

A single pair of pharyngeal neurons functions as a commander to reject high salt in *Drosophila melanogaster*

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Abstract

Salt is an essential nutrient for survival, while excessive NaCl can be detrimental. In the fruit fly, *Drosophila melanogaster*, internal taste organs in the pharynx are critical gatekeepers impacting the decision to accept or reject a food. Currently, our understanding of the mechanism through which pharyngeal gustatory receptor neurons (GRNs) sense high salt are rudimentary. Here, we found that a member of the ionotropic receptor family, *Ir60b*, is expressed exclusively in a pair of GRNs activated by high salt. Using a two-way choice assay (DrosoX) to measure ingestion volume, we demonstrate that IR60b and two coreceptors IR25a and IR76b, are required to prevent high salt consumption. Mutants lacking external taste organs but retaining the internal taste organs in the pharynx exhibit much higher salt avoidance than flies with all taste organs but missing the three IRs. Our findings highlight the vital role for IRs in a pharyngeal GRN to control ingestion of high salt.

eLife assessment

This **valuable** study on the molecular and cellular mechanisms of ingestion avoidance of high salt in insects is focused in scope, but the authors present **convincing** evidence that a specific subset of gustatory receptors in a pair of pharyngeal taste neurons are necessary and sufficient for avoiding ingestion of high salt during feeding. This work will be of interest to *Drosophila* neuroscientists interested in taste coding and feeding behavior.

Introduction

The sense of taste enables animals to find nutritious food while avoiding potentially harmful substances in their environment. Most animals have evolved sophisticated systems to detect and steer clear of consuming levels of substances that are toxic. Salts such as NaCl are essential for a wide array of physiological functions. However, consumption of excessive salt can contribute to

various health issues in mammals, including hypertension, osteoporosis, gastrointestinal cancer, autoimmune diseases, and can lead to death ¹⁻⁶. Therefore, high concentrations of salt are rejected by most animals.

Multiple studies have delved into how Na^+ is sensed in the *Drosophila* taste system, shedding light on the mechanisms behind the attraction to low salt and aversion to high salt ⁷⁻¹⁴. The largest taste organs in flies are two bilaterally symmetrical labella, each of which is decorated with 31 gustatory bristles (sensilla). These sensilla are defined based on size (small, S; intermediate, I; large, L). The I-type sensilla harbor two gustatory receptor neurons (GRNs), while the S- and L-sensilla contain four. These GRNs fall into five classes (A-E) based on their response profiles ¹⁵. These include Class A GRNs (formerly sugar GRNs; marked by *Gr5a* or *Gr64f*) ^{16,17}, which respond to attractive compounds such as low salt, sugars, glycerol, fatty acids and carboxylic acids, Class B GRNs (formerly bitter GRNs; marked by *Gr66a*) ^{16,17}, which are activated by high Na^+ , bitter compounds, acids, polyamines, tryptophan, and L-canavanine, Class C GRNs respond to water (marked by *ppk28*) ¹⁸, Class D GRNs detect high levels of cations such as Na^+ , K^+ and Ca^{2+} (marked by *ppk23*) ^{11,19}, and Class E GRNs sense low Na^+ levels and pheromones (marked by *Ir94e*) ^{11,20}.

Several of the 66 ionotropic receptor (IR) family members function in the sensation of low and high salt. These include IR76b and IR25a, which are IR-coreceptors, and therefore have broad roles in sensing many taste stimuli including low and high Na^+ (also referred to in this work as salt) ^{7,8,10,12}, Ca^{2+} ¹⁹, several carboxylic acids ^{21,23}, fatty acids ²⁴⁻²⁷, amino acids ²⁸, and carbonation ²⁹. A subset of Class A GRNs, as well as glutamatergic Class E GRNs are responsible for sensing low salt ^{8,11,12,15}, and this sensation depends on IR56b working together with the broadly expressed co-receptors IR25a and IR76b ⁸. Conversely, detection of high salt depends on Class B GRNs and Class D GRNs, and IR7c, in conjunction with IR25a and IR76b ⁷. Additionally, two Pickpocket channels, Ppk11, Ppk19, and Sano have been associated with high salt aversion ^{30,31}.

In addition to the labellum and taste bristles on other external structures, such as the tarsi, fruit flies are endowed with hairless sensilla on the surface of the labellum (taste pegs), and three internal taste organs lining the pharynx, the labral sense organ (LSO), the ventral cibarial sense organ (VCSO), and the dorsal cibarial sense organ (DCSO), which also function in the decision to keep feeding or reject a food ³²⁻³⁶. A pair of GRNs in the LSO express a member of the gustatory receptor family, *Gr2a*, and knockdown of *Gr2a* in these GRNs impairs the avoidance to slightly aversive levels of Na^+ ¹⁴. Pharyngeal GRNs also promote the aversion to bitter tastants, Cu^{2+} , L-canavanine, and bacterial lipopolysaccharides ³⁷⁻⁴⁰. Other pharyngeal GRNs are stimulated by sugars and contribute to sugar consumption ^{32,36,41}. Remarkably, a pharyngeal GRN in each of the two LSOs functions in the rejection rather the acceptance of sucrose ⁴².

In this work, we investigated whether IRs function in pharyngeal GRNs for avoidance of high Na^+ . We found that IR60b, along with co-receptors IR25a and IR76b function in a taste organ in the pharynx for limiting high salt consumption. *Ir60b* is expressed exclusively in a pair of pharyngeal GRNs in the LSO ⁴², and we found that these neurons respond to high Na^+ . While these *Ir60b* GRNs are narrowly tuned, surprisingly, they have previously been shown to respond to sucrose and glucose ⁴². Introduction of the rat capsaicin receptor, TRPV1 ⁴³, into *Ir60b* GRNs induces aversion towards capsaicin, supporting the conclusion that *Ir60b*-positive GRNs are sufficient for instinctive avoidance. To validate these findings further, we used a two-way choice Drosophila assay ⁴⁴ to measure actual ingestion levels. We found that the three *Ir* mutants consumed high salt at levels similar to sucrose over an extended period, emphasizing the critical role of this single pair of pharyngeal GRNs in controlling harmful ingestion of high salt.

Results

Ir60b functions in the repulsion to high salt

To identify potential salt sensors in *Drosophila melanogaster*, we conducted binary food choice assays using 30 *Ir* mutants (**Figure 1A** and **Figure 1—figure supplement 1A**). Through screens in which we gave flies a choice between 2 mM sucrose alone or 2 mM sucrose plus a low, attractive level of salt (50 mM NaCl), we confirmed that *Ir76b*¹², *Ir25a*, and *Ir56b*⁸, are essential for detecting low salt (**Figure 1—figure supplement 1A**). Moreover, using tip recordings to assay tastant-induced action potentials, we confirmed a previous report⁸ that loss of *Ir56b* nearly eliminated spikes in S-type and L-type sensilla in response to low salt (**Figure 1—figure supplement 1B**). Using *Ir56b*-*GAL4* to drive *UAS-mCD8::GFP*, we also confirmed that the reporter was restricted to a subset of Class A GRNs, which were marked with *LexAop-tdTomato* expressed under the control of the *Gr64f*-*LexA* (**Figure 1—figure supplement 1D—F**). We generated a *UAS-Ir56b* transgene which restored normal frequencies of action potentials in *Ir56b*-expressing GRNs (**Figure 1—figure supplement 1B**). Moreover, ectopic expression of *UAS-Ir56b* in GRNs that typically have minimal responses to low salt, caused a large increase in salt-induced action potentials (**Figure 1—figure supplement 1C**).

In our behavioral screen for *Ir* mutants required for avoiding high salt (300 mM NaCl), we found that in addition to *Ir7c*, *Ir25a*, and *Ir76b* as previously described⁷, *Ir60b* was also required (**Figure 1A**). The *Ir60b* mutant, *Ir60b*³, was generated by removing 768 base pairs, which spanned from 44 base pairs upstream of the predicted transcription start site to the region coding for the N-terminal 241 residues of the 577-amino acid protein (**Figure 1—figure supplement 2A—C**). We verified the impairment in high salt avoidance using a previously described mutant, *Ir60b*¹ (**Figure 1—figure supplement 2D**)⁴². We conducted dose-response behavioral assays using the *Ir60b* mutants, as well as *Ir25a*, *Ir76b* and *Ir7c* mutants and found that all four exhibited significant deficiencies in avoiding salt concentrations ranging from 200 mM to 500 mM (**Figure 1B**). Nevertheless, all of the mutants exhibited a strong aversion to an extremely high salt concentration (1000 mM), a level twice as concentrated as sea water. This very high level of NaCl could potentially trigger activation of nociceptive neurons, serving as a protective mechanism to prevent potential tissue and organ damage.

Activation of *Ir60b* neurons inhibits motivation to feed

To investigate whether activation of *Ir60b* GRNs induces aversive behavior, we used both chemogenetic and optogenetic approaches. Capsaicin, a ligand for the mammalian TRPV1 channel⁴³, does not normally elicit responses in flies (**Figure 1C**) as described previously⁴⁵. Therefore, we expressed *UAS-trpV1* under the control of the *Ir60b*-*GAL4*, and presented the flies with a choice between a 2 mM sucrose and a 2 mM sucrose laced with 100 μ M capsaicin. We found that the transgenic flies actively avoided capsaicin (**Figure 1C**), whereas expression of TRPV1 in Class A (sweet) GRNs (*Gr64f*-*GAL4* and *UAS-trpV1*) induced a preference for capsaicin (**Figure 1C**). These findings support the idea that the activation of *Ir60b* neurons leads to gustatory avoidance.

To further test the proposal that *Ir60b*-positive GRNs elicit aversive behavior, we expressed CsChrimson, a light-activated cation channel⁴⁶ in the *Ir60b* GRN. As controls we drove *UAS-CsChrimson* expression using either the *Gr5a*-*GAL4* or the *Gr66a*-*GAL4*. Upon stimulation with red lights and 2% sucrose, nearly all of the control flies (*UAS-CsChrimson* only) or flies expressing *UAS-CsChrimson* in Class A GRNs (*Gr5a*-*GAL4*) extended their proboscis (**Figure 1D**). In contrast, the PER was notably diminished in flies expressing *UAS-CsChrimson* in the Class B GRNs (*Gr66a*-*GAL4*) or in the *Ir60b* GRN (*Gr66a*-*GAL4*; $56.7 \pm 4.2\%$ and *Ir60b*-*GAL4*; $55.0 \pm 5.0\%$, respectively; **Figure 1D**). These results are fully consistent with a previous study showing optogenetic activation of the *Ir60b* GRN reduces consumption of a sugar⁴². Together, these findings provide compelling evidence that stimulation of the *Ir60b* GRN induces behavioral aversion.

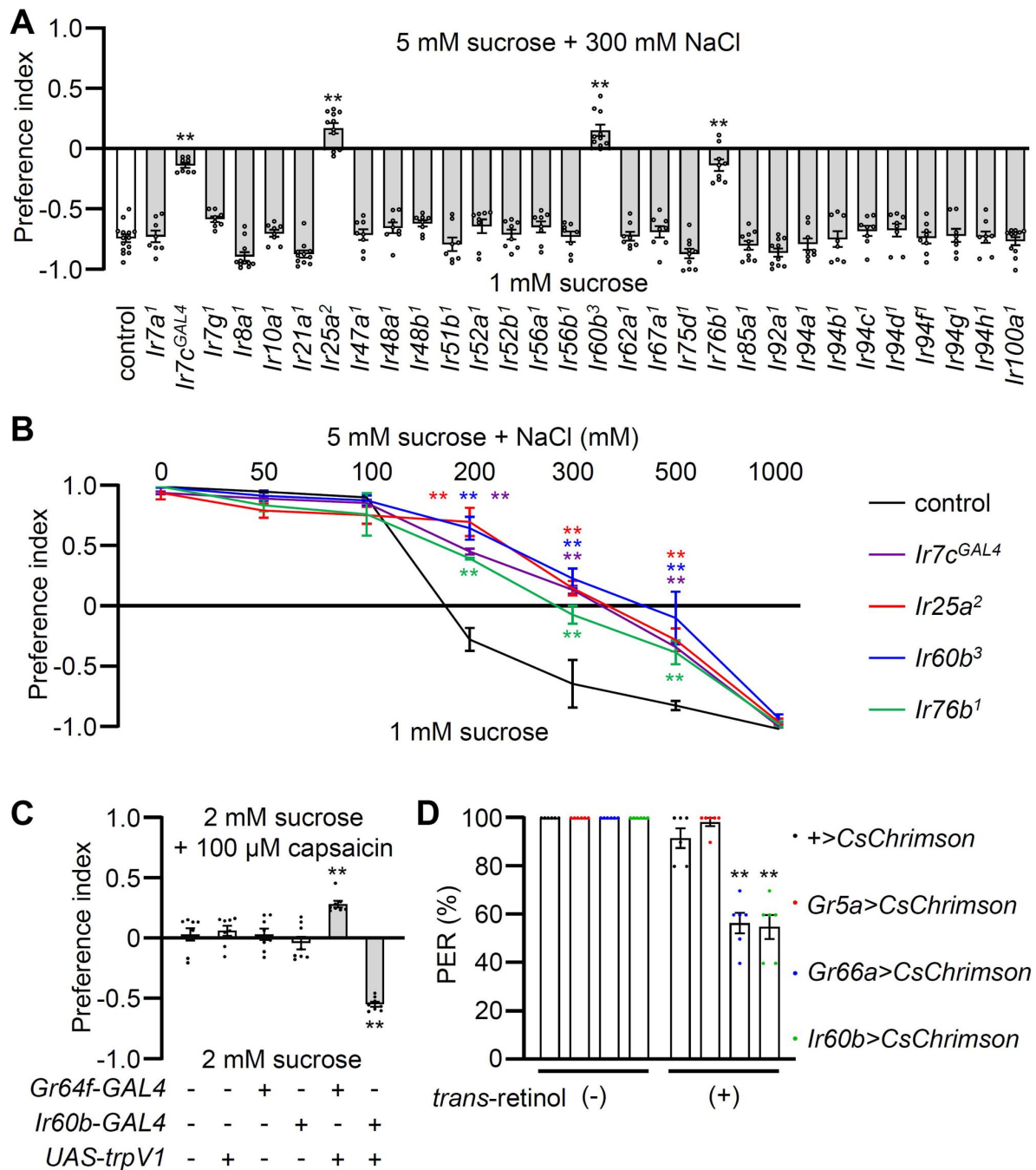


Figure 1.

