing GRNs and glutamatergic *Ppk23* GRNs (Jaeger et al. 2018; Lee et al. 2017; McDowell et al. 2022). Ir76b, a widely expressed co-receptor, appears to function with different sets of Irs in different GRNs to mediate both attractive and repulsive taste responses to salt (Dweck et al. 2022; Jaeger et al. 2018; Lee et al. 2017; McDowell et al. 2022; Zhang et al. 2013). Receptors such as *ppk11*, *ppk19*, and *sano* have also been linked to high salt aversion (Alves et al. 2014; Liu et al. 2003). *ppk19* and *ppk11*, 2 members of *pickpocket* (*ppk*) gene family, are expressed in the taste-sensing terminal organ of larvae and in taste sensilla of the adult labellum, wing margins, and tarsi. In response to low and high salt concentrations, the Ppk19 and Ppk11 receptors are linked to appetitive and aversive behavior respectively (Liu et al. 2003). *sano*, on the other hand, is co-expressed with the *Gr66a* bitter co-receptor in the terminal organs of third-instar larvae and is required to avoid high salt (Alves et al. 2014).

We found that high salt can inhibit sugar-evoked calcium activity in *Gr43a*-labeled GRNs in the pharynx (Chen et al. 2019). We therefore considered the possibility that salt-mediated inhibition of appetitive GRNs may be relevant for behavioral responses to salt. However, pharyngeal taste neurons are not accessible for high-resolution activity analyses and present limitations in correlating salt-mediated inhibition of GRNs with behavioral outcomes. Here we characterize the action of salt on sugar-evoked activity of labellar taste GRNs in flies with an intact taste system.

We find that salt elicits a bimodal dose-dependent response in *Gr64f* GRNs, activating at low concentrations and inhibiting at high concentrations, mirroring the dose-dependent valence of behavioral preference to salt. In addition, high salt blocks sugar-evoked activity of *Gr64f* GRNs in a manner that correlates with how the presence of salt alters feeding responses to sugar. We find that the inhibitory interaction appears to be selective—it depends on the identity of the salt and of the GRN; bitter-tastant evoked activity of *Gr66a* GRNs is not affected by salt. Comparison of the action of different salts indicates that the cationic moiety rather than the anionic component is a determining feature. Together, our results characterize a key mechanism in *Gr64f* GRNs that may contribute to reducing intake of harmful levels of salts.

Materials and methods

Fly stocks

Fly stocks were maintained at 22–25°C on standard cornmeal-dextrose diet. Wild-type flies were w^{1118} (BL5905). *Ir76b*^{1} (BL51309), *Gr66a-Gal4* (BL28801) and *UAS-Kir2.1* (BL6596), *Ppk23-Gal4* (BL93026), *Ppk28-Gal4* (BL93020), *Ir94e-Gal4* (BL81246) flies were obtained from the Bloomington *Drosophila* Stock Center.

Chemicals

$CaCl_2$ (C3881), denatonium (D5765), $FeCl_3$ (157740), indigocarmine (I8130), $MgCl_2$ (M3634), Na_2SO_4 (239313), sucrose (84097), and sulforhodamine B (230162) were obtained from Sigma. NaCl was obtained from Macron Fine chemicals (7581-06), while KCl was obtained from Mallinckrodt (6858). For extracellular tip recordings, solutions were dissolved in 30 mM tricholine citrate (Sigma, T0252).

Proboscis extension response assay

Proboscis extension response (PER) assays were performed as previously described with certain modifications. Briefly, 0- to 2-day-old flies were collected in standard food vials in groups of 10 males plus 10 females and maintained at 25°C for 5 days. Subsequently, they were starved for 24–26 h by transferring to vials containing 2 Kimwipes soaked with 6 mL of water. For the assay, single flies were immobilized in P200 pipette tips. Each tip was cut at the narrow end in a manner such that only the fly's head could protrude from it; the other end of the tip was sealed with clay. Paper wicks prepared from 6 mm wide strips of Kimwipe were used to deliver tastants to the labellum. Contact between the Kimwipe and labellar taste hairs was allowed for 2 s and proboscis extension was recorded within the next 7 s. Flies were water satiated prior to the assay and also between stimuli. After every proboscis extension we ascertained that the response was specific to the stimulus by confirming a negative response to water immediately after. Each fly was also tested with 100 mM sucrose at the beginning and the end of the assay; those that failed to respond to sucrose were discarded from the analysis. Proboscis extension was scored as follows: full extension = 1, partial extension = 0.5, and no extension = 0, and a mean PER index was scored for each tested stimulus.

Binary choice assay

Binary choice assays were performed as previously described. Briefly, 0–2 days old flies were collected in groups of 10 males plus 10 females in standard food vials and were maintained in a 25°C incubator with 50% humidity and 12:12 h light dark cycle. Flies were tested when they were 5–7 days old and were starved for 24 h prior to the assay. For the choice assays, taste stimuli were prepared in 0.75% agarose containing either 0.25 mg/ml of indigo carmine or 0.5 mg/ml of sulforhodamine B. Equal numbers of trials were performed with dyes swapped for the 2 stimuli. Nine drops of 10 µl of each of the 2 stimuli were spotted in tight-fit Petri dishes. Flies were introduced into these petri dishes, which were then placed in humid Styrofoam chambers for 2 h at 25°C, following which they were frozen and scored for the color of their abdomen. Only plates in which >50% flies had survived and >50% flies participated were used for analysis. A tastant preference index was calculated using the following formula:

Preference Index (PI) = (# Tastant 1 – # Tastant 2)/(# Tastant 1 + # Tastant 2 + # both)

Survival assay

Zero- to two-day-old flies were collected in vials containing 2 Kimwipes soaked with 6 mL of the desired solution. Flies

were transferred to fresh vials every other day. The number of dead flies in each vial was counted every 24 h.

Consumption assay

Five- to seven-day-old mated female flies were sorted into vials with 1% agar ± 100 mM sucrose and indicated salts and 1% sulforhodamine B (pink dye). Flies were allowed to feed in these vials for 2 h in a dark humid chamber at 25°C after which they were frozen at −80°C and dissected to remove the gustatory tracts into 5 µl of water in 50 µl PCR tubes. The tubes were vortexed and spun in a mini centrifuge for 30 s. The supernatant was collected and its absorbance was recorded at 565 nm using a Nanodrop 2000c Spectrometer. Dye concentration in gut extracts was determined from a standard curve generated for sulforhodamine B and used to calculate ingested food volume.

