Activity and state dependent modulation of salt taste behavior in *Drosophila melanogaster*

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Abstract

Sodium present in NaCl is a fundamental nutrient required for many physiological processes. In animals including *Drosophila* low-salt concentrations induce attraction and high-salt concentrations evoke aversive behavior. Although high salt detection pathways have been studied in great details but mechanisms that regulate high salt consumption in animals are largely undetermined. We looked into the neural mechanisms of high NaCl consumption in adult *Drosophila* by which flies modify their acceptance of high salt as a function of diet where a long-term high-salt exposure increases taste sensitivity of pharyngeal LSO neurons and enhance high salt intake. Exposing flies to high NaCl diet for three days show decline in high salt aversion under starvation. Additionally, genetic suppression of LSO pharyngeal neurons in high NaCl fed flies inhibit excessive salt intake. We observed this modulation requires functional LSO neurons and a starvation state or dopamine. Multiple independent taste receptor neurons and pathways are involved in such a modulation. Silencing any one of multiple LSO neuronal types inhibits excessive salt intake. Our study suggests flies can adapt to the amount of salt ingested over several days, indicating the presence of a critical mechanism to reset the salt appetite and related neural circuits.

Introduction

Like sugars, animals have an innate liking for low levels of salt (NaCl). Sodium present in table salt (NaCl) is among the more limiting essential nutrients and its intake must be carefully regulated to maintain ionic homeostasis, nerve signaling, fluid balance and cardiovascular activity. Since, animals cannot metabolically create sodium themselves, they ingest it from external food sources to carry out various physiological functions. Presence of low sodium in the body triggers specific appetite signals by brain that drives the consumption of sodium. Recently, identification of a small population of neurons in the mouse hindbrain that controls the drive to consume sodium suggest a role of chemosensory signals in its appetite regulation ¹.

Low salt drives appetitive behavior in animals including flies while higher concentrations drive aversion ². Molecularly and anatomically distinct taste pathways are involved in mediating these two-opposing behavioral responses. In mice, the epithelial sodium channel (ENaC) functions as low salt receptor ^{3,4}. Interestingly, high salt does not activate its own taste population in mice, but rather it co-opt other taste pathways ² by recruiting additional pathways including bitter and acid (sour)-sensing TRCs (Taste receptor cells). In *Drosophila* larva, *Pickpocket* (*ppk*) gene family (DEG/ENaC channels) members, *ppk11* and *ppk19* are involved in sensing low- and high-NaCl concentrations ⁵ but not in adult flies ⁶. Analogous to the mammalian system, bitter GRNs (gustatory receptor neurons) have also been shown to respond to high concentrations of salt in flies ^{7,8}.

Low- and high-NaCl concentrations are detected by GRNs in L-type and S-type sensilla in *Drosophila* labellum^{6,9-12}. *Drosophila* pharyngeal *Gr2a* taste neurons mediate aversion to high salt¹³. Additionally, *Drosophila* *dpr* locus (for defective proboscis extension response), a member of the Ig superfamily, is required for the aversive response to high salt¹⁴. TMC-1 (*trans*-membrane channels like) is also reported to be a sodium channel that controls high salt aversion in *C.elegans*¹⁵. *Ir76b* has been shown to detect low and high-NaCl concentrations^{6,12}. Moreover, a complex code for salt taste has been documented in flies recently¹⁶. Also, it has been observed that adult female *Drosophila* switch from sugar feeding to fermenting yeast to get protein and micronutrients for egg development^{17,18}.