Testing requirements for *Irs* for avoiding high salt-containing food, and chemogenetic and optogenetic control of *Ir60b* GRNs. (A) Binary food choice assays (1 mM sucrose versus 5 mM sucrose and 300 mM NaCl) comparing 30 *Ir* mutants to the control strain (*w¹¹¹⁸*) for high salt avoidance, n=8–12. (B) Preferences of the indicated flies for 1 mM sucrose versus 5 mM sucrose and 0–1000 mM NaCl. n=8–12. (C) Testing the effects of 100 μ M capsaicin after expressing the rat TRPV1 channel (*UAS-trpV1*) either in Class A GRNs or *Ir60b* GRNs under the control of the *Gr64f-GAL4* or the *Ir60b-GAL4*, respectively. The flies were given a choice between 2 mM sucrose or 2 mM sucrose plus 100 μ M capsaicin. The presence or absence of the various transgenes is indicated by "+" and "-", respectively. n=8. (D) Testing the effects of light activation of various classes of GRNs. *UAS-CsChrimson* was expressed in Class A GRNs (driven by the *Gr5a-GAL4*), Class B GRNs (driven by the *Gr66a-GAL4*) or in *Ir60b* GRNs (driven by the *Ir60b-GAL4*). The flies were then simultaneously exposed to red lights (650 nm; WP-5700, 3M, USA) for 5 sec while 2% sucrose was applied to labellum and the percent PER was recorded. n=6. Data were compared using single-factor ANOVA coupled with Scheffe's *post hoc* test. Statistical significance compared with the control. Means $\pm$ SEMs. **p < 0.01.

***Ir60b* is not required in the labellum to sense high salt**

To investigate the physiological responses of labellar sensilla to high salt (300 mM), we conducted tip recordings on each of the 31 sensilla (**Figure 2A**). Five sensilla, including three S-type (S3, S5, and S7) and two L-type (L3 and L4), exhibited the strongest responses to high salt (**Figure 2—figure supplement 1A**). These responses were largely dependent on the IR25a and IR76b co-receptors, as well as IR7c (**Figure 2B, C** and **Figure 2—figure supplement 1B**) as reported⁷. Interestingly, the *Ir60b*³ deletion mutant did not affect the neuronal responses to high salt in labellar taste bristles (**Figure 2B** and **C**). We inactivated individual GRNs by expressing the inwardly rectifying K⁺ channel (*UAS-Kir2.1*)⁴⁷ in Class A GRNs (*Gr64f-GAL4*)⁴⁸, Class B GRNs (*Gr66a-GAL4*)¹⁷, Class C GRNs (*ppk28-GAL4*)¹⁸, and Class D GRNs (*ppk23-GAL4*)¹⁹, and confirmed that the aversive behavior and neuronal responses to high salt primarily relied on Class B and D GRNs (**Figure 2D** and **E**) as described¹¹.

To examine the gustatory repulsion to high salt that is mediated through the labellum, we conducted proboscis extension response (PER) assays. Starved control and *Ir* mutant flies extend their proboscis when the labellum is lightly touched with a 100 mM sucrose probe (**Figure 2F**). Upon a second sucrose offering, the various fly lines exhibited slightly diminished responses (**Figure 2G**). When we added 300 mM salt to the sucrose, it significantly reduced the PER in the control group (**Figure 2H** and **I**; first offering 40.9 ± 4.0%; second offering 41.5 ± 3.7%). Both the *Ir25a*² and *Ir76b*¹ mutants also exhibited suppressed PERs, but the suppression was not as great as in the control (**Figure 2H** and **I**). In contrast, high salt reduced the PER by the *Ir60b*³ mutant to a similar extent as the control (**Figure 2H** and **I**; first offering 41.6 ± 6.5%; second offering 47.7 ± 6.7%). This indicates that the labellum of the *Ir60b*³ detects 300 mM salt normally, even though the mutant is impaired in avoiding high salt in a two-way choice assay (**Figure 1A**).

High salt sensor in the pharynx

The observations that *Ir60b* is required for the normal aversion to high salt, but does not appear to function in labellar bristles raise the possibility that *Ir60b* is required in the pharynx for salt repulsion. *Ir60b* is expressed in the pharynx where it plays a role in limiting sucrose consumption⁴². *Gr2a* is also expressed in the pharynx and contributes to the repulsion to moderate salt levels (150 mM)¹⁴. However, the *Gr2a*^{GAL4} mutant displays a normal response to high salt (450 mM)¹⁴. In our two-way choice assay, which focuses on 300 mM NaCl, we found that salt repulsion displayed by the *Gr2a*^{GAL4} mutant was also indistinguishable from the control (**Figure 2—figure supplement 2**).

To investigate a role for GRNs in the pharynx for high salt (300 mM) repulsion, we conducted tests on the *Poxn* mutant (*Poxn*^{70–28}/*Poxn*^{ΔM22–B5}) in which external chemosensory bristles have been converted to mechanosensory sensilla⁴⁹. The *Poxn* mutants retain GRNs in taste pegs, which are hairless sensilla³⁶. As a result, *Poxn* mutants only possess intact internal gustatory organs, as well as taste pegs. We found that the aversive behavior to high salt was reduced in the *Poxn* mutants relative to the control (**Figure 2J**), consistent with previous studies demonstrating roles for GRNs in labellar bristles in high salt avoidance^{7,11,12}. However, the diminished high salt avoidance of the *Poxn* mutant was significantly different from the *Poxn;Ir60b*³ double mutant, even though the response of *Poxn;Ir60b*³ was not significantly different from *Ir60b*³ (**Figure 2J**).

Quantification of increased high salt ingestion in *Ir60b* mutants

In a prior study, it was observed that the repulsion to high salt exhibited by the *Ir60b* mutant was indistinguishable from wild-type⁴². Specifically, the flies were presented with a drop of liquid (sucrose plus salt) at the end of a probe, and the *Ir60b* mutant flies fed on the food for the same period of time as control flies⁴². However, this assay did not discern whether or not the volume of the high salt-containing food consumed by the *Ir60b* mutant flies was reduced relative to control flies. Therefore, to assess the volume of food ingested, we used the Drosophila system, which we recently developed (**Figure 3—figure supplement 1A**)⁴⁴. This system consists of a set of five

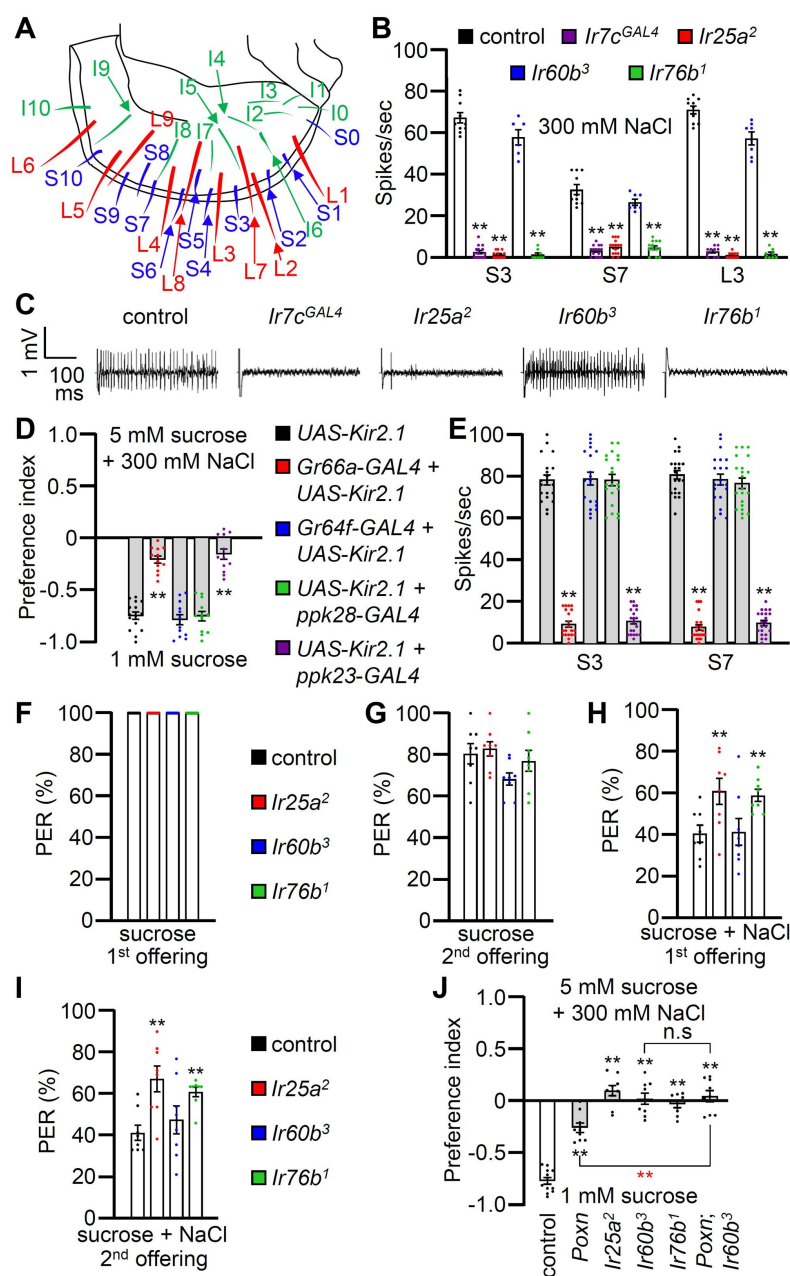


Figure 2.

Contributions of different classes of GRNs to high salt avoidance. **(A)** Schematic showing the names of sensilla bristles on the labellum ⁶⁵. **(B)** Tip recordings conducted on S3, S7, and L3 sensilla using 300 mM NaCl and the indicated flies. $n=10-16$. **(C)** Representative traces obtained from S3 sensilla. **(D)** Binary food choice assays (1 mM sucrose versus 5 mM sucrose and 300 mM NaCl) after inactivating different classes of GRNs with *UAS-Kir2.1*, driven by the indicated *GAL4* drivers: Class A (*Gr64f*; blue), Class B (*Gr66a*; red), Class C (*ppk28*; green) and Class D (*ppk23*; purple). Significances were determined by comparing to the *UAS-Kir2.1* only control (black). $n=12$. **(E)** Tip recordings conducted by stimulating S3 and S7 sensilla with 300 mM NaCl from flies with different classes of GRNs inactivated with *UAS-Kir2.1*. See panel **D** for legend. $n=16-20$. **(F–I)** PER assays performed using the control strain (w^{1118} ; black) and *Ir25a*² (red), *Ir60b*³ (blue), *Ir76b*¹ (green). $n=8-10$. **(F)** PER percentages induced by 2% sucrose (1st offering). **(G)** PER percentages induced by 2% sucrose (2nd offering). **(H)** PER percentages induced by 2% sucrose with 300 mM NaCl (1st offering). **(I)** PER percentages induced by 2% sucrose with 300 mM NaCl (2nd offering). **(J)** Binary food choice assays for 300 mM salt avoidance were conducted with the control strain, *Poxn* (*Poxn*⁷⁰⁻²⁸/*Poxn*^{ΔM22-B5}), *Ir25a*², *Ir60b*³, *Ir76b*¹, and *Poxn;Ir60b*³. $n=9-12$. Data were compared using single-factor ANOVA coupled with Scheffé's *post hoc* test. Statistical significances compared with the control flies or the *Poxn* mutant are denoted by black and red asterisks, respectively. Means $\pm$ SEMs. $**p < 0.01$.

separately housed flies, each of which is exposed to two capillary tubes with different liquid food options. One capillary contained 100 mM sucrose and the other contained 100 mM sucrose mixed with 300 mM NaCl. The volume of food consumed from each capillary was then monitored automatically over the course of 6 hours and recorded on a computer. We found that control flies consuming approximately four times more of the 100 mM sucrose than the sucrose mixed with 300 mM NaCl (**Figure 3A**). In contrast, the *Ir25a*, *Ir60b*, and *Ir76b* mutants consumed approximately two-fold less of the sucrose plus salt (**Figure 3A**). Consequently, they ingested similar amounts of the two food options (**Figure 3B**; ingestion index). Thus, while the *Ir60b* mutant and control flies spend similar amounts of time in contact with high salt-containing food when it is the only option⁴², the mutant consumes considerably less of the high salt food when presented with a sucrose option without salt.

To further investigate the requirement for *Ir25a*, *Ir60b*, and *Ir76b*, we performed genetic rescue experiments. We introduced their respective wild-type cDNAs under the control of their cognate *GAL4* drivers, which resulted in a conversion from salt-insensitive behavior to the salt-sensitive behavior observed in wild-type flies (**Figure 3C–H**). In addition, the defects in the *Ir25a*² and *Ir76b*¹ mutants were fully rescued by expressing the wild-type *Ir25a* and *Ir76b* transgenes, respectively, in the pharynx using the *Ir60b-GAL4* (**Figure 3I–L**). This suggests that both *IR25a* and *IR76b* act as coreceptors in the *Ir60b* GRNs. Furthermore, we investigated whether the expression of *UAS-Ir60b* driven by *Ir25a-GAL4* or *Ir76b-GAL4* could rescue the defects observed in *Ir60b*³. Despite the broad expression of *Ir60b* using these *GAL4* drivers, the *Ir60b* salt ingestion defect was rescued (**Figures 3M** and **N**).

Next, we addressed whether *Ir60b* is required specifically for regulating ingestion of high salt. To investigate this, we assessed the volumes of caffeine, strychnine, and coumarin consumed by *Ir60b*³ flies. We found that the *Ir60b*³ mutant displayed similar consumption patterns to the wild-type control flies for these bitter compounds (**Figure 3O, P** and **Figure 3—figure supplement 1B–E**). This is in contrast to the impairments exhibited by the *Gr66a*^{ex83} mutant (**Figure 3O, P** and **Figure 3—figure supplement 1B–E**), which displays defects in sensing many bitter chemicals. This indicates that *Ir60b* is involved in regulating the avoidance of high salt ingestion rather than general avoidance responses to toxic compounds. Nevertheless, the role of *Ir60b* in suppressing feeding is not limited to high salt, since *Ir60b* also functions in the pharynx in inhibiting the consumption of sucrose⁴².

To investigate the aversion induced by high salt in the absence of a highly attractive sugar, such as sucrose, we combined 300 mM salt with 100 mM sorbitol, which is a tasteless but nutritive sugar^{50,51}. Using two-way choice assays, we found that the *Ir25a*, *Ir60b*, and *Ir76b* mutants exhibited substantial reductions in high salt avoidance (**Figure 3—figure supplement 2A**). In addition, we performed Drosophila assays using 100 mM sorbitol alone or sorbitol mixed with 300 mM NaCl. Sorbitol alone provoked less feeding than sucrose since it is a tasteless sugar (**Figure 3—figure supplement 2B and C**). Nevertheless, addition of high salt to the sorbitol reduced food consumption (**Figure 3—figure supplement 2B and C**).