CAFE assay

Zero- to two-day-old mated female flies were collected and housed in plastic dram vials (10 females each) containing 2% agar as a base. The vials were fitted with plastic caps in which four small openings were made. Borosilicate glass capillaries (1B100F-4) were filled with tastant solutions containing sulforhodamine using capillary action and inserted into the openings in the vial cap. An empty vial with capillaries was set up as an evaporation control. The meniscus was marked in each capillary at the start of the experiment and tracked every 6 hours when capillaries were replaced with new ones. The following formula was used to calculate total intake: $\pi r^2 h$, where r = radius of the capillary, h = distance of meniscus traveled. To correct for evaporation, we subtracted the mean change in the control vials. Experiments were done in 12 h light/dark cycle at room temperature.

Extracellular tip recordings

Extracellular tip recordings were performed as described previously from 5- to 7-day-old flies. Genotypes being compared with each other were tested in parallel. All tastants were dissolved in 30 mM tricholine citrate, which served as electrolyte. To quantify neuronal responses, action potentials obtained in the first 500 ms following contact were counted.

Statistical analysis

Sample size of each experiment was determined based on previous literature. All statistical analyses were performed using Graphpad Prism Version 9. Kruskal–Wallis with Dunn's post hoc multiple comparison test was done for consumption assay. Two-way ANOVA followed by Sidak's post hoc analysis to generate *P* values for pairwise comparisons was used for binary choice assays. Friedman Test with Dunn's post hoc analysis was used for PER assays. Results of extracellular tip recording experiments were compared using either 1-way ANOVA or 2-way ANOVA adjusted for repeated measures followed by Tukey's *post hoc* test for pairwise comparisons or Unpaired *t*-test with Welch's correction as appropriate. For all column and line graphs, error bars indicate s.e.m.

RESULTS

A high salt diet is known to cause a range of physiological dysfunction in fruit flies, such as impaired neuronal plasticity and neurotransmitter release, accelerated cardiac aging, and fragmented sleep (Murashov et al. 2021; Naikkhwah and O'Donnell 2011; Stergiopoulos et al. 2009; Xie et al. 2019). Due to these deleterious health effects, we anticipated that chronic exposure to dietary salt would impact the life expectancy of flies. To test this, we placed zero- to two-day-old flies in vials containing 100 mM sucrose alone or 100 mM sucrose mixed with salt. We tested three different concentrations of salt (100, 250, and 500 mM). Flies given water alone, which we tested as a control, had 100% mortality by day 6, whereas those given 100 mM sucrose alone had 100% survival even at day 10 (Fig. 1A). The addition of 100 mM NaCl to 100 mM sucrose had no effect on the survival of flies during the 10-day observation period. However, higher salt concentrations caused significant dose-dependent reduction in survival. With a mixture of sucrose and 500 mM NaCl, flies survived only as long as they did on water alone. Thus, the effect of high salt on survival is likely to be via suppression of food intake even as flies are starving and highly motivated to consume.

We tested this idea directly by comparing intake of sucrose with that of sucrose-salt mixtures. Flies starved for 24 h were allowed to feed on selected tastant solutions containing sulforhodamine B for 2 h, following which we quantified consumption by measuring the amount of dye present in gut extracts (Fig. 1B). In agreement with the survival curves, we found no significant difference in the intake of sucrose alone versus sucrose mixed with 100 mM salt. However, flies consumed far less of mixtures containing 250 or 500 mM NaCl.

To test whether a similar pattern was observed over a longer time frame, we employed a CAFE assay to supply each stimulus as the sole food source and monitored consumption as well as fly survival over 8 days. We found that the presence of high concentrations of NaCl suppressed intake throughout the 6- to 8-day period that the flies were able to survive (Fig. 1C). While flies increased consumption of water at 3–5 days, those with access only to sucrose mixed with either 250 or 500 mM sucrose did not do so.

The observed anti-feedant effect of high concentrations of NaCl may be limited to NaCl-containing food. Alternatively, the presence of high salt may have a general effect on feeding suppression. To distinguish between these possibilities, we tested flies in binary choice assays in Petri dishes containing equal numbers of spots containing either 1 mM sucrose alone or 5 mM sucrose mixed with a selected concentration of NaCl. In the absence of NaCl, 5 mM sucrose was the preferred tastant (Fig. 1D). However, the preference shifted to 1 mM sucrose as increasing amounts of NaCl were mixed with 5 mM sucrose. As expected, 100 mM NaCl did not alter feeding preference in any way, whereas the higher concentrations significantly reduced the palatability of the mixture containing 5 mM sucrose (Fig. 1D). Notably, even in these experiments, flies exhibited robust participation and feeding on 1 mM sucrose, consistent with selective avoidance of foods containing high salt as opposed to an overall shift in feeding behavior in the presence of salt. We confirmed that the intake of mixtures with high salt was in fact lower than that of sucrose alone using consumption assays (Fig. 1E).

Ir76b, a widely expressed ionotropic receptor, is required for opposing behavioral responses to low salt and high salt (Dweck et al. 2022; Lee et al. 2017; McDowell et al. 2022; Zhang et al. 2013). In binary choice assays to assess feeding preference between 5 mM sucrose mixed with salt versus 1 mM sucrose, $Ir76b^1$ mutants showed deficits in aversion to the mixture with 250 mM NaCl, and potentially in attraction