Although high salt detection pathways have been studied in great detail but mechanisms that regulate high salt consumption in animals are largely undetermined. In a recent study, functional and behavioral experiments were performed to study the role of different subsets of pharyngeal neurons in governing avoidance responses to taste stimuli including high salt¹⁹. To understand how pre-exposure to high NaCl diet modulates subsequent taste behavior, we looked into the molecular mechanisms of high NaCl consumption and its impact on the feeding behavior using adult *Drosophila* as a model system. We studied, the neural mechanism by which flies modify their acceptance of high salt as a function of diet where a long-term high-salt exposure increases taste sensitivity of pharyngeal LSO neurons and enhance high salt intake. We also found that wildtype flies are attracted to low NaCl levels and show aversion towards higher concentrations. We discovered that exposing flies to high NaCl diet for three days modify their feeding preferences to high levels of salt. High NaCl fed flies show decline in high salt

aversion under starvation. Genetic suppression of LSO pharyngeal neurons in high NaCl fed flies inhibit excessive salt intake. We observed this modulation requires functional LSO neurons and starvation state. This modulation is also dependent on dopamine. Multiple independent taste receptor neurons and pathways are involved in such a modulation. Silencing any one of multiple LSO neuronal types inhibit excessive salt intake. Our data support the idea that high dietary salt modulates and reshapes salt and other taste curves to promote over consumption of food in flies. Our study suggest flies can adapt to the amount of salt ingested over several days, indicating the presence of a critical mechanism to reset the salt appetite and related neural circuits.

Results

Wildtype flies show aversion to high NaCl concentrations

To understand how flies, respond to varying concentrations of NaCl, we first tested wildtype (*CsBz*) flies for their feeding preferences in a feeding assay (Figure 1a). For examining the salt feeding behavior in feeding assays, batches of flies (20 flies each plate, 10 males + 10 females) were presented with a choice between water and varying concentrations of salt. In our two-hour feeding assay, flies were allowed to make a choice between water and varying concentrations of salt in an unbiased manner in the dark. Blue spots in the feeding plate consisted of just water and agar. NaCl added with agar and water was presented as red spots (Figure 1a). % flies showing preference for NaCl (eating red) were scored based on abdomen color. We found that wildtype flies show highest attraction to 50mM NaCl concentration exhibiting highest feeding preference at this concentration (Figure 1b). But with the

increase in salt concentration, flies show reduced feeding preferences towards high NaCl concentrations 100mM, 200mM and 500mM. Flies showed least feeding preference at 500mM concentration (Figure 1b). Our dye switching experiment (NaCl presented in blue spots) also showed highest feeding preference at 50mM NaCl (Supplementary figure 1a).

Similar feeding preferences were observed when mated males and females were tested separately in the feeding assays. Although, female flies showed slightly high preference for NaCl (Figure 1d) compared to males (Figure 1c) but in both the cases highest feeding preference was observed at 50mM NaCl concentration. Later feeding results were confirmed by performing spectrophotometry on mated males and females (Figure 1e and f) separately. Our spectrophotometry data also showed similar results as feeding assays (Figure 1b, c and d) suggesting 50mM acts as an attractive concentration. The absorbance values (Figure 1e and f) for mated females were found higher compared to mated males suggesting mated females need more sodium to invest in the progeny.

We then used an independent assay to evaluate further and tested if flies really prefer lower concentrations of NaCl and show aversive responses at higher NaCl concentrations. We performed tarsal PER (Proboscis Extension Reflex assay, Figure 2a, b and c), where the tarsal taste hairs of wildtype (*CsBz*) flies were stimulated with a series of NaCl concentrations ranging from 10-500mM (same concentrations that were used for the feeding assays). Just like our feeding results, we observed highest proboscis extension responses (~ 65%) in wild-type flies (mix of males and females) at 50mM NaCl concentration (Figure 2c, Supplementary video 1). The same experiment was repeated by performing the labellum PER (Figure 2d) where the