A single neuron in the LSO depends on *Ir25a*, *IR60b* and *Ir76b* for responding to both high salt and sucrose

In addition to *Ir60b*, two broadly required *Irs* (*Ir25a* and *Ir76b*) also function in repulsion to high salt^{11,12}. Moreover, we found that we could rescue the *Ir25a*, *Ir60b* or *Ir76b* Drosophila phenotypes using the same *Ir60b-GAL4* to drive expression of the cognate wild-type transgenes in the corresponding mutant backgrounds. These findings imply that all three *Irs* are coexpressed in the *Ir60b* GRN in the pharynx. Therefore, we examined the relative expression patterns of the *Ir60b-GAL4* reporter with the *Ir25a* and *Ir76b* reporters. We observed that the *Ir76b-QF* reporter was expressed in two cells within the labral sensory organ (LSO), one of which colocalized with the *Ir60b* reporter (**Figure 4A**). Additionally, the expression pattern of the *Ir25a-GAL4* perfectly

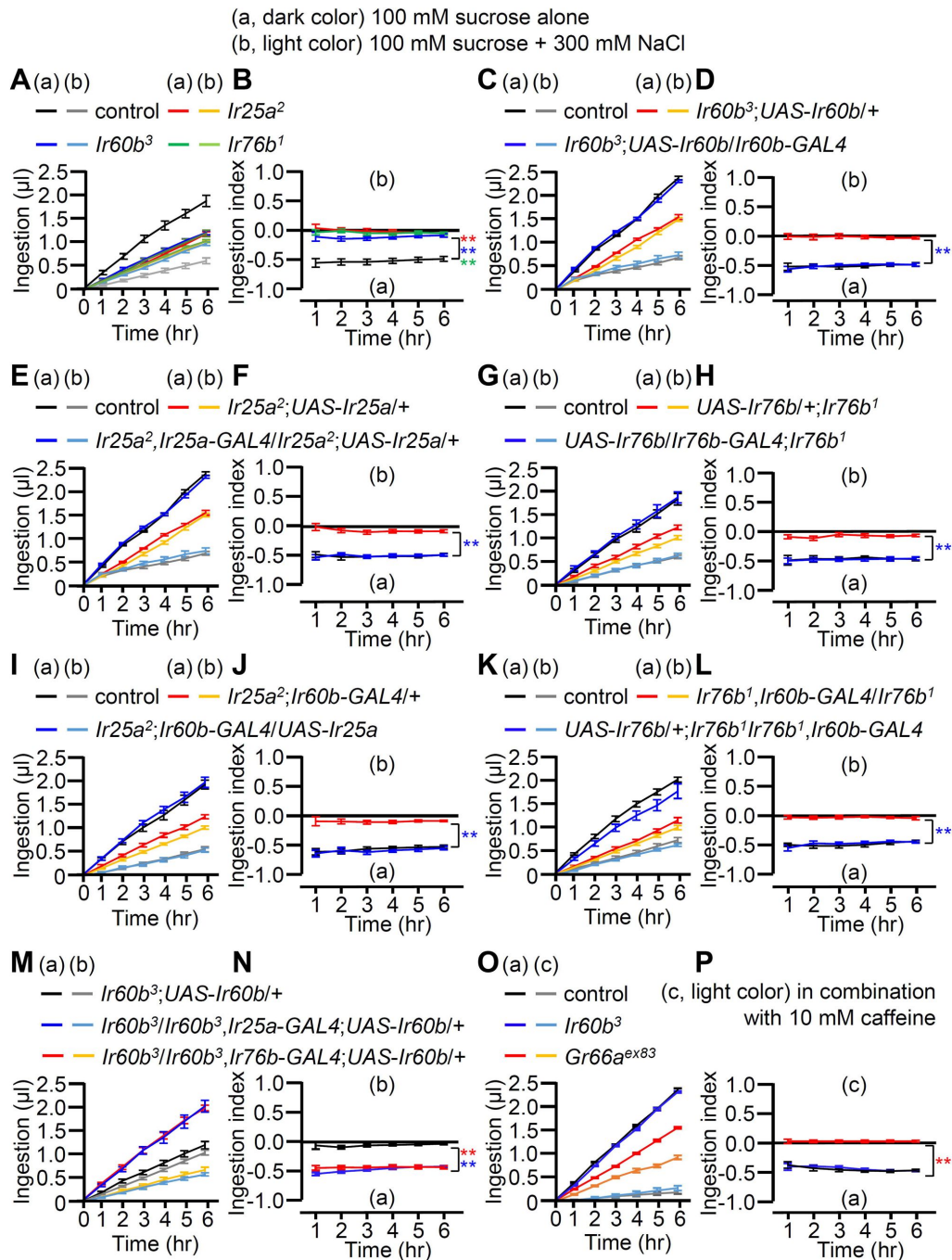


Figure 3.

Measuring volume of food intake in *Ir* mutants using the Drosophila system. (**A–N**) Each fly was exposed to two capillaries, one of which contained 100 mM sucrose (a), and the other contained 100 mM sucrose and 300 mM NaCl (b). (**O and P**) Each fly was exposed to two capillaries, one of which contained 100 mM sucrose (c), and the other contained 100 mM sucrose and 10 mM caffeine (d). (**A, C, E, G, I, K, M and O**) Volumes of the two food options consumed by the indicated flies over the course of 6 hrs. (**B, D, F, H, J, L, N and P**) Ingestion indexes to indicate the relative consumption of the two foods. Ingestion indexes were calculated in each time point using the following equation: $[(\text{Ingestion volume of 100 mM sucrose and 300 mM NaCl or 10 mM caffeine}) - (\text{Ingestion volume of 100 mM sucrose})] / [(\text{Ingestion volume of 100 mM sucrose and 300 mM NaCl or 10 mM caffeine}) + (\text{Ingestion volume of 100 mM sucrose})]$ $n=12$. Multiple sets of data were compared using single-factor ANOVA coupled with Scheffe's *post hoc* test. Statistical significances were relative to the control and determined for the Ingestion indexes only. In all panels, the controls were *w¹¹¹⁸*. The colors of the asterisks match the colors of the genotypes in the corresponding panels. Means $\pm$ SEMs. $**p < 0.01$.

overlapped with that of *Ir76b-QF* in the LSO (**Figure 4B**). Thus, we suggest that *Ir25a*, *Ir60b* and *Ir76b* function in the same GRN in the LSO to limit consumption of high salt. We attempted to induce salt activation in the I-type sensilla by ectopically expressing *Ir60b*, under control of the *Gr33a-GAL4*. *Gr33a* is co-expressed with *Gr66a*⁵², which has been shown to be co-expressed with *Ir25a* and *Ir76b*^{19,53}. When we performed tip recordings from I5 and I9 sensilla, we did not observe a significant increase in action potentials in response to 300 mM NaCl (**Figure 4—figure supplement 1A**), indicating that ectopic expression of *Ir60b* in combination with *Ir25a* and *Ir76b* is not sufficient to generate a high salt receptor.

To determine whether the *Ir60b* GRN in the LSO is activated by high salt, we examined Ca^{2+} responses in the LSO using *UAS-GCaMP6f*, expressed under the control of each *GAL4* driver. In the wild-type LSO, we identified a single cell that responded to 300 mM NaCl (**Figure 4C**), indicating that the GRN in the LSO that expresses all three reporters responds to high salt. Moreover, this GRN responded robustly to 300 mM to 1000 mM Na^+ but not to a low level of Na^+ (50 mM; **Figure 4E**). We then examined the Ca^{2+} responses in the *Ir25a*², *Ir60b*³, and *Ir76b*¹ mutants, and found that each of them failed to respond to NaCl (**Figure 4D and E**). Additionally, we rescued the deficits in the GCaMP6f responses exhibited by each mutant by expressing a wild-type transgene under control of the corresponding *GAL4* driver (**Figure 4F**). We also tested other Cl^- salts (CaCl_2 , MgCl_2 , and KCl) to determine if Cl^- rather than Na^+ induced responses in the *Ir60b* GRN. None of these other salts affected these neurons at the 50 mM, 300 mM, and 500 mM concentrations tested (**Figure 4G**). In contrast, NaBr induced GCaMP6f responses (**Figure 4H**). Thus, the *Ir60b* GRN is responsive to Na^+ and not Cl^- . Due to the effects of NaBr on the *Ir60b* GRN, we used the Drosophila assay to determine whether 300 mM NaBr suppressed ingestion of sucrose. We found that the impact of NaBr on sucrose ingestion was similar to that with NaCl (**Figure 4—figure supplement 1B and C**). We also found that the *Ir60b* GRN did not respond to bitter compounds such as quinine, caffeine, strychnine, lobeline, denatonium, and coumarin at the 5 mM and 50 mM concentrations (**Figure 4I**).

It has been shown previously that *Ir60b* is required in a single GRN in the LSO for suppressing sucrose feeding, and this neuron responds to sucrose⁴². Therefore, we tested whether the same GRN in the LSO that responds to salt also responds to sucrose. Using GCaMP6f, we found that the *Ir60b* GRN was responsive to sucrose in the LSO of control flies, but not in the *Ir25a*, *Ir60b* and *Ir76b* mutants (**Figure 4J**). Furthermore, we used GCaMP6f to compare the Ca^{2+} responses exhibited by the *Ir60b* GRN to 100 mM sucrose alone, 300 mM NaCl alone, and a combination of 100 mM sucrose and 300 mM NaCl. We found that the Ca^{2+} responses were significantly higher when we exposed the *Ir60b* GRN to 300 mM NaCl alone, compared with the response to 100 mM sucrose alone (**Figure 4—figure supplement 1D**). However, the GCaMP6f response was not higher when we presented 100 mM sucrose in combination with 300 mM NaCl, compared with the response to 300 mM NaCl alone. We conclude that the same LSO neuron depends on the same three receptors (*Ir25a*, *Ir60b*, and *Ir76b*) for suppressing feeding in response to high salt or to sucrose.

Both Class B and Class D GRNs in labellar bristles respond to high salt¹⁵, and the Class D GRNs (marked by *ppk23*) depend on *Ir7c* as well as *Ir25a* and *Ir76b* for responding to high salt⁷. Consequently, we investigated whether *Ir7c* plays a role in the *Ir60b* GRN in the LSO. We expressed *UAS-GCaMP6f* under control of the *Ir60b-GAL4* in either or *Ir7c*^{GAL4} mutant background or in a heterozygous (*Ir7c*^{GAL4/+}) control. We then stimulated the *Ir60b* GRN in the LSO with 300 mM NaCl and found that the responses elicited by the mutant and control were the same (**Figure 4—figure supplement 2A**). Consistent with these findings, we did not detect *Ir7c* reporter expression in the *Ir60b* GRNs (**Figure 4—figure supplement 2B–D**).

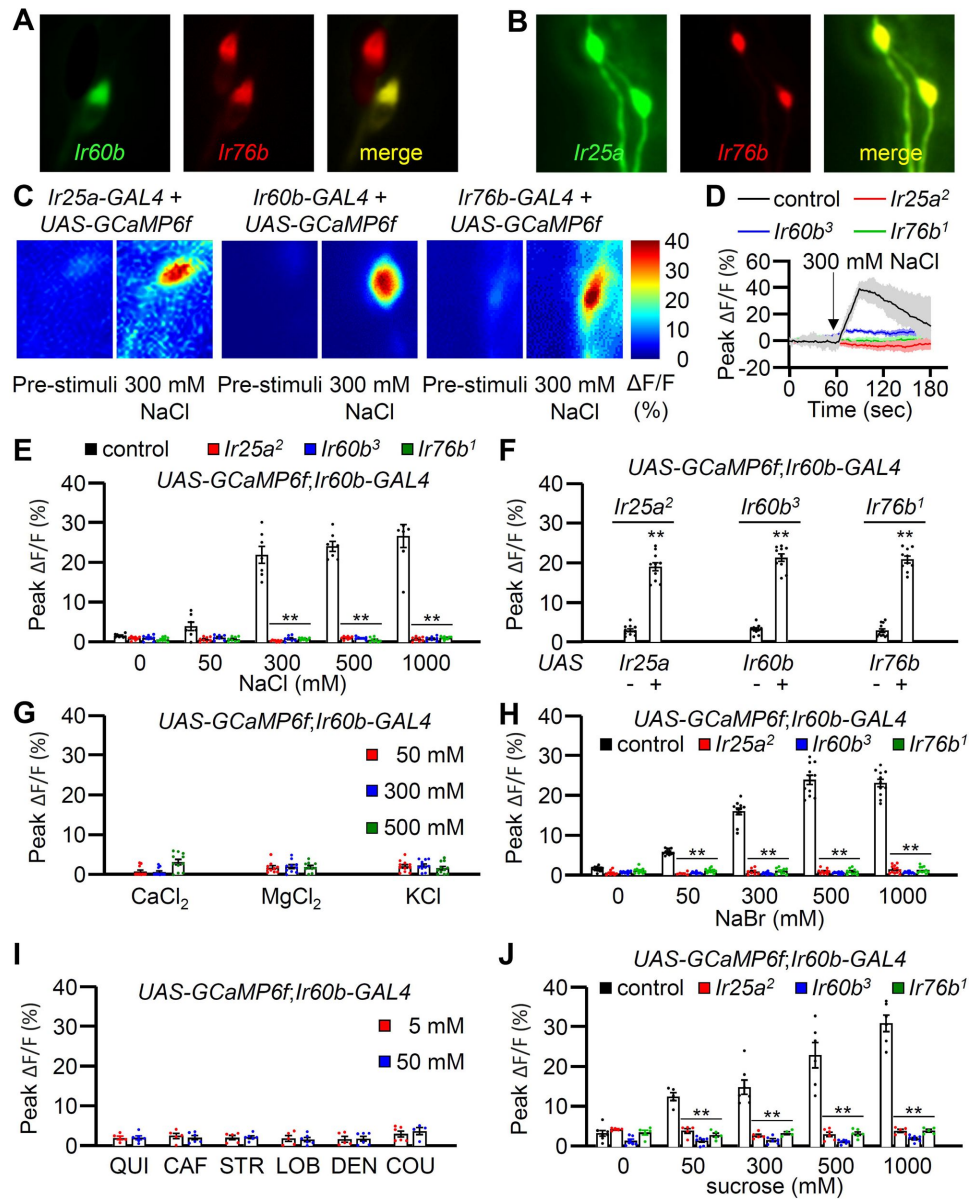


Figure 4.