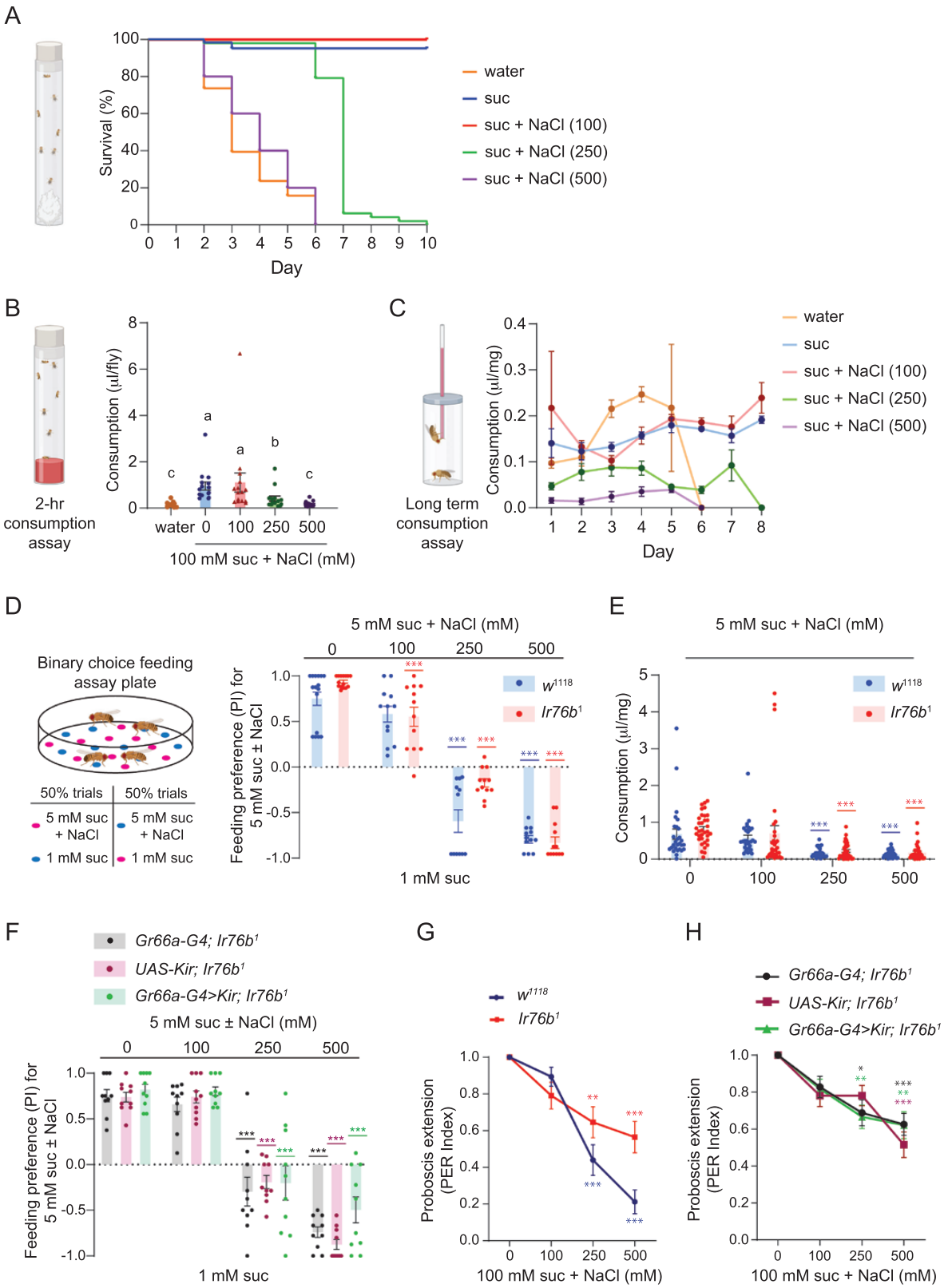

Fig. 1. Silencing of high salt taste neurons does not abolish salt avoidance in *Drosophila*. A) Survival of mated female w^{1118} flies on the indicated tastants. Log-rank (Mantel-Cox) test. *n*=60 flies for each tastant. B) Ingested volume (µl) of 100 mM sucrose mixed with indicated concentration (mM) of NaCl, normalized to body weight (mg) in mated female w^{1118} flies tested in 1-choice consumption assays. Kruskal-Wallis with Dunn's post hoc multiple comparison test. *n* = 11–15. C) Daily ingested volume (µl) of 100 mM sucrose mixed with indicated NaCl concentrations (mM) per mated female w^{1118} flies tested in CAFE assays. *n* = 60 for each tastant. D) Feeding preference of mated females of the named genotypes, tested in binary choice assays with the indicated stimuli. Two-way ANOVA with Sidak's post hoc multiple comparison test. *n* = 16–12. Asterisks indicate significant differences from preference for 5 mM sucrose alone for the same genotype. E) Ingested volume (µl) of 100 mM sucrose laced with indicated NaCl concentrations (mM) normalized with body weight (mg) in mated female flies. Asterisks indicate significant differences from intake of 5 mM sucrose (0) for the same genotype. Kruskal–Wallis with Dunn's post hoc multiple comparison test. *n* = 28–34. F) Feeding preference of mated females tested in binary choice

to the mixture with 100 mM NaCl (PI is significantly different for 5 mM sucrose versus 5 mM sucrose + 100 mM NaCl for *Ir76b*^{1} (P = 0.001) but not for control w^{1118} (P = 0.2687)) (Fig. 1D). However, in 1-choice consumption assays with control flies we did not observe enhanced intake of sucrose with the addition of 100 mM NaCl, suggesting that the assay conditions may not be sensitive enough to evaluate salt attraction in the presence of sucrose. By contrast, both control and *Ir76b*^{1} mutant flies were able to suppress intake of high salt mixtures with sucrose (Fig. 1E). We expected that *Gr66a* GRNs would be responsible for the high salt aversion observed in the *Ir76b*^{1} mutants, since a previous study found that these and glutamatergic *ppk23* neurons are both involved in high salt taste (Jaeger et al. 2018). While *ppk23* neurons require *Ir76b* to detect NaCl, bitter-sensing neurons detect NaCl independently of *Ir76b* (Jaeger et al. 2018). We silenced bitter GRNs by expressing *Kir 2.1*, an inward rectifying potassium channel, using *Gr66a-Gal4* in an *Ir76b*^{1} background. Surprisingly, even these flies exhibited feeding avoidance of high salt mixtures with sucrose, although a role for *Gr66a* GRNs was consistent with a small deficit in aversion to 500 mM NaCl when compared with *UAS* control flies (P = 0.0309) but not the *GAL4* control flies (P = 0.4159) (Fig. 1F).

Together, these results invoke the presence of bitter- and high salt GRN-independent mechanisms for suppressing intake of high salt. We next tested whether such auxiliary mechanisms occur via taste input by performing proboscis extension response (PER) assays, which allowed us to measure taste acceptance of mixed stimuli without any confounding post-ingestive effects of salt intake. For these experiments, labellar taste hairs of flies starved for 24–26 h were stimulated with 100 mM sucrose, a highly appetitive stimulus, laced with a range of NaCl concentrations. We found that sucrose alone elicited a strong PER in all wild-type flies that we tested (Fig. 1G). The PER index fell with increasing concentrations of NaCl mixed with the sugar solution (Fig. 1G). *Ir76b*^{1} flies that were tested in parallel also exhibited a robust response to sucrose, as expected, as well as reduced PER with increasing concentrations of NaCl in the stimulus, although not to the same degree as observed for wild-type flies (Fig. 1G). In fact, *Ir76b*^{1} mutants had similar PER to mixtures with 250 mM and 500 mM NaCl ($P > 0.9999$), suggesting different plateaus of high salt sensitivity in the 2 genotypes (Fig. 1G). Notably, the PER of *Ir76b*^{1} flies in which *Gr66a* GRNs were also silenced was indistinguishable from that of *Ir76b*^{1} mutants (P = 0.4946) (Fig. 1H). These results indicate that while *Ir76b* and *Gr66a* GRN-mediated pathways suppress acceptance of high salt mixtures, additional taste mechanisms are also involved.