drop of salt solution was presented to the fly labellum. During this assay flies were not allowed to drink the taste solution. Also, while performing the assay flies were monitored on screen to make sure that they were not drinking the solution. We observed highest PER responses at 50mM NaCl concentration (~70%). At 10mM NaCl, both tarsal and labellum PER responses of flies were very similar to 50mM (Figure 2c and d). We observed 25-40% +ve PER responses in case of tarsal (Figure 2c) and labellum PER (Figure 2d) at 200 and 500 mM (Supplementary video 2) concentrations suggesting aversion at high concentrations. Snapshot of tarsal PER responses at 50mM (positive PER response showing extended proboscis- left image; Supplementary video 1) and 200mM NaCl concentrations (-ve PER- right image showing no proboscis extension, Figure 2b and Supplementary video 2). Taken together, these results suggest that flies show attractive behavior to low concentration (50mM) of NaCl and avoid NaCl as the concentration increases. During the PER experiments sugar was tested in the beginning and at the end of the experiments to make sure flies were not losing sensitivity after testing various salt concentrations. We only considered and plotted responses of those flies that responded to sucrose before and after presenting the NaCl. % flies that showed positive PER responses were between 85-95% in case of sucrose (Supplementary figure 1b and 1c).

High NaCl fed flies show decline in high salt aversion under starvation

To understand, if prior exposure to high NaCl alters the feeding behavior for preferred level of salt, wild type flies were fed continuously for 3 days on high salt diet (200 mM NaCl mixed with normal fly food; Figure 3a). Following starvation

period of 24 hrs, flies were tested in a feeding assay for their feeding preferences for different concentrations of NaCl (Figure 3a and b). Flies kept on normal media for 3 days were treated as control flies (Figure 3a and b). Flies kept on high salt media maintained their preferences for high NaCl concentrations and showed increased feeding preferences for 100 and 200mM NaCl concentrations (Figure 3b). No difference in feeding were observed at lower concentrations tested (10 and 50mM, Figure 3b). In high salt fed flies, we observed a shift in feeding preferences, preferring higher concentrations of NaCl (black bars -100 and 200mM, Figure 3b). We found, the aversion towards high NaCl reduced when flies were pre-exposed to high NaCl diet. For our high NaCl paradigm, we decided 3 days' time window to pre-expose flies to high salt concentration based on our feeding results obtained for 1 day and 2 days (Supplementary figure 2a and b).

In our intensity based spectrophotometry experiments, assays done with two different wildtype flies (*CsBz* and *w¹¹¹⁸*) also showed similar results when compared between flies pre-exposed to high NaCl and normal media (Figure 3c and d). Our results suggest repulsion towards high NaCl reduced in flies pre-exposed to high NaCl.

To test if flies were actually feeding on high salt during their pre-exposure period, we next tested the effect of high NaCl feeding on the body weight of mated male and female flies separately. After eclosion followed by 3 days of mating, 4 day old flies were separated as males and females, and were grown on different concentrations of NaCl (mixed with normal media) for the next 3 days. Weight of the flies was taken before and after consuming salt media (Supplementary figure 2c and d) and normal media. In case of mated females, we observed slight increase in weight at 100mM

NaCl concentration (Supplementary figure 2c) but no differences were observed for other concentrations. We found no difference or reduction in mean weight of mated males (Supplementary figure 2d). Our results suggest that both mated females and males were feeding on high salt media in the vials during pre-exposure period.

We also tested if change in the internal state is responsible for modulating the salt taste behavior in the high salt fed flies. For this, we tested flies kept on high NaCl media for 3 days and on normal media conditions (control flies) without any starvation (Supplementary figure 3a). In the fed state, we observed no significant differences in feeding preferences between normal media and high salt fed flies (Supplementary figure 3a). Even under no starvation condition, flies showed highest attraction to salt at 50mM concentration. The overall feeding preferences were found reduced compared to starved flies (Supplementary figure 3a grey bars). Our data suggest that internal state guides the fly feeding decisions.

High NaCl fed flies show no alteration in consumption of potassium under starvation

Sodium works alongside potassium in animals for conducting electrical impulses, muscle contraction, and to maintain many other important physiological functions. Animals including human body requires daily need of potassium to support these key processes. To understand if flies feeding on high NaCl media for 3 days also needed high potassium in the body to blunt the effects of high sodium, we tested flies feeding on normal and high NaCl diet to determine their feeding preferences for different concentrations of KCl (Supplementary figure 3b). We found high NaCl fed flies did

not consume higher amount of KCl at any tested concentration. We found no significant differences in the feeding preferences of flies kept on normal media and high NaCl media (Supplementary figure 3b). Our data suggest that feeding on high sodium diet does not require high potassium to blunt the effects of sodium.