GCaMP6f responses of *Ir60b* GRNs to NaCl and other chemicals. **(A)** Relative staining of the *Ir60b* reporter (green; anti-GFP) and the *Ir76b* reporter (red; anti-dsRed) in the LSO of *UAS-mCD8::GFP/Ir76b-QF2;Ir60b-GAL4/QUAS-tdTomato* flies. Merge is to the right. **(B)** Relative staining of the *Ir25a* reporter (green; anti-GFP) and the *Ir76b* reporter (red; anti-dsRed) in the LSO of *Ir25a-GAL4/Ir76b-QF2;UAS-mCD8::GFP/QUAS-tdTomato*. Merge is to the right. **(C–J)** Peak GCaMP6f responses ($\Delta F/F$) of *Ir60b* GRNs in flies expressing UAS-GCaMP6f under control of the indicated GAL4 driver. **(C)** Heat map images illustrating changes in GCaMP6f fluorescence before and after stimulation with 300 mM NaCl using the indicated flies. **(D)** Sample traces depicting GCaMP6f responses to 300 mM NaCl. The traces are from the indicated flies expressing UAS-GCaMP6f driven by the *Ir60b*-GAL4. *n*=10–14. **(E)** GCaMP6f responses to various concentrations of NaCl in the indicated flies. UAS-GCaMP6f was driven by the *Ir60b*-GAL4. *n*=10–14. **(F)** GCaMP6f responses to 300 mM NaCl in the indicated mutants and in the absence or presence of the corresponding rescue transgene indicated by “-” and “+”, respectively. *n*=10. **(G)** GCaMP6f responses to 50 mM, 300 mM, and 500 mM of CaCl₂, MgCl₂, and KCl in control flies. *n*=10–14. **(H)** GCaMP6f responses of *Ir60b* GRNs from the indicated flies to various concentrations of NaBr. *n*=10–14. **(I)** GCaMP6f responses to 5 mM and 50 mM concentrations of bitter compounds (quinine, caffeine, strychnine, lobeline, denatonium, and coumarin). *n*=8–10. **(J)** GCaMP6f responses to various concentrations of sucrose in *Ir60b* GRNs from the indicated flies. *n*=10–14. Multiple sets of data were compared using single-factor ANOVA coupled with Scheffe’s *post hoc* test. Statistical significance compared with the controls. Means $\pm$ SEMs. ***p* < 0.01.

Discussion

The taste organs lining the walls of the pharynx represent the final gatekeepers that flies use to decide whether to continue feeding or to reject the food. We found that activation of a single pharyngeal GRN by high salt depends on three IRs, two of which are widely expressed in other GRNs (*Ir25a* and *Ir76b*) and were previously shown to function in salt taste sensation in external labellar bristles. In addition, we identified a third IR (*Ir60b*) that contributes to salt rejection. Consistent with a previous study⁴², *Ir60b* is expressed in a single GRN in each of two labral sense organs (LSOs) lining the pharynx. In addition, we demonstrated that *Ir60b* is co-expressed with *Ir25a* and *Ir76b* in this one GRN in each LSO.

Multiple observations in this study underscore the important role of the *Ir60b* GRN in sensing high Na^+ levels and in promoting salt rejection. Mutation of *Ir60b* eliminates the flies ability to reject sucrose laced with high salt over another option with sucrose only. This result is especially notable in view of the observation that flies that are missing all GRNs in labellar bristles in the *Poxn* mutant exhibit a less pronounced defect than the *Ir60b* mutant, since *Poxn* mutant flies still retain some bias for sucrose over sucrose laced with high salt. The observation that one pair of pharyngeal GRNs is sufficient to induce rejection of high salt underscores the profound role of internal taste neurons in protecting flies from ingesting dangerous levels of this mineral. In addition, activation of the *Ir60b* GRN suppresses feeding since optogenetic activation of these neurons effectively suppresses the proboscis extension response (PER) when the flies are presented with a highly attractive tastant (sucrose). Conversely, Yang et al. demonstrated that activation of *Ir60b* neurons can induce the activation of IN1 neurons, potentially leading to heightened feeding⁵⁴. However, our research reveals a specific activation pattern for *Ir60b* neurons. Instead of being generalists, they exhibit specialization for certain sugars, such as sucrose and high salt. As a result, while *Ir60b* GRNs activate IN1 neurons⁵⁴, we posit that there are other neurons in the brain responsible for inhibiting feeding.

A finding that is seemingly conflicting with the optogenetic results is that mutation of *Ir60b* does not reduce the suppression of the PER when the flies are presented with sucrose laced with high Na^+ levels. The PER assay under high salt conditions only permits flies to taste the food without allowing ingestion. Consequently, the internal sensor may not exhibit suppression of the PER. In contrast, optogenetic activation has the capability to directly stimulate the internal sensor, leading to the induction of PER suppression. We propose that high Na^+ is effective in suppressing the PER by the *Ir60b* mutant since these flies still have functional high salt receptors in aversive GRNs in labellar bristles. In support of this conclusion, loss of either *Ir25a* or *Ir76b* reduces the suppression of the PER by high salt, and this occurs because *Ir76b* and *Ir25a* are required in both high salt activated GRNs in labellar bristles^{11,12} and in the *Ir60b* GRN in the pharynx.

We also found that the requirement for *Ir60b* appears to be different when performing binary liquid capillary assay (DrosoX), versus solid food binary feeding assays. When we employed the DrosoX assay to test mutants that were missing salt aversive GRNs in labellar bristles but still retained functional *Ir60b* GRNs, the flies behaved the same as wild-type flies (e.g. **Figure 3J** and **L**). However, using solid food binary assays, *Poxn* mutants, which are missing labellar taste bristles but retain *Ir60b* GRNs³⁶, displayed repulsion to high salt food that was intermediate between control flies and the *Ir60b* mutant (**Figure 2J**). *Poxn* mutants still possess taste pegs³⁶, and these hairless taste organs become exposed to food only when the labial palps open. We suggest that there are high-salt sensitive GRNs associated with taste pegs, which are accessed when the labellum contacts a solid substrate, but not when flies drink from the capillaries used in DrosoX assays. This explanation would also account for the findings that the *Ir60b* mutant is indifferent to 300 mM NaCl in the DrosoX assay (**Figure 3B**), but prefers 1 mM sucrose alone over 300 mM NaCl and 5 mM sucrose in the solid food binary assay (**Figure 1B**). Alternatively, the different behavioral responses might be due to the variation in sucrose concentrations in each

of these two assays, which employed 5 mM sucrose in the solid food binary assay, as opposed to 100 mM sucrose in the Drosophila assay. The disparity in attractive valence between these two concentrations of sucrose might consequently impact feeding amount and preference.

Another unresolved question is why does the same pharyngeal GRN respond to sucrose and high Na^+ . It has been pointed out that since flies prefer over ripe fruit, which has lower sucrose levels than ripe fruit, then activation of the *Ir60b* GRN by sucrose might serve to favor consumption of fruit at an advanced state of ripeness³⁷. In contrast to sugar, Na^+ levels tend to remain constant during the ripening process⁵⁵ and remain at relatively low levels (50–100 mM), which is below the level that is aversive to flies. Thus, in contrast to sucrose, assessing the concentration of Na^+ does not aid in the distinction of ripe versus overripe fruit, but may be important in avoiding consuming non-vegetarian sources of food that are high in Na^+ .

Based on Ca^{2+} imaging results with GCaMP6f, we conclude that the *Ir60b* GRN directly responds to NaCl, and this depends on the presence of *Ir25a*, *Ir60b* and *Ir76b*. The *Ir60b* GRN responds to high NaCl, but is not stimulated by CaCl_2 , MgCl_2 and KCl. Thus, the *Ir60b* GRN is a Na^+ sensor not a Cl^- sensor. In further support of this conclusion, we found that the *Ir60b* GRN also responds to NaBr.

An open question is the subunit composition of the pharyngeal high Na^+ receptor, and whether the sucrose/glucose and Na^+ receptors in the *Ir60b* GRN are the same or distinct. Our results indicate that the high salt sensor in the *Ir60b* GRN includes IR25a, IR60b and IR76b since all three IRs are required in the pharynx for sensing high levels of NaCl. I-type sensilla do not elicit a high salt response, and we were unable to induce salt activation in I-type sensilla by ectopically expressing *Ir60b*, under control of the *Gr33a-GAL4*. This indicates that IR25a, IR60b and IR76b are insufficient for sensing high Na^+ . The inability to confer a salt response by ectopic expression of *Ir60b* was not due to absence of *Ir25a* and *Ir76b* in *Gr33a* GRNs since *Gr33a* and *Gr66a* are co-expressed⁵², and *Gr66a* GRNs express *Ir25a* and *Ir76b*^{19,53}. Thus, the high salt receptor in *Ir60b* GRNs appears to require an additional subunit. Given that Na^+ and sugars are structurally unrelated, we suggest that the Na^+ and sucrose/glucose receptors do not include the identical set of subunits, or that they activate a common receptor through disparate sites.

Finally, it is remarkable that the single *Ir60b* GRN in the LSO of the pharynx is also stimulated by sucrose and to a lesser extent to glucose⁴², since this GRN is otherwise very narrowly tuned. We did not detect GCaMP signals upon application any of six bitter tastants tested. The *Ir60b* GRN is also unresponsive to trehalose and glycerol. Based on behavioral experiments, mutation of *Ir60b* does not impact on consumption of an array of amino acids, low pH, bitter chemicals and other sugars³⁷. It is also surprising that the pharyngeal GRN that responds to Na^+ is unresponsive to other cations such as Ca^{2+} , which is toxic at high levels¹⁹. The fact that there are relatively few pharyngeal GRNs, yet one is narrowly tuned to sucrose, glucose and Na^+ underscores the critical role of limiting Na^+ consumption in flies, which could otherwise lead to dehydration, and dysfunction of many homeostatic processes impacted by excessive levels of Na^+ ⁵⁶.

Materials and methods

Key resources table

Reagent type	Designation	SOURCE	IDENTIFIER
Antibody	Mouse anti-GFP	Molecular probes	Cat # A11120; RPID: AB_221568
Antibody	Rabbit anti-DsRed	Clontech	Cat # 632496; RPID: AB_10013483
Antibody	Goat Anti-mouse Alexa Fluro 488	Invitrogen	Cat # A32723 ; RRID: AB_2633275
Antibody	Goat anti-rabbit Alexa Fluor 568	Invitrogen	Cat # A11011 ; RPID: AB_143157
Chemical compound	Sucrose	Sigma-Aldrich	Cat # 9378S
Chemical compound	Tricholine citrate	Sigma-Aldrich	Cat # T0252
Chemical compound	Sulforhodamine B	Sigma-Aldrich	Cat # 230162
Chemical compound	Capsaicin	Sigma-Aldrich	Cat # M2028
Chemical compound	Caffeine	Sigma-Aldrich	Cat # C02750
Chemical compound	CaCl ₂ dihydrate	Sigma-Aldrich	Cat # C3881
Chemical compound	KCl	Sigma-Aldrich	Cat # P9541
Chemical compound	Quinine	Sigma-Aldrich	Cat # Q1125
Chemical compound	Strychnine	Sigma-Aldrich	Cat # S8753
Chemical compound	Lobeline	Sigma-Aldrich	Cat # 141879

Chemical compound	Denatonium	Sigma-Aldrich	Cat # D5765
Chemical compound	Coumarin	Sigma-Aldrich	Cat # C4261
Chemical compound	Brilliant blue FCF	Wako Pure Chemical Industry	Cat # 027-12842
Chemical compound	Paraformaldehyde	Electron Microscopy Sciences	Cat # 15710
Chemical compound	NaCl	LPS solution	Cat # NACL01
Chemical compound	MgCl ₂ hexahydrate	SAMCHUN	Cat # M0038
Chemical compound	NaBr	DUKSAN	Cat # S2531
Chemical compound	Goat Serum, New Zealand origin	Gibco	Cat # 16210064
Genetic reagent (<i>Drosophila melanogaster</i>)	<i>w¹¹¹⁸</i>	Bloomington <i>Drosophila</i> Stock Center (BDSC)	BDSC:5905
Genetic reagent (<i>Drosophila melanogaster</i>)	<i>Ir7a¹</i>	Dr. Y. Lee	23
Genetic reagent (<i>Drosophila melanogaster</i>)	<i>Ir7g¹: y¹w¹Mi{y⁺mDintZ=MIC}Ir7g^{M106687}</i>	BDSC	BDSC:42420
Genetic reagent (<i>Drosophila melanogaster</i>)	<i>Ir7c^{GAL4}</i>	Dr. M. D. Gordon	7
Genetic reagent (<i>Drosophila melanogaster</i>)	<i>Ir8a¹: w¹TI{w[+m*]=TI}Ir8a¹;BI¹L²/CyO</i>	BDSC	BDSC:23842
Genetic reagent (<i>Drosophila melanogaster</i>)	<i>Ir10a¹: w¹¹¹⁸₀₃₂₇₃ Mi{GFP^{E:3XP3}=ET1}Ir10a^{MB}</i>	BDSC	BDSC:41744
Genetic reagent (<i>Drosophila melanogaster</i>)	<i>Ir21a¹: w¹¹¹⁸;PBac{w^{+mC}=PB}Ir21a^{CU2720}</i>	BDSC	BDSC:10975
Genetic reagent (<i>Drosophila melanogaster</i>)	<i>Ir25a²</i>	Dr. L. VossHall	57
Genetic reagent (<i>Drosophila melanogaster</i>)	<i>Ir47a¹</i>	Dr. Y. Lee	23
Genetic reagent (<i>Drosophila melanogaster</i>)	<i>Ir48a¹: w¹¹¹⁸_{B09217};Mi{GFP^{E:3XP3}=ET1}Ir48a^M</i>	BDSC	BDSC:26453
Genetic reagent (<i>Drosophila melanogaster</i>)	<i>Ir48b¹: w¹¹¹⁸_{B02315};Mi{GFP^{E:3XP3}=ET1}Ir48b^M</i>	BDSC	BDSC:23473
Genetic reagent (<i>Drosophila melanogaster</i>)	<i>Ir51b¹: w¹¹¹⁸_{c00387};PBac{w^{+mC}=PB}row^{CU0387} I</i>	BDSC	BDSC:10046
Genetic reagent (<i>Drosophila melanogaster</i>)	<i>Ir52a¹</i>	Dr. Y. Lee	23
Genetic reagent (<i>Drosophila melanogaster</i>)	<i>Ir52b¹: w¹¹¹⁸_{B02231}/SM6a;Mi{GFP^{E:3XP3}=ET1}Ir52b^M</i>	BDSC	BDSC:25212