We therefore wished to test whether high salt can inhibit the activity of labellar sugar-sensing *Gr64f* GRNs, as found for pharyngeal *Gr43a/Gr64f* GRNs (Chen et al. 2019). Given that salt acts on all taste neurons in labellar sensilla, we devised a genetic strategy to isolate the activity of *Gr64f* GRNs. We generated flies with *Gr64f solo* L-type sensilla, in which the three other GRNs marked by Ir94e, Ppk23, and Ppk28, were genetically silenced by expression of *Kir2.1* (Fig. 2A). L7-9 sensilla in *Gr64f solo* flies exhibited normal responses to sucrose, but not to high salt (500 mM NaCl) or water (1 mM KCl) (Fig. 2B). Since *Gr64f* GRNs are known to be activated by salt (Dweck et al. 2022; Jaeger et al. 2018), we next characterized the response dynamics of *Gr64f solo* sensilla to a concentration range of NaCl. We recorded from L1 and L7 sensilla (Fig. 2A), because of reported differences in salt sensitivity between the two sensilla (Dweck et al. 2022). Our results from *Gr64f solo* sensilla were similar, in that NaCl elicited a response from both sensilla and moreover, the response of the L7 sensillum was consistently lower than that of the L1 sensillum. Surprisingly, in both L1 and L7 *Gr64f solo* sensilla, the dose-response curves exhibited a bimodal pattern with higher responses to low salt (~20–60 spikes per second) and depressed responses to high salt (≤10 spikes per second) (Fig. 2C and D). As reported previously (Dweck et al. 2022), this was not the case in wild-type L1 and L7 sensilla, in which the peak response was observed at higher NaCl concentrations (Fig. 2C and D). It is possible that this response reflects the summed activity of *Gr64f* and *Ppk23*^{glut} GRNs, since the latter is activated with high salt (Jaeger et al. 2018).

Similarly, opposite effects were observed in *Gr64f solo* sensilla using mixtures of low salt (10 mM) or high salt (500 and 1000 mM) with sucrose. With 10 mM NaCl, the mixture evoked a stronger response than sucrose (Fig. 2E and F). However, with high salt mixtures the responses were lower than those obtained with sucrose (Fig. 2G and H). Importantly, subsequent recordings with sucrose showed responsiveness that was no different from sucrose recordings taken prior to stimulation with NaCl, indicating that the presence of salt only temporarily depressed neuronal response (Fig. 2H). Elevation of sucrose concentration in the mixture quelled the negative effect of high salt—we found no significant difference in the response to 250 mM sucrose tested alone and in a mixture with 500 mM NaCl (Fig. 2I and J).

To determine if salt-mediated activation of *Gr64f* GRNs is required for the response patterns observed with sucrose-high salt mixtures, we examined the responses to mixtures in *Ir76b* mutants (Fig. 3A). Previous studies have found that *Ir76b* is essential for salt responsiveness, and functions in both *Gr64f* and *Ppk23* neurons (Dweck et al. 2022; Jaeger et al. 2018; Lee et al. 2017; Zhang et al. 2013). Consistent with these observations, we found that response to 500 mM NaCl was virtually eliminated in L sensilla of *Ir76b*^{1} flies (Fig. 3B and C). Spike trains of uniform action potentials were observed when L hairs in *Ir76b*^{1} flies were stimulated with sugar-salt mixtures, and we inferred that these spikes originated from *Gr64f* GRNs (Fig. 3D). We conducted recordings using 10 mM sucrose mixed with a range of NaCl concentrations. Even in the

assays with the indicated stimuli. Genotypes were: *Gr66a-Gal4; Ir76b*^{1} (*Gr66a-G4*; *Ir76b*^{1}), *UAS-Kir2.1; Ir76b*^{1} (*UAS-Kir*; *Ir76b*^{1}) and *Gr66a-Gal4/UAS-Kir2.1*; *Ir76b*^{1}/*Ir76b*^{1} (*Gr66a-G4>Kir*; *Ir76b*^{1}). Asterisks indicate significant differences versus preference for 5 mM sucrose (0) for the same genotype. Two-way ANOVA with Sidak's post hoc multiple comparison test. n = 10. G) Proboscis extension response index obtained from mated females upon labellar stimulation with indicated stimuli. Asterisks indicate significant differences from PER to 100 mM sucrose (0) for the same genotype. Friedman Test with Dunn's post hoc multiple comparison test. n = 31–33. H) Proboscis extension response index obtained from mated female upon labellar stimulation with indicated stimuli. Genotypes as in (F). Asterisks indicate significant differences from PER to 100 mM sucrose (0) for the same genotype. Friedman Test with Dunn's post hoc multiple comparison test. n = 32–33. For all graphs, *P < 0.05, **P < 0.01, ***P < 0.001. Error bars = s.e.m. Schematics in A–D created with Biorender.com.

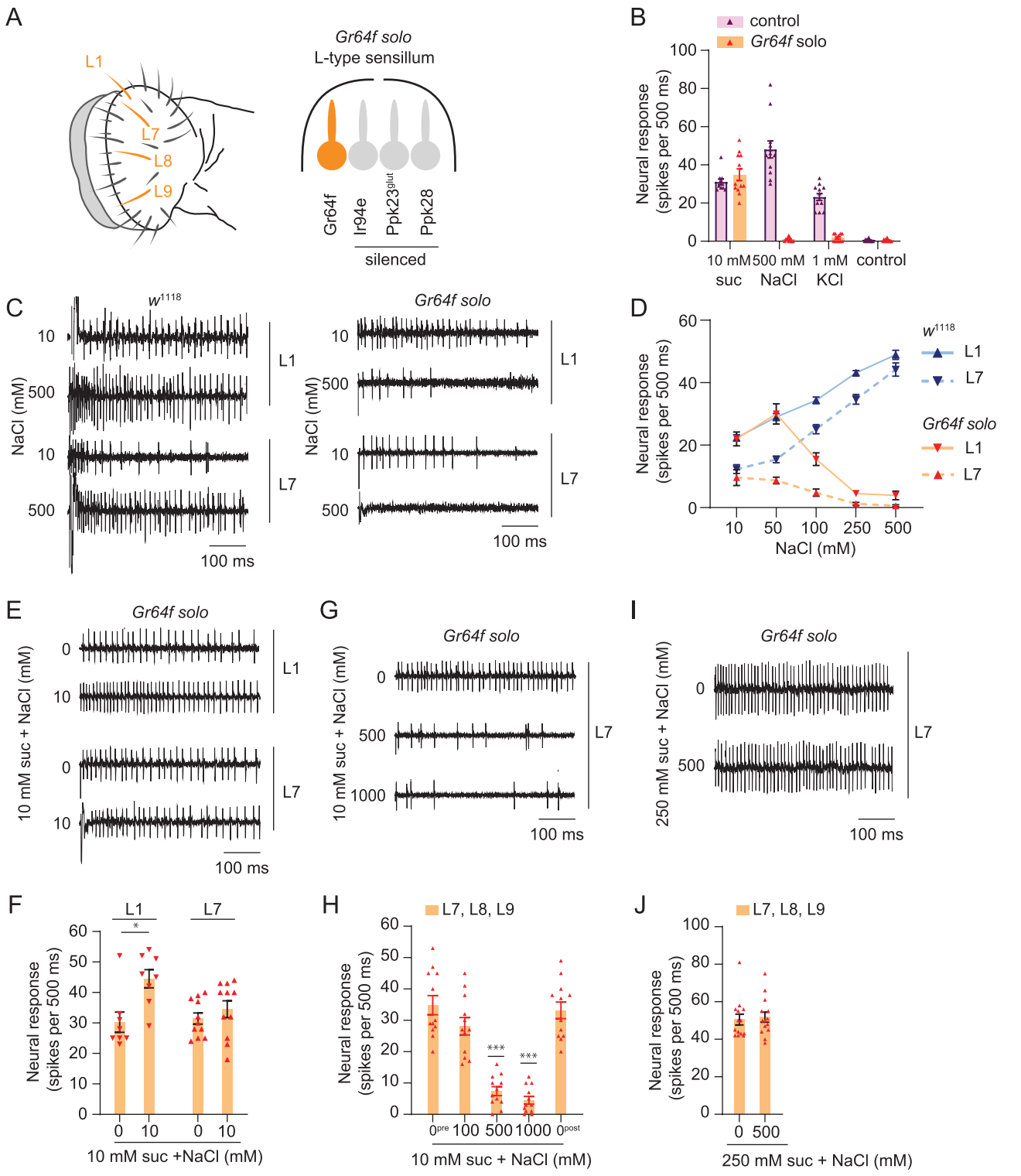

Fig. 2. High NaCl inhibits *Gr64f* sugar-sensing taste neurons. A) Schematic of taste hairs of the *D. melanogaster* labellum indicating in orange the L-type sensilla that were selected for recordings (left). Shown on the right are GRNs in *Gr64f solo* L-type sensilla, in which all except the *Gr64f* GRN are silenced by expression of Kir2.1. *Gr64f solo* genotype is: *ppk23-Gal4/UAS-Kir 2.1; ppk28-Gal4/Ir94e-Gal4*. For all experiments, recordings were taken from mated females aged 5-7 days. B) Mean responses of *Gr64f solo* L7-9 sensilla in the first 500-ms period upon stimulation with the indicated tastant dissolved in 30 mM tricholine citrate (control). Unpaired *t*-test with Welch's correction. $n = 12$ sensilla from 4 flies. C) Representative traces from wild type (w^{1118}) and *Gr64f solo* L1 and L7 sensilla stimulated with NaCl. D) Mean responses of wild type (w^{1118}) and *Gr64f solo* L1 and L7 sensilla in the first 500-ms period upon stimulation with indicated concentration of NaCl. $n = 9$–10 sensilla from 5 to 6 flies. E) Representative traces from *Gr64f solo* L1 and L7 sensilla stimulated with sucrose ± low NaCl. F) Mean responses of *Gr64f solo* L1 and L7 sensilla in the first 500-ms period upon stimulation with sucrose alone (0) or in a mixture with 10 mM NaCl (10). $n = 8$–11 sensilla from 5 to 6 flies. G) Representative traces from *Gr64f solo* L7 sensilla stimulated with 10 mM sucrose ± high NaCl. H) Mean responses of *Gr64f solo* L7-9 sensilla in the first 500-ms period upon stimulation with

absence of *Ir76b*, the firing frequency decreased with NaCl in a concentration-dependent manner, with 100 mM NaCl having the smallest effect and 1 M the largest effect (Fig. 3E).

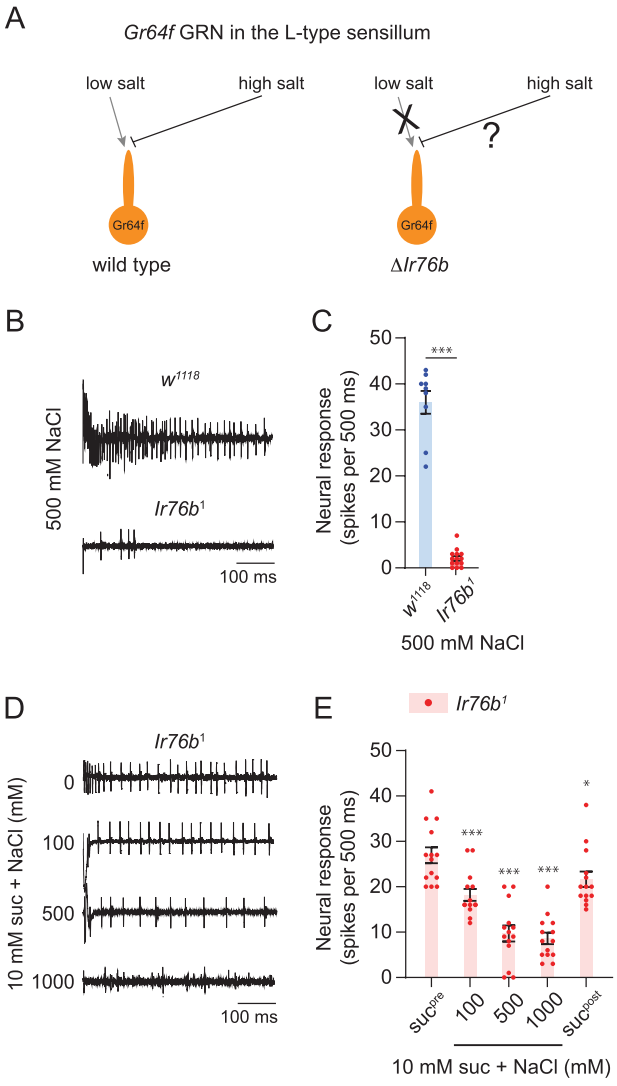

Figure 3. *Ir76b* is not necessary for inhibition of *Gr64f* sugar-sensing taste neurons by high salt. A) Experimental strategy to test if high salt inhibition of *Gr64f* neurons is independent of Ir76b-mediated response to NaCl. B) Representative traces of recordings from w^{1118} and $Ir76b^1$ labellar L7 sensilla stimulated with salt. C) Mean responses of labellar L7-9 sensilla to 500 mM NaCl in mated females of indicated genotypes. Unpaired *t*-test with Welch's correction. n = 9–14 sensilla from 4 flies. D) Representative traces of recordings from $Ir76b^1$ labellar L7 sensilla stimulated with indicated tastant mixtures. E) Mean responses of $Ir76b^1$ labellar L7-9 sensilla to indicated stimuli. Asterisks indicate significant differences from 10 mM sucrose (0^{pre}), which was tested first. Sensillar responses to sucrose were confirmed at the end of each series of recordings (0^{post}). One-way ANOVA for repeated measures followed by Tukey's post hoc test for multiple comparisons. n = 14 sensilla from 5 flies. For all graphs, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Error bars = s.e.m.