Genetic reagent (<i>Drosophila melanogaster</i>)	<i>Ir52c</i> ¹ : <i>w</i> ¹¹⁷⁸ ;Mi{GFP ^{E.3xP3} =ET1} <i>Ir52c</i> ^{MB} 04402	BDSC	BDSC:24580
Genetic reagent (<i>Drosophila melanogaster</i>)	<i>Ir56a</i> ¹	Dr. Y. Lee	23
Genetic reagent (<i>Drosophila melanogaster</i>)	<i>Ir56b</i> ¹ : <i>w</i> ¹¹⁷⁸ ;Mi{GFP ^{E.3xP3} =ET1} <i>Ir56b</i> ^M B09950	BDSC	BDSC:27818
Genetic reagent (<i>Drosophila melanogaster</i>)	<i>Ir56d</i> ¹ : <i>w</i> [*] ; <i>Ir56d</i> ¹	BDSC	BDSC:81249
Genetic reagent (<i>Drosophila melanogaster</i>)	<i>Ir60b</i> ¹	Dr. J. Carlson	42
Genetic reagent (<i>Drosophila melanogaster</i>)	<i>Ir60b</i> ³	Dr. Y. Lee	In this study
Genetic reagent (<i>Drosophila melanogaster</i>)	<i>Ir62a</i> ¹ : <i>y</i> ¹ <i>w</i> [*] ;Mi{y ^{+mDintZ} =MIC} <i>Ir62a</i> ^{M10089} 5Im1 ^{M100895} /TM3, Sb ¹ Ser ¹	BDSC	BDSC:32713
Genetic reagent (<i>Drosophila melanogaster</i>)	<i>Ir67a</i> ¹ : <i>y</i> ¹ <i>w</i> [*] ;Mi{y ^{+mDintZ} =MIC} <i>Ir67a</i> ^{M11128} 8	BDSC	BDSC:56583
Genetic reagent (<i>Drosophila melanogaster</i>)	<i>Ir75d</i> ¹ : <i>w</i> ¹¹⁷⁸ ;Mi{GFP ^{E.3xP3} =ET1} <i>Ir75d</i> ^M B04616	BDSC	BDSC:24205
Genetic reagent (<i>Drosophila melanogaster</i>)	<i>Ir76b</i> ¹	Dr. C. Montell	12
Genetic reagent (<i>Drosophila melanogaster</i>)	<i>Ir85a</i> ¹ : <i>w</i> ¹¹⁷⁸ ;Mi{GFP ^{E.3xP3} =ET1} <i>Ir85a</i> ^M B04613 Pif1A ^{MB04613}	BDSC	BDSC:24590
Genetic reagent (<i>Drosophila melanogaster</i>)	<i>Ir92a</i> ¹ : <i>w</i> ¹¹⁷⁸ ;Mi{GFP ^{E.3xP3} =ET1} <i>Ir92a</i> ^M B03705	BDSC	BDSC:23638
Genetic reagent (<i>Drosophila melanogaster</i>)	<i>Ir94a</i> ¹	Dr. Y. Lee	23
Genetic reagent (<i>Drosophila melanogaster</i>)	<i>Ir94b</i> ¹ : <i>w</i> ¹¹⁷⁸ ;Mi{GFP ^{E.3xP3} =ET1} <i>Ir94b</i> ^{MBU} 2190	BDSC	BDSC:23424
Genetic reagent (<i>Drosophila melanogaster</i>)	<i>Ir94c</i> ¹	Dr. Y. Lee	23
Genetic reagent (<i>Drosophila melanogaster</i>)	<i>Ir94d</i> ¹ : <i>y</i> ¹ <i>w</i> [*] ;Mi{y ^{+mDintZ} =MIC} <i>Ir94d</i> ^{M101659} CG17380 ^{M101659}	BDSC	BDSC:33132
Genetic reagent (<i>Drosophila melanogaster</i>)	<i>Ir94f</i> ¹ : <i>y</i> ¹ <i>w</i> [*] ;Mi{y ^{+mDintZ} =MIC} <i>Ir94f</i> ^{M100928}	BDSC	BDSC:33095
Genetic reagent (<i>Drosophila melanogaster</i>)	<i>Ir94g</i> ¹ : <i>w</i> ¹¹⁷⁸ ;Mi{GFP ^{E.3xP3} =ET1} <i>Ir94g</i> ^M B07445	BDSC	BDSC:25551

Genetic reagent (<i>Drosophila melanogaster</i>)	<i>Ir94h</i> ¹	Dr. Y. Lee	23
Genetic reagent (<i>Drosophila melanogaster</i>)	<i>Ir100a</i> ¹ ; <i>w</i> ¹¹¹⁸ ; <i>P</i> { <i>w</i> ^{+mC} =EP} <i>Ir100a</i> ^{G19846} <i>CG42233</i> ^{G19846}	BDSC	BDSC:31853
Genetic reagent (<i>Drosophila melanogaster</i>)	<i>UAS-mCD8::GFP</i>	BDSC	BDSC:5137
Genetic reagent (<i>Drosophila melanogaster</i>)	<i>UAS-mCD8::GFP</i>	BDSC	BDSC:32184
Genetic reagent (<i>Drosophila melanogaster</i>)	<i>UAS-Kir2.1</i>	BDSC	BDSC:6595
Genetic reagent (<i>Drosophila melanogaster</i>)	<i>UAS-Ir25a</i>	Dr. Y. Lee	19
Genetic reagent (<i>Drosophila melanogaster</i>)	<i>UAS-Ir60b</i>	Dr. Y. Lee	In this study
Genetic reagent (<i>Drosophila melanogaster</i>)	<i>UAS-Ir76b</i>	Dr. C. Montell	12
Genetic reagent (<i>Drosophila melanogaster</i>)	<i>Ir25a-GAL4</i>	Dr. L. Vosshall	57
Genetic reagent (<i>Drosophila melanogaster</i>)	<i>Ir60b-GAL4</i>	Dr. C. Montell	42
Genetic reagent (<i>Drosophila melanogaster</i>)	<i>Ir76b-GAL4</i>	Dr. C. Montell	12
Genetic reagent (<i>Drosophila melanogaster</i>)	<i>ppk23-GAL4</i>	Dr. K. Scott	58
Genetic reagent (<i>Drosophila melanogaster</i>)	<i>ppk28-GAL4</i>	Dr. H. Amrein	18
Genetic reagent (<i>Drosophila melanogaster</i>)	<i>Gr66a-GAL4</i>	Dr. H. Amrein	17
Genetic reagent (<i>Drosophila melanogaster</i>)	<i>Gr64f-GAL4</i>	Dr. A. Dahanukar	19
Genetic reagent (<i>Drosophila melanogaster</i>)	<i>Ir76b-QF</i>	BDSC	BDSC:51312
Genetic reagent (<i>Drosophila melanogaster</i>)	<i>QUAS-tdTomato</i> : <i>y</i> ¹ <i>w</i> ¹¹¹⁸ ; <i>P</i> { <i>QUAS</i> -mt <i>dTomato</i> -3xHA}26	BDSC	BDSC:30005
Genetic reagent (<i>Drosophila melanogaster</i>)	<i>Poxn</i> ^{ΔMZZ-B5, 0113} : <i>y</i> ¹ <i>w</i> ^{57C23} ; <i>Mi</i> {ET1} <i>Poxn</i> ^{MBU}	BDSC	BDSC:22701

Genetic reagent (<i>Drosophila melanogaster</i>)	<i>Poxn</i> ^{7U-28} : <i>Poxn</i> ^{7U} /CyO; <i>twi</i> -Gal4, <i>UAS</i> <i>-2XEGFP</i>	BDSC	BDSC:60688
Software	Origin Pro Version	Dr. Y. Lee	https://www.originlab.com
Software	GraphPad Prism	Dr. Y. Lee	https://www.graphpad.com
Software	Autospike 3.1 software	Dr. Y. Lee	https://www.syntech.co.za/
Software	Fiji/ImageJ software	Dr. Y. Lee	https://fiji.sc
Software	ZEN lite 2.5 blue	Dr. Y. Lee	https://www.zeiss.com/

Generation of *Ir60b*³ and *UAS-Ir60b* lines

The *Ir60b*³ mutant was generated by ends-out homologous recombination ⁵⁹. For generating the construct for injections, approximately two 3-kb genomic fragments were amplified by PCR, and subcloned into *NotI* and *BamHI* sites of the pw35 vector ⁵⁹. The resulting mutation deleted the region from -44 to +724 (the A of the ATG initiation codon is defined at +1). The construct was injected into *w*¹¹¹⁸ embryos by Best Gene Inc. We outcrossed the mutant to *w*¹¹¹⁸ for six generations.

To generate the *UAS-Ir60b* transgenic line, we amplified the full-length *Ir60b* cDNA by reverse transcription polymerase chain reaction (RT-PCR) using mRNA prepared from whole adult flies and the following primer pair: 5'-GAGAATTCAACTCGAAAATGAGGCGG-3' and 5'-ATGCGGCCGCAATGCTAATTTTG-3'. The *Ir60b* cDNA was subcloned between the *EcoRI* and *NotI* sites of the pUAST vector ⁶⁰, and verified by DNA sequencing. The p*UAS-Ir60b* vector was introduced into *w*¹¹¹⁸ embryos by P-element mediated germline transformation (Korea Drosophila Resource Center, Republic of Korea).

Chemical reagents

The following chemicals were purchased from Sigma-Aldrich (USA): sucrose (CAS No. 57-50-1), tricholine citrate (TCC) (CAS No. 546-63-4), sulforhodamine B (CAS No. 3520-42-1), capsaicin (CAS No. 404-86-4), caffeine (CAS No. 58-08-2), CaCl₂ dihydrate (CAS No. 10035-04-8), KCl (CAS No. 7447-40-7), quinine (CAS No. 6119-47-7), strychnine (CAS No. 1421-86-9), lobeline (CAS No. 134-63-4), denatonium (CAS No. 6234-33-6), and coumarin (CAS No. 91-64-5). Brilliant blue FCF (CAS No. 3844-45-9) was purchased from Wako Pure Chemical Industry (Japan).

Paraformaldehyde (CAS No. 30525-89-4) was purchased from Electron Microscopy Sciences (USA). NaCl (CAS No. 7647-14-5) was purchased from LPS solution (Korea). NaBr (CAS No. 7647-15-6) was purchased from DUKSAN (Korea). Goat serum, New Zealand Origin was purchased from Gibco (USA).

Binary food choice assay using microtiter dishes

We conducted binary food choice assays as described ⁶¹. Briefly, two mixtures were prepared, one of which consisted of 1% agarose, the indicated concentration of NaCl and 5 mM sucrose and red food dye (sulforhodamine B, 0.1 mg/ml). The second mixture contained 1% agarose, 1 mM sucrose, and blue food dye (Brilliant Blue FCF, 0.125 mg/ml). The same phenotypes in **Figure 1B** ⁶¹ were verified by swapping the tastants/dye combinations. The two foods were distributed in alternating wells of a 72-well microtiter dish (Cat # 438733, Thermo Fisher Scientific, USA) in a zigzag pattern. 40–50 flies (3–6 days old) were starved for 18-hour on 1% agarose and transferred to the microtiter dish, which was placed in a dark and humid chamber for 90 mins. The flies were then frozen at -20°C, and the colors of their abdomen to determine the number of flies with blue (N_B), red (N_R), and purple (N_P) abdomens.

Preference indexes (P.I.s) were calculated using the following equation: (N_R - N_B)/(N_R + N_B + N_P) or (N_B - N_R)/(N_R + N_B + N_P), depending on the specific dye/tastant combinations. A P.I. of -1.0 or 1.0 indicates a complete preference for either 5 mM sucrose with the indicated concentration of NaCl or 1 mM sucrose alone, respectively. A P.I. of 0.0 indicates no preference between the two food alternatives.

Proboscis extension response (PER) assays

PER assays were conducted as described ⁶² with minor modifications. Briefly, 20–25 flies (3–6 days old) were deprived of food for 18–20 hrs in vials containing Kimwipe paper wet with tap water. After briefly anesthetizing the flies on ice, they were carefully trapped inside a pipette tip with a volume of 200 µL yellow tip. To expose their heads, the edge of the pipette tip was gently cut

using a paper cutter blade. The protruded head and proboscis were used to deliver stimuli. Total 15–20 flies were prepared for the next step. To eliminate any potential biases due to thirst, water was initially provided to the flies with Kimwipe paper until they no longer responded to water. For both the positive control and initial stimulation, a 2% sucrose solution was used. Flies that did not exhibit a response to the sucrose during the initial exposure were excluded from the experiment. The same conditions as the initial exposures were maintained for the second exposure. Therefore, 10–18 flies were selected for the next step. The tastant stimuli, consisting of either 2% sucrose or 2% sucrose mixed with 300 mM NaCl, were presented using Kimwipe paper. We scored the PER as 1.0 with both complete extensions and partial extensions. PER was calculated using the following equation: (PER flies)/(selected flies). Each test round included 10–18 flies.

DrosoX binary capillary feeding assay

DrosoX is a recently developed modification of the Expresso technique, which quantifies the amount of feeding by fruit flies [63](#). We conducted DrosoX assays essentially as described [44](#). Each sensor bank of the DrosoX system is composed of a printed circuit board housing five Linear Optical Array Sensors (TAOS, TSL1406). Each sensor consists of 768 photodiodes and a microcontroller, establishing a connection to a computer through a Universal Serial Bus port. In the DrosoX setup, when a fly consumes liquid food from a glass capillary, a decrease in the liquid level is identified by a photodiode, enabling the calculation of instantaneous food ingestion. Photodiodes are semiconductor devices, which generate photocurrents upon absorbing light. To ensure light-tight conditions, the sensor bank is enclosed in a box made of black acrylic sheets using precision cutting. A computer reads the electrical signal generated by each photodiode, and the microcontroller (STMicroelectronics, STM32 F103RCBT6) on a development board (Scitech Korea) connected to the DrosoX sensor bank samples the light intensity at each pixel in the array at a rate of 8 Hz. Liquid level readings can be obtained at sample rates ranging from 0.1 to 2 Hz. The data acquisition software records the time vs. liquid level data for multiple sensor banks into a single file using the Hierarchical Data Format (HDF5) (<http://www.hdfgroup.org/HDF5/>). The obtained data is then analyzed using software (DrosoX_gui) supplied by Scitech Korea.

To conduct the DrosoX assays, we inserted the system in a controlled incubator (25°C, 60% humidity). To quantify ingestion, a mixture comprising 100 mM sucrose and the specified concentration of chemicals was injected into a glass tube (Cat # 53432-706; VWR International, USA) using a syringe (KOVAX-SYRINGE 1 mL 26G; KOREA VACCINE, Korea) and needle (Cat # 90025; Hamilton, Switzerland). DrosoX was equipped with five glass tubes containing a solution, while DrosoXD was equipped with another set of five glass tubes containing different solutions (**Figure 3—figure supplement 1A**). DrosoX and DrosoXD were cross-tested. Each cuvette contained flies (3–6 days old) and was physically isolated to prevent them from consuming the solution prior to the experiment. Each experiment was conducted for a duration of 6 hours, from 9 AM to 3 PM. The ingestion amount at time X (X h) was calculated as the difference between the initial solution amount (0 h) and the solution amount at time X.