At the conclusion of each series of recordings, sensilla were tested with 10 mM sucrose, which showed that the effects of high salt were reversible and the GRN remained capable of responding to sucrose (Fig. 3E). These results suggest that NaCl, which activates the *Gr64f* GRN, also depresses its response to sucrose via an independent mechanism.

We next tested sodium sulfate (Na_2SO_4) to determine if a different sodium salt had the same effect. In consumption assays, we found that intake of sucrose containing 500 mM Na_2SO_4 was drastically reduced compared with sucrose alone (Fig. 4A). Independent taste behavior assays lent support to the idea that Na_2SO_4 also acts by blocking sucrose response. First, flies avoided food containing 250 mM Na_2SO_4 in binary choice assays (Fig. 4B). Second, addition of 500 mM Na_2SO_4 reduced PER of wild-type flies to 100 mM sucrose (Fig. 4C). In both cases, the effect of Na_2SO_4 was independent of the function of the *Ir76b* receptor and of *Gr66a* bitter GRNs (Fig. 4B and C; Supplementary Fig. S1A and B). We next recorded from L-type sensilla of $Ir76b^1$ flies. First, we confirmed that no spikes were elicited with 500 mM Na_2SO_4 alone (Fig. 4D and E). However, inclusion of 500 mM Na_2SO_4 with 10 mM sucrose evoked fewer spikes than the number observed with 10 mM sucrose (Fig. 4F and G; Supplementary Fig. S1C). Thus, Ir76b-independent depression of sucrose response is observed with at least 2 different sodium salts.

High concentrations of salts with other cations, including potassium, magnesium, and iron, are also detrimental to general health (Kobayashi et al. 2018; McLaughlin and McKinney 1998), arguing that flies may have evolved taste mechanisms to reject foods containing harmful levels of these. We determined whether 500 mM of KCl, $MgSO_4$, $CaCl_2$, or $FeCl_3$ could suppress food intake using the single-choice consumption assay described above. In all instances, flies consumed little of the mixtures containing any of the salts, whereas age-matched flies tested in parallel fed normally on 100 mM sucrose (Fig. 4A). The results of binary choice assays to measure preference for 1 mM sucrose versus 5 mM sucrose mixed with salt were similar—flies preferred the higher concentration of sucrose in the absence of salt but avoided mixtures with salt (Fig. 4B). Rejection of the sucrose-salt mixtures was maintained in *Ir76b* mutants, as well as in *Ir76b* mutants in which *Gr66a* bitter GRNs were silenced (Figures 4B, Supplementary Fig. S1A). Similarly, in proboscis extension assays, each of the salts suppressed acceptance of sucrose (Figures 4C, Supplementary Fig. S1B). This was also the case for $Ir76b^1$ mutants, and for $Ir76b^1$, *Gr66a*-silenced flies.

We next examined the action of these salts on sugar-evoked response of labellar GRNs. We recorded from L-type sensilla with 500 mM solutions of each salt. As observed for NaCl, we observed Ir76b-dependent spiking activity in response to these salts (Fig. 4D and E). However, salts were sensed in the context of sugar-salt mixtures in $Ir76b^1$ mutants. Each of the mixtures evoked fewer spikes than sucrose alone, although there were some differences in the inhibitory strength of the different salts that were tested (Fig. 4F and G). Importantly, *Gr64f* GRNs remained viable and responded to subsequent

sucrose alone (0) or in a mixture with indicated concentration of NaCl. Each sensillum was tested with sucrose before (0^{pre}) and after (0^{post}) the series of recordings with NaCl. Asterisks indicate significance versus 10 mM sucrose (0^{pre}), one-way ANOVA for repeat measures followed by Tukey's post hoc test for multiple comparisons. n = 12 sensilla from 4 flies. I) Representative traces from *Gr64f solo* L7 sensilla stimulated with 250 mM sucrose ± high NaCl. J) Mean responses of *Gr64f solo* L7-9 sensilla in the first 500-ms period upon stimulation with 250 mM sucrose alone (0) or in a mixture with high NaCl (500). There is no significant difference between the 2, unpaired *t*-test with Welch's correction. n = 14 sensilla from 4 flies. For all graphs, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Error bars = s.e.m.