The ingestion index (I.I) was calculated at each time point using the following equation: $(\text{Ingestion volume}_{\text{DrosoX}} - \text{Ingestion volume}_{\text{DrosoXD}}) / (\text{Ingestion volume}_{\text{DrosoX}} + \text{Ingestion volume}_{\text{DrosoXD}})$ or $(\text{Ingestion volume}_{\text{DrosoXD}} - \text{Ingestion volume}_{\text{DrosoX}}) / (\text{Ingestion volume}_{\text{DrosoXD}} + \text{Ingestion volume}_{\text{DrosoX}})$, depending on the specific tastant combinations. A I.I. at 0.0 indicated no preference based on their ingestion between the two food alternatives.

Tip recordings to assay tastant-induced action potentials

To measure tastant-induced action potentials, we performed tip recordings as previously described [64](#). We immobilized 3–6 day-old flies by exposing them to ice. We immobilized a fly by inserting a reference glass electrode filled with Ringer's solution through the back thorax all the way into the proboscis. The recording glass electrode (tip diameter 10–20 μm) contained the NaCl or aversive compounds dissolved in distilled water with 30 mM tricholine citrate (TCC) or 1 mM

KCl as the electrolyte. Reference glass electrode and recording glass electrode were created by processing Standard Glass Capillaries (Cat # IB150F-3, World Precision Instruments, USA) with glass puller. The recording electrode was placed over a bristle on the labellum and connected to a pre-amplifier (Taste PROBE, Syntech, Germany), which amplified the signals by a factor of 10 using a signal connection interface box (Syntech) and a 100-3,000 Hz band-pass filter. The recorded action potentials were acquired at a sampling rate of 12 kHz and analyzed using Autospike 3.1 software (Syntech). The average frequencies of action potentials (spikes/s) were based on spikes occurring between 50 and 550 msec after contact of the recording electrode. The sensilla bristles were defined as described ⁶⁵[\[65\]](#).

Immunohistochemistry

We performed immunohistochemistry as previously described ⁶⁶[\[66\]](#) with slight modifications. Labella were dissected from 6–8 day-old flies and fixed in a solution containing 4% paraformaldehyde (Electron Microscopy Sciences, Cat # 15710) and 0.2% Triton X-100 for 15 minutes at room temperature. The tissues were then washed three times with PBST (1x PBS and 0.2% Triton X-100), bisected using a razor blade, and incubated in blocking buffer (0.5% goat serum in 1x PBST) for 30 minutes at room temperature. To detect the target protein, primary antibodies (mouse anti-GFP; Molecular Probes, Cat # A11120; diluted 1:1,000 and rabbit anti-dsRed; Clontech, Cat # 632496; diluted 1:1,000) were added to fresh blocking buffer and incubated with the samples overnight at 4 °C. The tissues were then washed three times with PBST, incubated with the secondary antibodies (goat anti-mouse Alexa Fluor 488; Invitrogen, Cat # A32723; diluted 1:200 and goat anti-rabbit Alexa Fluor 568; Invitrogen, Cat # A11011; diluted 1:200) for 4 hours at 4 °C, washed three times with PBST, and placed in 1.25x PDA mounting buffer (containing 37.5% glycerol, 187.5 mM NaCl, and 62.5 mM Tris pH 8.8). The signals were visualized using a Leica Stellaris 5 confocal microscope.

Ex vivo Ca²⁺ imaging using GCaMP6f

Ex vivo Ca²⁺ imaging was performed as previously described ⁶⁷[\[67\]](#) with slight modifications using 6–8 day-old flies expressing *UAS-GCaMP6f* driven by *Ir25a-GAL4*, *Ir60b-GAL4* or *Ir76b-GAL4*, which were incubated at 25 °C, under 12 hr light/12 hr dark cycles and 50-60% humidity. 0.5% low melting agarose was applied to a confocal dish (Cat # 102350, SPL LIFE SCIENCE, Korea). After solidification of the low-melting agarose, a blade was used to cut and create a shallow well for sample fixation. Fly heads were carefully decapitated using sharp razor blades, followed by excising a small portion of the extended proboscis to facilitate tastant access to the pharyngeal organs. The tissue sample was then carefully fixed in an inverted position in the pre-prepared well.

Adult hemolymph (AHL: 108 mM NaCl, 5 mM KCl, 8.2 mM MgCl₂, 2 mM CaCl₂, 4 mM NaHCO₃, 1 mM NaH₂PO₄, and 5 mM HEPES pH 7.5) was used as *Drosophila* imaging saline. After recording 1 minute as a pre-stimulus (20 µL AHL), we imaged the Ca²⁺ dynamics following the application of a specific tastant (5-fold higher concentration in 5 µL AHL). GCaMP6f fluorescence was observed using a fluorescence microscope (Axio Observer 3; Carl Zeiss) with a 20x objective, specifically focusing on the relevant area of the pharynx. Videos were recorded at a speed of two frames per second. Neuronal fluorescent activity changes were recorded for 5 minutes following stimulus application. We did not use a perfusion system to wash the stimulus. Fiji/ImageJ software (<https://fiji.sc> ⁶⁸[\[68\]](#)) was used to measure fluorescence intensities. A region of interest (ROI) was drawn around the cell bodies, and the Time-Series Analyzer Plugin, developed by Balaji, J. (<https://imagej.nih.gov/ij/plugins/time-series.html> ⁶⁹[\[69\]](#)), was used to measure the average intensity for the ROIs during each frame. The average pre-stimulation value before chemical stimulation was calculated. $\Delta F/F$ (%) was determined using the formula $(F_{\max} - F_0)/F_0 \times 100\%$, where F_0 represents the baseline value of GCaMP6f averaged for 10 frames immediately before stimulus application, and $F_{\max}$ is the maximum fluorescence value observed after stimulus delivery.

Statistical analyses

Error bars indicate the standard error of the means (SEMs), while the dots represent the number of trials conducted for the experiment. To compare multiple datasets, we used single-factor ANOVA coupled with Scheffe's analysis as a *post hoc* test. Pairwise comparisons were conducted using unpaired Student's *t*-tests. Statistical significance is denoted by asterisks (**p* < 0.05, ***p* < 0.01). We performed all statistical analyses using Origin Pro 8 for Windows (ver. 8.0932; Origin Lab Corporation, USA).

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Author contributions

J.S. and S.D. performed most of the genetic studies and physiology. J.S. and Y.L. conceived and designed the experiments. B.S. confirmed optogenetics and ectopic experiments. D.N. worked on low salt sensors. Y.K. generated most new reagents and conducted screening for high salt sensor in the initial stage. A.G. worked on the initial calcium imaging. C.M. and Y.L. supervised the project and wrote the manuscript.

Additional files

Data availability

Source data for all figures contained in the manuscript and SI have been deposited in 'figshare' (<https://doi.org/10.6084/m9.figshare.23939394>).

Supplementary files

Figure 1—figure supplement 1. Requirements for *Irs* for preferring low salt-containing food.

Figure 1—figure supplement 2. Gene structure of *Ir60b* locus, generation of *Ir60b*³ and behavioral defect of *Ir60b*¹ in high salt avoidance.

Figure 2—figure supplement 1. Assaying action potentials induced by different labellar bristles in response to 300 mM salt using tip recordings.

Figure 2—figure supplement 2. Two-way solid-food choice assay to assess whether the *Gr2a*^{GAL4} mutant exhibits a deficit in avoidance of high salt.

Figure 3—figure supplement 1. DrosoX system and measurement of food intake using strychnine and coumarin.

Figure 3—figure supplement 2. Two-way solid-food choice assay and DrosoX binary capillary feeding assay using 100 mM sorbitol with or without 300 mM NaCl.

Figure 4—figure supplement 1. Testing whether ectopic expression of *Ir60b* confers responses to 300 mM NaCl, measurement of intake of sucrose plus 300 mM NaBr using the DrosoX assay, and Ca²⁺ response of *Ir60b* GRNs.

Figure 4—figure supplement 2. GCaMP6f responses evoked by 300 mM NaCl in *Ir60b* GRNs of control and *Ir7c*^{GAL4} flies, and relative expression of *Ir60b* and *Ir7c* reporters in the LSO.

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Editors

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Reviewer #1 (Public Review):

Summary:

In this manuscript, Sang et al. proposed a pair of IR60b-expressing pharyngeal neurons in *Drosophila* use IR25a, IR76b, and IR60b channels to detect high Na⁺ and limit its consumption. Some of the key findings that support this thesis are: 1) animals that lacked any one of these channels - or with their IR60b-expressing neurons selectively silenced - showed much reduced rejection of high Na⁺, but restored rejection when these channels were reintroduced back in the IR60b neurons; 2) animals with TRPV artificially expressed in their IR60b neurons rejected capsaicin-laced food whereas WT did not; 3) IR60b-expressing neurons exhibited increased Ca²⁺ influx in response to high Na⁺ and such response went away when animals lacked any of the three channels.

The experiments were thorough and well designed and further improved after revision. The results are compelling and support the main claim. The development and the use of the Drosophila two-choice assay put forward for a more quantitative and automatic/unbiased assessment for ingestion volume and preference.

<https://doi.org/10.7554/eLife.93464.2.sa2>

Reviewer #2 (Public Review):

Summary:

In this paper, Sang et al. set out to identify gustatory receptors involved in salt taste sensation in *Drosophila melanogaster*. In a two-choice assay screen of 30 Ir mutants, they identify that Ir60b is required for avoidance of high salt. In addition, they demonstrate that activation of Ir60b neurons is sufficient for gustatory avoidance using either optogenetics or TRPV1 to specifically activate Ir60b neurons. Then, using tip recordings of labellar gustatory sensory

neurons and proboscis extension response behavioral assays in Ir60b mutants, the authors demonstrate that Ir60b is dispensable for labellar taste neuron responses to high salt and the suppression of proboscis extension by high salt. Since external gustatory receptor neurons (GRNs) are not implicated, they look at Poxn mutants, which lack external chemosensory sensilla but have intact pharyngeal GRNs. High salt avoidance was reduced in Poxn mutants but was still greater than Ir60b mutants, suggesting that pharyngeal gustatory sensory neurons alone are sufficient for high salt avoidance. The authors use a new behavioral assay to demonstrate that Ir60b mutants ingest a higher volume of sucrose mixed with high salt than control flies do, suggesting that the action of Ir60b is to limit high salt ingestion. Finally, they identify that Ir60b functions within a single pair of gustatory sensory neurons in the pharynx, and that these neurons respond to high salt but not bitter tastants.

Strengths:

A great strength of this paper is that it rigorously corroborates previously published studies that have implicated specific Irs in salt taste sensation. It further introduces a new role for Ir60b in limiting high salt ingestion, demonstrating that Ir60b is necessary and sufficient for high salt avoidance and convincingly tracing the action of Ir60b to a particular subset of gustatory receptor neurons. Overall the authors have achieved their aim by identifying a new gustatory receptor involved in limiting high salt ingestion. They use rigorous genetic, imaging, and behavioral studies to achieve this aim, often confirming a given conclusion with multiple experimental approaches. They have further done a great service to the field by replicating published studies and corroborating the roles of a number of other Irs in salt taste sensation.

<https://doi.org/10.7554/eLife.93464.2.sa1>

Reviewer #3 (Public Review):

Sang et al. successfully demonstrate that a set of single sensory neurons in the pharynx of *Drosophila* promotes avoidance of food with high salt concentrations, complementing previous findings on Ir7c neurons with an additional internal sensing mechanism. The experiments are well-conducted and presented, convincingly supporting their important findings and extending the understanding of internal sensing mechanisms.

The authors convincingly demonstrate the avoidance phenotype using different behavioral assays, thus comprehensively analyzing different aspects of the behavior. The experiments are straightforward and well-contextualized within existing literature.

<https://doi.org/10.7554/eLife.93464.2.sa0>

Author Response

The following is the authors' response to the original reviews.

Public Reviews:

Reviewer #1 (Public Review):

Summary:

*In this manuscript, Sang et al. proposed a pair of IR60b-expressing pharyngeal neurons in *Drosophila* use IR25a, IR76b, and IR60b channels to detect high Na⁺ and limit its consumption. Some of the key findings that support this thesis are: 1) animals that lacked any one of these channels - or with their IR60b-expressing neurons selectively*

silenced - showed much reduced rejection of high Na⁺, but restored rejection when these channels were reintroduced back in the IR60b neurons; 2) animals with TRPV artificially expressed in their IR60b neurons rejected capsaicin-laced food whereas WT did not; 3) IR60b-expressing neurons exhibited increased Ca²⁺ influx in response to high Na⁺ and such response went away when animals lacked any of the three channels.

Strengths:

The experiments were thorough and well designed. The results are compelling and support the main claim. The development and the use of the Drosophila two-choice assay put forward for a more quantitative and automatic/unbiased assessment for ingestion volume and preference.

Weaknesses:

There are a few inconsistencies with respect to the exact role by which IR60b neurons limit high salt consumption and the contribution of external (labellar) high-salt sensors in regulating high salt consumption. These weaknesses do not significantly impact the main conclusion, however.

Reviewer #2 (Public Review):

Summary:

*In this paper, Sang et al. set out to identify gustatory receptors involved in salt taste sensation in *Drosophila melanogaster*. In a two-choice assay screen of 30 *Ir* mutants, they identified that *Ir60b* is required for avoidance of high salt. In addition, they demonstrate that activation of *Ir60b* neurons is sufficient for gustatory avoidance using either optogenetics or TRPV1 to specifically activate *Ir60b* neurons. Then, using tip recordings of labellar gustatory sensory neurons and proboscis extension response behavioral assays in *Ir60b* mutants, the authors demonstrate that *Ir60b* is dispensable for labellar taste neuron responses to high salt and the suppression of proboscis extension by high salt. Since external gustatory receptor neurons (GRNs) are not implicated, they look at *Poxn* mutants, which lack external chemosensory sensilla but have intact pharyngeal GRNs. High salt avoidance was reduced in *Poxn* mutants but was still greater than *Ir60b* mutants, suggesting that pharyngeal gustatory sensory neurons alone are sufficient for high salt avoidance. The authors use a new behavioral assay to demonstrate that *Ir60b* mutants ingest a higher volume of sucrose mixed with high salt than control flies do, suggesting that the action of *Ir60b* is to limit high salt ingestion. Finally, they identify that *Ir60b* functions within a single pair of gustatory sensory neurons in the pharynx, and that these neurons respond to high salt but not bitter tastants.*

Strengths:

*A great strength of this paper is that it rigorously corroborates previously published studies that have implicated specific *Irs* in salt taste sensation. It further introduces a new role for *Ir60b* in limiting high salt ingestion, demonstrating that *Ir60b* is necessary and sufficient for high salt avoidance and convincingly tracing the action of *Ir60b* to a particular subset of gustatory receptor neurons. Overall, the authors have achieved their aim by identifying a new gustatory receptor involved in limiting high salt ingestion. They use rigorous genetic, imaging, and behavioral studies to achieve this aim, often confirming a given conclusion with multiple experimental approaches. They have further done a great service to the field by replicating published studies and corroborating the roles of a number of other *Irs* in salt taste sensation. An aspect of this study that merits further investigation is how the same gustatory receptor neurons and *Ir* in the pharynx*

can be responsible for regulating the ingestion of both appetitive (sugar) and aversive tastants (high salt).