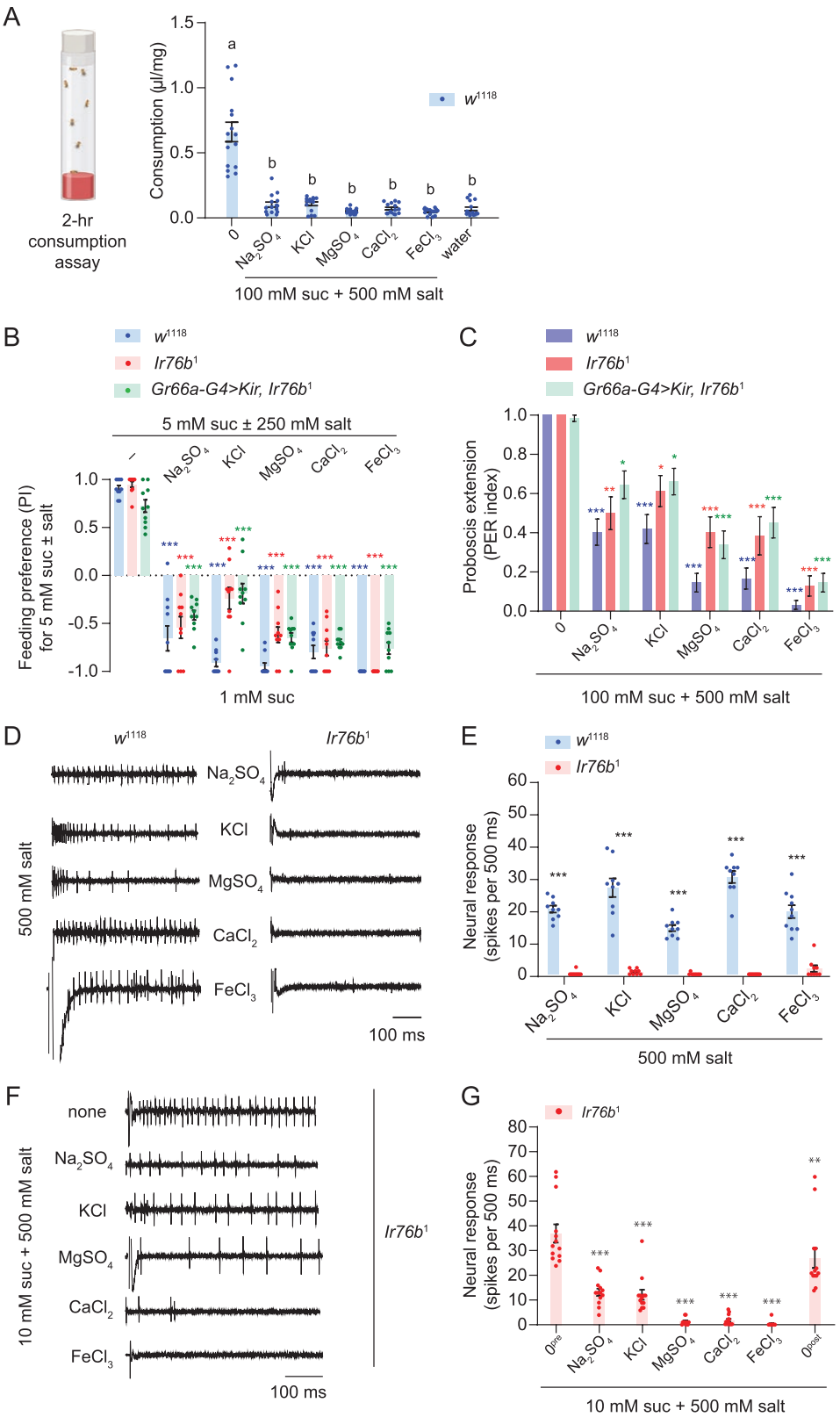

Figure 4. Cationic moieties of salts determine inhibition of *Gr64f* sugar-sensing taste neurons. A) Ingested volume (µl) of solutions of 100 mM sucrose mixed with 500 mM of indicated salt, normalized to body weight (mg). Genotype was w^{1118}. Kruskal–Wallis with Dunn's post hoc test for multiple comparison. $n = 15$. Diagram created with Biorender.com. B) Preference of mated females tested in binary choice assays with indicated stimuli. Genotypes were: w^{1118}, $Ir76b^1/Ir76b^1$ ($Ir76b^1$) and *Gr66a-Gal4/UAS-Kir*, $Ir76b^1/Ir76b^1$ (*Gr66a-G4>Kir*, $Ir76b^1$). Asterisks indicate significant differences from preference for 5 mM sucrose (–) in flies of the same genotype. Two-way ANOVA with Sidak's post hoc test for multiple comparison. $n = 10$. C) Proboscis extension response upon labellar stimulation with tastant solutions. Genotypes as in B. Asterisks indicate significant differences from PER to 100 mM sucrose (0) in flies of the same genotype. Friedman Test with Dunn's post hoc test for multiple comparison. $n = 26$–31. D) Representative traces of recordings from labellar L7 sensilla stimulated with 500 mM of indicated salt. E) Mean responses of labellar L7-9 sensilla to indicated stimuli.

stimulation with sucrose (Fig. 4G, Supplementary Fig. S1D). Together, these results are consistent with the notion that high concentrations of a variety of salts can directly suppress the activity of *Gr64f* GRNs.

The observed neuro-inhibitory function of high salt could be restricted to sugar-sensing GRNs to suppress feeding. Alternatively, it could be a more generalized phenomenon that also acts on other GRNs. To test one other GRN, we selected bitter-sensing GRNs from S-b sensilla, which are activated by various bitter tastants including denatonium. These sensilla were chosen because neuronal firing to 500 mM NaCl was almost obliterated in *Ir76b*^{1} flies (Fig. 5A and B), precluding any confounding effects from salt-evoked activation. We performed recordings with 10 mM denatonium alone and in a mixture with 500 mM NaCl. We observed a robust firing frequency in response to denatonium, which was not altered by the presence of NaCl (Fig. 5C and D). Together, these data suggest that salt has a direct and selective effect on *Gr64f* GRNs, inhibiting its response to sugar and thereby reducing the palatability of mixtures that contain high salt along with sugar (Fig. 5E).

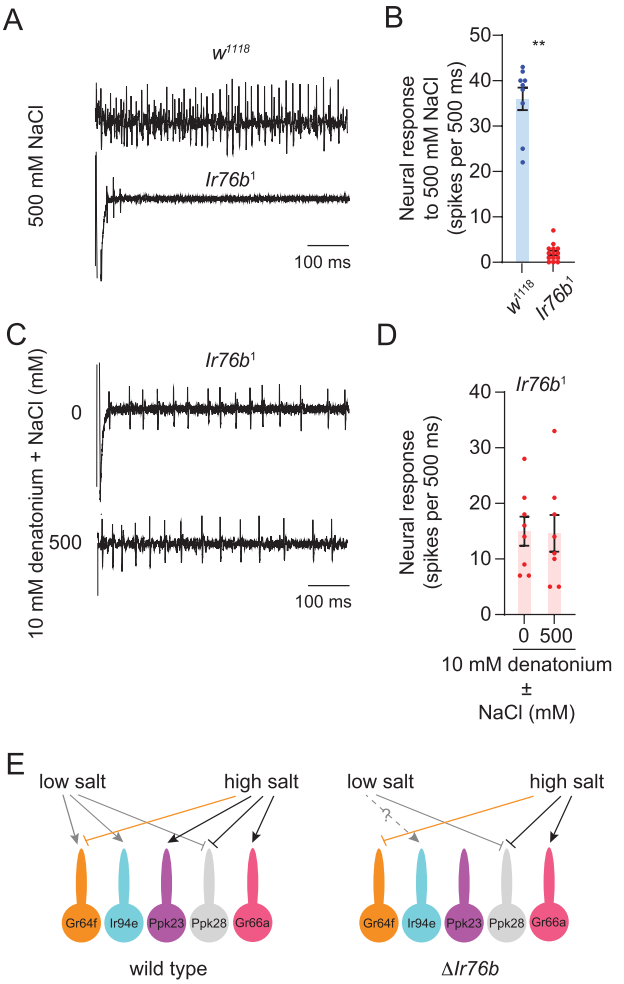

Fig. 5. High salt does not inhibit *Gr66a* bitter taste neurons. A) Representative traces of recordings from labellar Sb sensilla stimulated with 500 mM NaCl. B) Mean responses of labellar Sb sensilla stimulated with 500 mM NaCl. Unpaired *t*-test with Welch's correction. $n = 9$ sensilla from 4 flies. C) Representative traces of recordings from labellar Sb sensilla in *Ir76b*^{1} flies, stimulated with 10 mM denatonium ± high salt. D) Mean responses of labellar Sb sensilla in *Ir76b*^{1} flies stimulated with indicated tastants. Unpaired *t*-test with Welch's correction. n=8 from 4 flies. For all graphs, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Error bars = s.e.m. E) Schematic summarizing the action of salt on labellar GRNs in wild type and *Ir76b* mutant flies. The inhibitory arrow added from this work is in orange.