A previous report published in eLife from John Carlson's lab (Joseph et al, 2017) showed that the Ir60b GRN in the pharynx responds to sucrose resulting in sucrose repulsion. Thus, stimulation of this pharyngeal GRN results in gustatory avoidance only, not both attraction and avoidance. (lines 205-207)

Weaknesses:

There are several weaknesses that, if addressed, could greatly improve this work.

(1) The authors combine the results and discussion but provide a very limited interpretation of their results. More discussion of the results would help to highlight what this paper contributes, how the authors interpret their results, and areas for future study.

We agree and have now separated the Results and Discussion, and in so doing have greatly expanded discussion of the results.

(2) The authors rename previously studied populations of labellar GRNs to arbitrary letters, which makes it difficult to understand the experiments and results in some places. These GRN populations would be better referred to according to the gustatory receptors they are known to express.

One of the corresponding authors (Craig Montell) introduced this alternative GRN nomenclature in a review in 2021: Montell, C. (*Drosophila* sensory receptors—a set of molecular Swiss Army Knives. *Genetics* 217, 1-34) (Montell, 2021). We are not fans of referring to different classes of GRNs based on the receptors that they express since it is not obvious which receptors to use. For example, the GRNs that respond to bitter compounds all express multiple GR co-receptors. The same is true for the GRNs that respond to sugars. The former system of referring to GRNs simply as sugar, bitter, salt and water GRNs is also not ideal since the repertoire of chemicals that stimulates each class is complex. For example, the Class A GRNs (formerly sugar GRNs) are also activated by low Na⁺, glycerol, fatty acids, and acetic acid, while the B GRNs (former bitter GRNs) are also stimulated by high Na⁺, acids, polyamines, and tryptophan. In addition, there are five classes of GRNs. At first mention of the Class A—E GRNs, we mention the most commonly used former nomenclature of sugar, bitter, salt and water GRNs. In addition, for added clarity, we now also include a mention of one of the receptors that mark each class. (lines 51-59)

(3) The conclusion that GRNs responsible for high salt aversion may be inhibited by those that function in low salt attraction is not well substantiated. This conclusion seems to come from the fact that overexpression of Ir60b in salt attraction and salt aversion sensory neurons still leads to salt aversion, but there need not be any interaction between these two types of sensory neurons if they act oppositely on downstream circuits.

We did not make this claim.

(4) The authors rely heavily on a new Drosophila behavioral apparatus that is not sufficiently described here or in the previous paper the authors cite. This greatly limits the reader's ability to interpret the results.

We expanded the description of the apparatus in the Drosophila assay section of the Materials and Methods. (lines 588-631)

Reviewer #3 (Public Review):

Summary:

Sang et al. successfully demonstrate that a set of single sensory neurons in the pharynx of Drosophila promotes avoidance of food with high salt concentrations, complementing previous findings on Ir7c neurons with an additional internal sensing mechanism. The experiments are well-conducted and presented, convincingly supporting their important findings and extending the understanding of internal sensing mechanisms. However, a few suggestions could enhance the clarity of the work.

Strengths:

The authors convincingly demonstrate the avoidance phenotype using different behavioral assays, thus comprehensively analyzing different aspects of the behavior. The experiments are straightforward and well-contextualized within existing literature.

Weaknesses:

Discussion

While the authors effectively relate their findings to existing literature, expanding the discussion on the surprising role of Ir60b neurons in both sucrose and salt rejection would add depth. Additionally, considering Yang et al. 2021's (<https://doi.org/10.1016/j.celrep.2021.109983>) result that Ir60b neurons activate feeding-promoting IN1 neurons, the authors should discuss how this aligns with their own findings.

Yang et al. demonstrated that the activation of Ir60b neurons can trigger the activation of IN1 neurons akin to pharyngeal multimodal (PM) neurons, potentially leading to enhanced feeding (Yang et al, 2021). However, our research reveals a specific pattern of activation for Ir60b neurons. Instead of being generalists, they are specialized for certain sugars, such as sucrose and high salt. Consequently, while Ir60b GRNs activate IN1 neurons, we contend that there are other neurons in the brain responsible for inhibiting feeding. (lines 412-417)

Lines 187: The discussion primarily focuses on taste sensillae outside the labellum, neglecting peg-type sensillae on the inner surface. Clarification on whether these pegs contribute to the described behaviors and if the Poxn mutants described also affect the pegs would strengthen the discussion.

We added the following to the Discussion section. “We also found that the requirement for Ir60b appears to be different when performing binary liquid capillary assay (DrosoX), versus solid food binary feeding assays. When we employed the DrosoX assay to test mutants that were missing salt aversive GRNs in labellar bristles but still retained functional Ir60b GRNs, the flies behaved the same as wild-type flies (e.g. Figure 3J and 3L). However, using solid food binary assays, Poxn mutants, which are missing labellar taste bristles but retain Ir60b GRNs (LeDue et al, 2015), displayed repulsion to high salt food that was intermediate between control flies and the Ir60b mutant (Figure 2J). Poxn mutants retain taste pegs (LeDue et al., 2015), and these hairless taste organs become exposed to food only when the labial palps open. We suggest that there are high-salt sensitive GRNs associated with taste pegs, which are accessed when the labellum contacts a solid substrate, but not when flies drink from the capillaries used in DrosoX assays. This explanation would also account for the findings that the Ir60b mutant is indifferent to 300 mM NaCl in the DrosoX assay (Figure 3B), but prefers 1 mM sucrose alone over 300 mM NaCl and 5 mM sucrose in the solid food binary assay (Figure 1B).”. (lines 430-444)

In line 261 the authors state: "We attempted to induce salt activation in the I-type sensilla by ectopically expressing Ir60b, similar to what was observed with Ir56b 8; however, this did not generate a salt receptor (Figures S6A)"

An obvious explanation would be that these neurons are missing the identified necessary co-receptors Ir76b and Ir25a. The authors should discuss here if the Gr33a neurons they target also express these co-receptors, if yes this would strengthen their conclusion that an additional receptor might be missing.

We clarified this point in the Discussion section as follows, "An open question is the subunit composition of the pharyngeal high Na⁺ receptor, and whether the sucrose/glucose and Na⁺ receptors in the Ir60b GRN are the same or distinct. Our results indicate that the high salt sensor in the Ir60b GRN includes IR25a, IR60b and IR76b since all three IRs are required in the pharynx for sensing high levels of NaCl. I-type sensilla do not elicit a high salt response, and we were unable to induce salt activation in I-type sensilla by ectopically expressing Ir60b, under control of the Gr33a-GAL4. This indicates that IR25a, IR60b and IR76b are insufficient for sensing high Na⁺. The inability to confer a salt response by ectopic expression of Ir60b was not due to absence of Ir25a and Ir76b in Gr33a GRNs since Gr33a and Gr66a are co-expressed (Moon et al, 2009), and Gr66a GRNs express Ir25a and Ir76b (Li et al, 2023). Thus, the high salt receptor in Ir60b GRNs appears to require an additional subunit. Given that Na⁺ and sugars are structurally unrelated, we suggest that the Na⁺ and sucrose/glucose receptors do not include the identical set of subunits, or that they activate a common receptor through disparate sites". (lines 464-477)

Methods

The description of the Drosophila assay seems to be missing some details. Currently, it is not obvious how the two-choice is established. Only one capillary is mentioned, I assume there were two used? Also, the meaning of the variables used in the equation (DrosoX and DrosoXD) are not explained.

We expanded the description of the apparatus in the Drosophila assay section of the Materials and Methods. (lines 588-631)

The description of the ex-vivo calcium imaging prep. is unclear in several points:

(1) It is lacking information on how the stimulus was applied (was it manually washed in? If so how was it removed?).

We expanded the description of the apparatus in the ex vivo calcium imaging section of the Materials and Methods. (lines 682-716)

(2) The authors write: "A mild swallow deep well was prepared for sample fixation." I assume they might have wanted to describe a "shallow well"?

We deleted the word "deep." (line 691)

(3) "...followed by excising a small portion of the labellum in the extended proboscis region to facilitate tastant access to pharyngeal organs." It is not clear to me how one would excise a small portion of the labellum, the labellum depicts the most distal part of the proboscis that carries the sensillae and pegs. Did the authors mean to say that they cut a part of the proboscis?

Yes. We changed the sentence to “...followed by excising a small portion of the extended proboscis to facilitate tastant access to the pharyngeal organs.”.(lines 693)-695

Recommendations for the authors:

Reviewer #1 (Recommendations For The Authors):

In this manuscript, Sang et al. proposed a pair of IR60b-expressing pharyngeal neurons in Drosophila use IR25a, IR76b, and IR60b channels to detect high Na⁺ and limit its consumption. Some of the key findings that support this thesis are: 1) animals that lacked any one of these channels - or with their IR60b-expressing neurons selectively silenced - showed much reduced rejection of high Na⁺, but restored rejection when these channels were reintroduced back in the IR60b neurons; 2) animals with TRPV artificially expressed in their IR60b neurons rejected capsaicin-laced food whereas WT did not; 3) IR60b-expressing neurons exhibited increased Ca²⁺ influx in response to high Na⁺ and such response went away when animals lacked any of the three channels. In general, I find the collective evidence presented by the authors convincing. But I feel the MS can benefit from having a discussion session and a few simple experiments. Below I listed some inconsistencies I hope the authors can address or at least discuss.

We have now added a Discussion section, and expanded the discussion.

(1) The role of IR60b neurons on suppressing PER appeared inconsistent. On the one hand, optogenetic activation of these neurons suppressed PER (Fig 1D), on the other hand, IR60b mutants were as competent to suppress PER in response to high salt as WT (Fig 2G). Are pharyngeal neurons expected to modulate PER? It might be worth including a retinal-free or genotype control to ascertain the PER suppression exhibited by IR60b>CsChrimson is genuine.

Please note that Figure 2G is now Figure 2H.

Our interpretation is that activation of aversive GRNs by high salt either in labellar bristles or in the pharynx is sufficient to inhibit repulsion to high salt. Consistent with this conclusion, optogenetic activation of Ir60b GRNs, which are specific to the pharynx, is sufficient to reduce the PER to sucrose containing food (Figure 1D). However, mutation of Ir60b has no impact on the PER to sucrose plus high (300 mM) NaCl since the high-salt activated GRNs in labellar bristles are not impaired by the Ir60b mutation. In contrast, Ir25a and Ir76b are required in both labellar bristles and in the pharynx to reject high salt. As a consequence, mutation of either Ir25a or Ir76b impairs the repulsion to high salt. Thus, there is no inconsistency between the optogenetics and PER results. We clarified this point in the Discussion section. In terms of controls for IR60b>CsChrimson, we show that UAS-CsChrimson alone or UAS-CsChrimson in combination with the Gr5a driver has no impact on the PER (Figure 1D). In addition, we now include a retinal free control (Figure 1D). These findings provide the key genetic controls and are described in the Results section. (lines 167-170)

(2) The role of labellar high-salt sensors in regulating salt intake appeared inconsistent. On the one hand, they appeared to have a role in limiting high salt consumption because poxn mutants were significantly more receptive to high salt than WT (Fig. 2J). On the other hand, selectively restoring IR76b or IR25a in only the IR60b neurons in these mutants - thus leaving the labellar salt sensors still defective - reverted the flies to behave like WT when given a choice between sucrose vs. sucrose+high salt (Fig 3J, L).

We now offer an explanation for these seemingly conflicting results in the Discussion section. When we employed the DrosoX assay with mutants with functional Ir60b GRNs, but were

missing salt aversive GRNs in labellar bristles, the flies behaved the same as control flies (e.g. Figure 3J and L). However, using solid food binary assays, Poxn mutants, which are missing labellar taste bristles but retain Ir60b GRNs (LeDue et al., 2015), display aversion high salt food intermediate between control and Ir60b mutant flies (Figure 2J). Poxn mutants retain taste pegs (LeDue et al., 2015), which are exposed to food substrates only when the labial palps open. We suggest that the taste pegs harbor high salt sensitive GRNs, and they may be exposed to solid substrates, but not to the liquid in capillary tubes used in the Drosophila assays. This explanation would also account for the findings that the Ir60b mutant is indifferent to 300 mM NaCl in the Drosophila assay (Figure 3B), but prefers 1 mM sucrose alone over 300 mM NaCl and 5 mM sucrose in the solid food binary assay (Figure 1B). (lines 433-444)

(3) The behavior sensitivity of Ir60b mutant to high salt again appeared somewhat inconsistent when assessed in the two different choice assays. Ir60b mutant flies were indifferent to 300 mM NaCl when assayed with Drosophila (Fig 3A, B) but were clearly still sensitive to 300 mM NaCl when assayed with "regular" assay - they showed much reduced preference for 5 mM sucrose over 1 mM sucrose when the 5 mM sucrose was adulterated with 300 mM NaCl (Fig 1B).

The explanation provided above may also account for the findings that the Ir60b mutant is indifferent to 300 mM NaCl in the Drosophila assay (Figure 3B), but not when selecting between 300 mM NaCl and 5 mM sucrose versus 1 mM sucrose in the solid food binary assay (Figure 1B). Alternatively, the different behavioral responses might be due to the variation in sucrose concentrations in each of these two assays, which employed 5 mM sucrose in the solid food binary assay, as opposed to 100 mM sucrose in the Drosophila assay. This disparity in attractive valence between these two concentrations of sucrose might consequently impact feeding amount and preference. This point is now also included in the Discussion section. (lines 441-449)

(4) Given the Ir60b neurons exhibited clear Ir60b/Ir25a/Ir76b-dependent sucrose sensitivity, too, I am curious how the various mutant animals behave when given a choice between 100 mM sorbitol vs. 100 mM sorbitol + 300 mM NaCl, a food choice assay not complicated by the presence of sucrose. Similarly, I am curious if the Ca²⁺ response of Ir60 neurons differs significantly when presented with 100 mM sucrose vs. when presented with 100 mM sucrose + 300 mM NaCl. In principle, the magnitude for the latter should be significantly larger than the former as animals appeared to be capable of discriminating these two choices solely relying on their Ir60b neurons.