DISCUSSION

Drosophila melanogaster are osmoregulators, meaning that they maintain a constant internal osmolality despite encountering variations in external osmolality. As a result, they are able to withstand high levels of salt in their diet. Maintaining a consistent hemolymph osmolality even when exposed to extremely concentrated salt solutions with high osmolality, on the other hand, likely necessitates a significant amount of energy expenditure. Furthermore, some ionic moieties can have a negative impact on normal physiological processes, as indicated by increased mortality in flies fed high sodium, calcium, or metal ions (Lee et al. 2017, 2018; Xiao et al. 2022).

As a result, flies must avoid the consumption of high concentrations of certain salts. The fly gustatory system has evolved numerous mechanisms to prevent ingestion of high salt. Previous studies have shown that salt activates GRNs that trigger deterrence, including *Gr66a* bitter and *Ppk23* high salt GRNs (Jaeger et al. 2018; McDowell et al. 2022). Salts inhibit the Ppk28 osmolality receptor expressed in water GRNs (Cameron et al. 2010). Our work here shows that high salt also inhibits *Gr64f* sweet GRNs, which signal appetitive value.

Sugar responses of *Gr64f* GRNs are inhibited by several different classes of tastants including organic acids (Charlu et al. 2013), bases (Clark et al. 2023), bitter compounds (French et al. 2015; Jeong et al. 2013), and metal ions (Xiao et al. 2022). One feature that distinguishes salt from these other inhibitors is that salt is also an activator of *Gr64f* GRNs. In fact, recent studies identified *Gr64f* GRNs as the classically described "low salt" cells that are activated by and mediate attraction to low salt (Dweck et al. 2022; Jaeger et al. 2018). Our electrophysiological analysis of *Gr64f* GRNs uncovers a bimodal concentration-dependent response to salt, which suggests that the *Gr64f* GRN firing pattern encodes high attraction to low salt as well as low attraction to high salt. Of note is that *Gr64f* GRNs display strong calcium transients upon stimulation with high NaCl (Jaeger et al. 2018). It is conceivable that the high salt response in *Gr64f* GRNs is modulated by the activity of other neurons in the sensillum. Another intriguing possibility is that high salt may block action potentials independent of Gr-mediated

Unpaired *t*-test with Welch's correction. n = 9–12 with 4 flies. F) Representative traces of recordings from *Ir76b*^{1} labellar L7 sensilla stimulated with indicated tastant mixtures. G) Mean responses of labellar L7-9 sensilla in *Ir76b*^{1} flies to indicated stimuli. Asterisks indicate significant differences from neuronal responses to 100 mM sucrose (0^{pre}), which was tested first. Sensillar responses to sucrose were confirmed at the end of each series of recordings (0^{post}). One-way ANOVA for repeated measures followed by Dunnet's post hoc test for multiple comparison. n = 10–13 with 4 flies. For all graphs, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Error bars = s.e.m.

sweet or Ir-mediated salt responses in these GRNs, and thus "inhibit" the *Gr64f* GRN taste response to salt itself. It will be interesting to determine whether responses of *Gr64f* GRNs to other classes of tastants, including polyamines (Hussain et al. 2016), amino acids (Ganguly et al. 2017), and fatty acids (Ahn et al. 2017; Tauber et al. 2017), are also depressed in the presence of high salt.

Evidence for salts as neuro-inhibitors is rare. Previous studies have reported that magnesium can reduce neuronal activity (Dodge and Rahamimoff 1967; Fomin et al. 2006; Frankenhaeuser and Meves 1958; Hille et al. 1975). High levels of sodium or potassium, on the other hand, are frequently associated with abnormally high firing patterns in neurons, which can cause epileptic seizures in mammals (Fröhlich et al. 2008; Traynelis and Dingledine 1988; Wengert and Patel 2021). The observation that high salt does not cause irreversible toxicity in *Gr64f* GRNs, and that it does not have a similar effect on *Gr66a* GRNs, lends support to the idea of a selective rather than a generalized mechanism for inhibiting tastant-evoked action potentials. However, further studies are needed to identify the potential sensors in *Gr64f* GRNs and resolve the underlying mechanisms. Candidate gene approaches could include screens of ligand-gated chloride channels expressed in the fly proboscis. Alternatively, unbiased approaches to identify all classes of transmembrane proteins that are expressed in *Gr64f* GRNs but not in *Gr66a* GRNs could lead to novel candidates.

Little is known about how various tastants inhibit *Gr64f* GRNs. The action of bitter tastants on these GRNs depends on an odorant-binding protein Obp49a, which is thought to associate with the sweet taste receptor, Gr64a, and potentially block its sensory function (Dahanukar et al. 2007; Jeong et al. 2013). The mechanism by which organic acids inhibit *Gr64f* GRNs is unknown, although the organic anionic moieties of these acids are known to be required for inhibition (Ganguly et al. 2021). Structurally, salts are quite distinct from the anionic moieties of organic acids. Therefore, it is highly probable that *Gr64f* GRN inhibition by high salt is mechanistically distinct from that mediated by other tastants.

Here, we also sought to identify the nature of the ionic moiety that might be the effector. Since both chloride and sulfate salts of sodium had similar outcomes, we surmised that the anion may not play an important role in inhibiting *Gr64f* GRNs. Different degrees of inhibition obtained with various cation salts, on the other hand, are consistent with the interpretation that they are the required determinants. In addition, there appears to be a relationship between the cation oxidation state and the level of inhibition. Iron (III) with an oxidation number of 3 elicited the strongest inhibition. This was followed by comparable actions of salts of magnesium and calcium, both of which have an oxidation number of 2. Finally, salts of sodium and potassium, with oxidation numbers of 1, were the weakest inhibitors. This novel phenomenon of oxidation state-dependent neuronal inhibition by cations may lead to the discovery of new classes of ion channels.

Supplementary material

Supplementary material is available at *Chemical Senses* online.

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Conflict of interest

The authors declare no conflict of interest.

Data availability

The data and statistical analyses underlying this article are available in Mendeley, at DOI: 10.17632/z97wmdkp5k.1.

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