To investigate the aversion induced by high salt in the absence of a highly attractive sugar, such as sucrose, we combined 300 mM salt with 100 mM sorbitol, which is a tasteless but nutritive sugar (Burke & Waddell, 2011; Fujita & Tanimura, 2011). Using two-way choice assays, we found that the Ir25a, Ir60b, and Ir76b mutants exhibited substantial reductions in high salt avoidance (Figure 3—figure supplement 2A). In addition, we performed Drosophila assays using 100 mM sorbitol alone, or sorbitol mixed with 300 mM NaCl. Sorbitol alone provoked less feeding than sucrose since it is a tasteless sugar (Figure 3—figure supplement 2B and C). Nevertheless, addition of high salt to the sorbitol reduced food consumption (Figure 3—figure supplement 2B and C). (lines 300-308)

We also conducted a comparative analysis of the Ca²⁺ responses within the Ir60b GRN, examining its reaction to various stimuli, including 100 mM sucrose alone, 300 mM NaCl alone, and a combination of 100 mM sucrose and 300 mM NaCl. We found that the Ca²⁺ responses were significantly higher when we exposed the Ir60b GRN to 300 mM NaCl alone, compared with the response to 100 mM sucrose alone (Figure 4—figure supplement 1D). However, the GCaMP6f responses was not higher when we presented 100 mM sucrose with

300 mM NaCl, compared with the response to 300 mM NaCl alone (Figure 4—figure supplement 1D). (lines 360-367)

Minor issues

(1) The labels of sucrose concentration on Figure 2D were flipped.

This has been corrected.

(2) The phrasing of the sentence that begins in line 196 (i.e., "This suggests the internal sensor ...") is not as optimal.

We changed the sentence to, "We found that the aversive behavior to high salt was reduced in the Poxn mutants relative to the control (Figure 2J), consistent with previous studies demonstrating roles for GRNs in labellar bristles in high salt avoidance (Jaeger et al, 2018; McDowell et al, 2022; Zhang et al, 2013).". (lines 217-219)

(3) In Line 231, I am not sure why the authors think ectopic expressing IR60b in labellar neurons would allow them to become activated by Na+. It seems highly unlikely to me, especially given IR60b also plays a role in sensing sugar.

We added the following paragraph to the Discussion addressing this point, "An open question is the subunit composition of the pharyngeal high Na⁺ receptor, and whether the sucrose/glucose and Na⁺ receptors in the Ir60b GRN are the same or distinct. Our results indicate that the high salt sensor in the Ir60b GRN includes IR25a, IR60b and IR76b since all three IRs are required in the pharynx for sensing high levels of NaCl. I-type sensilla do not elicit a high salt response, and we were unable to induce salt activation in I-type sensilla by ectopically expressing Ir60b, under control of the Gr33a-GAL4. This indicates that IR25a, IR60b and IR76b are insufficient for sensing high Na⁺. The inability to confer a salt response by ectopic expression of Ir60b was not due to absence of Ir25a and Ir76b in Gr33a GRNs since Gr33a and Gr66a are co-expressed (Moon et al., 2009), and Gr66a GRNs express Ir25a and Ir76b (Li et al., 2023). Thus, the high salt receptor in Ir60b GRNs appears to require an additional subunit. Given that Na⁺ and sugars are structurally unrelated, we suggest that the Na⁺ and sucrose/glucose receptors do not include the identical set of subunits, or that that they activate a common receptor through disparate sites.". (lines 464-477)

Reviewer #2 (Recommendations For The Authors):

Line 41, acutely excessive salt ingestion can lead to death, not just health issues

We now state that, "consumption of excessive salt can contribute to various health issues in mammals, including hypertension, osteoporosis, gastrointestinal cancer, autoimmune diseases, and can lead to death.". (lines 41-43)

Line 46, delete the comma after flies

Done. (line 47)

Lines 51-56: This description is unnecessarily confusing and does not cite proper sources. Renaming these GRNs arbitrarily can only create confusion, plus this description lacks nuance. If E GRNs are Ir94e positive, this description is out of date. Furthermore, If D GRNs are ppk23 and Gr66a positive then they will respond to both bitter and high salt.

Papers to consult: <https://elifesciences.org/articles/37167> 10.1016/j.cell.2023.04.038

We have now added citations. We prefer the A—E nomenclature, which was introduced in a 2021 Genetics review by one of the authors of this manuscript (Montell) (Montell, 2021) since naming different classes of GRNs on the basis of markers or as sweet, bitter, salt and water GRNs is misleading and an oversimplification. We cite the Genetics 2021 review, and for added clarity include both types of former names (markers and sweet, bitter, salt and water). Class D GRNs are not marked by Gr66a. The eLife reference cited above provided the initial rationale for stating that Class E GRNs are marked by Ir94e and activated by low salt. According to the Taisz et al reference (Cell 2023), the Class E GRNs, which are marked by Ir94e, are also activated by pheromones, which we now mention (Taisz et al, 2023). (lines 51-59)

| *Line 62, E GRNs are not required for low salt behaviors*

We do not state that E GRNs are required for low salt behaviors, only that they sense low Na⁺ levels. (line 58)

| *Line 70-81 - Great deal of emphasis on labellar GRNs but then no mention of how pharyngeal GRNs fit into categories A-E*

We devote the following paragraph to pharyngeal GRNs. We do not mention how they fit in with the A—E categories because it is not clear.

“In addition to the labellum and taste bristles on other external structures, such as the tarsi, fruit flies are endowed with hairless sensilla on the surface of the labellum (taste pegs), and three internal taste organs lining the pharynx, the labral sense organ (LSO), the ventral cibarial sense organ (VCSO), and the dorsal cibarial sense organ (DCSO), which also function in the decision to keep feeding or reject a food (Chen & Dahanukar, 2017, 2020; LeDue et al., 2015; Nayak & Singh, 1983; Stocker, 1994). A pair of GRNs in the LSO express a member of the gustatory receptor family, Gr2a, and knockdown of Gr2a in these GRNs impairs the avoidance to slightly aversive levels of Na⁺ (Kim et al, 2017). Pharyngeal GRNs also promote the aversion to bitter tastants, Cu²⁺, L-canavanine, and bacterial lipopolysaccharides (Choi et al, 2016; Joseph et al., 2017; Soldano et al, 2016; Xiao et al, 2022). Other pharyngeal GRNs are stimulated by sugars and contribute to sugar consumption (Chen & Dahanukar, 2017; Chen et al, 2021; LeDue et al., 2015). Remarkably, a pharyngeal GRN in each of the two LSOs functions in the rejection rather the acceptance of sucrose (Joseph et al., 2017).”. (lines 74-89)

| *Line 89, aversive --> aversion*

We changed this part.

| *Line 90, gain of aversion capsaicin avoidance suggests they are sufficient for avoidance, not essential for avoidance.*

We changed “essential” to “sufficient.”. (line 100)

| *Line 104, what are you recording from here? Labellar or pharyngeal GRNs*

We added “S-type and L-type sensilla” to the sentence. (line 119)

| *Line 107, How are A GRNs marked with tdTomato? It is important to mention how you are defining A GRNs.*

We modified the sentence as follows: “Using Ir56b-GAL4 to drive UAS-mCD8::GFP, we also confirmed that the reporter was restricted to a subset of Class A GRNs, which were marked

with LexAop-tdTomato expressed under the control of the Gr64f-LexA (Figure 1—figure supplement 1D—F).”. (lines 120-123)

| *Line 124, should read "concentrated as sea water."*

We made the change. (line 142)

| *Line 125, I am not sure what is meant by "alarm neurons"*

We changed “additional pain or alarm neurons” to “nociceptive neurons.”. (line 144)

| *Line 141, Are you definitely A GRNs as only labellar GRNs, i.e. the Gr5a-GAL4 pattern with labellar plus few pharyngeal GRNs? Or are the defining it as Gr64f-GAL4 (i.e. labellar plus many pharyngeal GRNs)*

We refer to the Class A—E GRNs as labellar GRNs. Therefore, in this instance, we removed the reference to A GRNs and B GRNs, and simply mention the drivers that we used (Gr5a-GAL4 and Gr66a-GAL4) to express UAS-CsChrimson. The modified sentence is, “As controls we drove UAS-CsChrimson under control of either the Gr5a-GAL4 or the Gr66a-GAL4.”. (lines 51-59, 160-161)

| *Line 180, labellar hairs--> labellar taste bristles*

We made the change. (line 204)

| *Line 190, possess only --> only possess*

We made the change. (line 216)

| *Line 202, Should this read increased?*

Yes. We changed “reduced” to “increased.”. (line 225)

| *Line 206, The information provided here and in reference 47 was not sufficient for me to understand how the Drosophila system works and whether it has been validated. Better diagrams and much more description is required for the reader to understand this system and assess its validity*

We now explain that the Drosophila “system consists of a set of five separately housed flies, each of which is exposed to two capillary tubes with different liquid food options. One capillary contained 100 mM sucrose and the other contained 100 mM sucrose mixed with 300 mM NaCl. The volume of food consumed from each capillary is then monitored automatically over the course of 6 hours and recorded on a computer.”. (lines 238-243)

| *Line 218-219, It would be helpful to expand on this to explain how the previous paper detected no difference. Is this because the contact time with the food is the same but the rate of ingestion is slower?*

Yes. This is correct. We now clarify this point by stating that, “In a prior study, it was observed that the repulsion to high salt exhibited by the Ir60b mutant was indistinguishable from wild-type (Joseph et al., 2017). Specifically, the flies were presented with drop of liquid (sucrose plus salt) at the end of a probe, and the Ir60b mutant flies fed on the food for the same period of time as control flies (Joseph et al., 2017). However, this assay did not discern whether or not the volume of the high salt-containing food consumed by the Ir60b mutant flies was

reduced relative to control flies. Therefore, to assess the volume of food ingested, we used the Drosophila system, which we recently developed (Figure 3—figure supplement 1A) (Sang et al., 2021). This system consists of a set of five separately housed flies, each of which is exposed to two capillary tubes with different liquid food options. One capillary contained 100 mM sucrose and the other contained 100 mM sucrose mixed with 300 mM NaCl. The volume of food consumed from each capillary was then monitored automatically over the course of 6 hours and recorded on a computer. We found that control flies consuming approximately four times more of the 100 mM sucrose than the sucrose mixed with 300 mM NaCl (Figure 3A). In contrast, the Ir25a, Ir60b, and Ir76b mutants consumed approximately two-fold less of the sucrose plus salt (Figure 3A). Consequently, they ingested similar amounts of the two food options (Figure 3B; ingestion index). Thus, while the Ir60b mutant and control flies spend similar amounts of time in contact with high salt-containing food when it is the only option (Joseph et al., 2017), the mutant consumes considerably less of the high salt food when presented with a sucrose option without salt.”. (lines 226-251)

Lines 231-235, Is this evidence for this, that Ir60b expression in the Ir25a or Ir76b pattern will induce high salt responses in the labellum? You should elaborate on this to clearly state what you mean rather than implying it. I do not think that overexpression of one Ir is enough evidence for this sweeping conclusion.

We agree. We eliminated this point. (lines 227-232)

Lines 261-263, Please elaborate here, how did you target the I-type sensilla and where are these neurons? So they already express Ir76b and Ir25a?

We now explain in the Results that, “We attempted to induce salt activation in the I-type sensilla by ectopically expressing Ir60b, under control of the Gr33a-GAL4. Gr33a is co-expressed with Gr66a (Moon et al., 2009), which has been shown to be co-expressed Ir25a and Ir76b (Li et al., 2023). When we performed tip recordings from I7 and I10 sensilla, we did not observe a significant increase in action potentials in response to 300 mM NaCl (Figure 4—figure supplement 1A), indicating that ectopic expression of Ir60b in combination with Ir25a and Ir76b is not sufficient to generate a high salt receptor.”. (lines 324-330)

Lines 300-303, The discussion needs to be greatly expanded. What is the proposed mechanism by which the same neurons/receptors can inhibit sucrose and high salt feeding? What is the author's interpretation of what this study adds to our understanding of taste aversion?

We have now added a Discussion section and greatly expanded the discussion.

Reviewer #3 (Recommendations For The Authors):

In line 73 there is a typo in "esophagus"

We changed this part.

In line 331, the use of a mixture of sucrose and "saponin" seems to be a mistake; "NaCl" is likely intended.

We made the correction. (lines 546 and 640)

On several occasions, the authors refer to the pharynx as a taste organ (for example 1st sentence of the abstract). I am not sure this is correct, the actual pharyngeal taste organs are the LSO, DSCO, and VSCO which are located in the pharynx.

We made the corrections. (lines 24, 90, 92, 93, and 356)

In line 155 the authors refer to Ir25a and Ir76b as "broadly tuned". I think it is not correct to refer to co-receptors this way, I'd suggest to just call them co-receptors.

We made the correction. (lines 177-178)

In line 182, stating "Gr2a is also expressed in the proboscis" is unclear. Clarify whether it refers to sensillae, pharyngeal taste organs, etc.

We clarified it refers to pharyngeal taste organs. (lines 206-207)

Line 253: "These finding imply that all three Irs are coexpressed in the pharynx." "The pharynx" is very unspecific, did the authors mean to say "the same neuron"?

We now clarify by saying "in the Ir60b GRN in the pharynx.". (line 317)

Figures & Legends

I found it confusing that the same color scale is being reused for different panels with different meanings repeatedly and in inconsistent ways. For example in Figure 2, red and blue are being used for Ir25a² mutants, while blue is also being used for Gr64f-Gal4 and S type sensilla. It is also not easily visible nor mentioned in the caption which of the 3 color scales presented belong to which panels.

We modified the colors in the figures so that they are used in a consistent way. We now also define the colors in the legends.

In Figure 2 F-I, indicating the stimulus sequence in each panel would enhance clarity. The color scale in Figure 3 could benefit from explicit explanations of different shades in the caption for easier interpretation.

For example: "The ingestion of (a, dark color) 100 mM sucrose alone and (b, light color) in combination with 300 mM"

We made the suggested modification.

In Figure 4a the authors highlight that Ir76b and Ir25a label 2 neurons in the LSO. Did the imaging in 4c also capture the second cell, and if so did it respond to their stimulation?

No, the focal plane differs, and the signal in Figure 4C is considerably weaker compared to the immunohistochemistry shown in Figure 4A. Notably, the other neuron did not exhibit a response to NaCl.

In Figure 4f a legend for the color scale is missing, or the color might not be necessary at all. Also, the asterisks seem to be shifted to the right.

We fixed the shifted asterisks and eliminated the color.

Figure 4i is mislabeled 4f

We made the correction.