High NaCl fed flies show preference for selective sugars

To understand if pre-exposure to high NaCl diet causes change in preferences to other taste modalities, we tested sweet taste. Varying concentrations (50, 100 and 200mM) of sucrose was presented to flies and tested in our feeding assay. We didn't observe any significant differences in feeding behavior between flies fed on normal media versus high NaCl media (Supplementary figure 4a). Only when lower concentrations of sucrose were presented to flies in the feeding assay and consumption was monitored using spectrophotometry analysis, we observed high absorbance values at 5, 10, 15mM sucrose concentrations in case of flies kept on high NaCl diet (Figure 4a). No change in NaCl consumption was observed at 1, 20 and 25mM sucrose (Figure 4a).

Later different sugars were provided in the feeding assay to check if flies show any specific preference for any particular sugar. We observed high absorbance values selectively for nutritive sugars like D-fructose and D-glucose in intensity based measurements done by spectrophotometry (Figure 4b). Both D-fructose and D-glucose are normally found in the fly hemolymph and in fruits. Surprisingly no significant difference was found in case of D-trehalose. We also didn't see any differences in feeding behavior for Galactose, non- nutritive sugars- Sucralose and L-(-) Glucose when tested at 100mM concentrations (Figure 4b). Our data suggest

modulation of sweet taste behavior in high NaCl fed flies. *Gr43a* is a receptor for fructose in flies²⁰, that detect fructose but sucrose is detected by multiple receptors. Sucrose receptor abundance may help flies to detect even the lower concentration of sucrose (less than 100mM). It is interesting to observe that feeding on high NaCl alters feeding preferences towards sugars perhaps to balance the nutrient requirement, a mechanism not fully understood yet.

We also tested many other salts. We didn't observe significant changes in feeding behavior between high NaCl fed and normal media fed flies (Supplementary figure 4b) for other categories of salts that we tested (lower and higher concentrations) including 25 and 100mM Sodium hydrogen carbonate, 25 and 100mM Di-sodium hydrogen O-phosphate, 10 and 100mM Magnesium chloride and 10 and 100mM Potassium di-hydrogen O-phosphate. Our data suggest high NaCl feeding does not alters feeding preference for other categories of salt in flies. We also didn't observe significant changes in feeding responses between high NaCl fed and normal media fed flies for bitter compound caffeine at 10mM concentration (Supplementary figure 4c).

Active and functional sweet LSO neurons of pharynx modulate feeding preferences after starvation in high NaCl fed flies

To identify the mechanism involved that causes high salt intake in flies pre-exposed to high NaCl diet, we started looking at different set of taste receptor neurons. After silencing the neuronal activity of the peripheral sweet taste neurons first, we

investigated its effect on high salt intake behavior. First we tested *Gr5a*+ve sugar receptor neurons (Figure 5a) that express in the peripheral sweet neurons of labellum only (not in the neurons of the internal taste organs of pharynx) ²¹. To reduce the neuronal activity in the *Gr5a* positive neurons, we used active form of tetanus toxin (*Gr5a-GAL4>UAS-TNT*). Expression of tetanus toxin make neurons lose their activity and neurotransmitter release ²². In our spectrophotometry and feeding assays, flies fed on high NaCl media were considered as test flies and flies fed on normal media for three days were considered as control flies (Figure 5b and supplementary figure 5a). Our high NaCl fed test flies (*Gr5a-GAL4>UAS-TNT*) showed increased NaCl consumption and preference as observed by high absorbance values and in feeding assays both at 200mM NaCl and at low NaCl (50mM) concentration (Figure 5b and supplementary figure 5a, black bars) when compared to normal media fed flies (Figure 5b and supplementary figure 5a- grey bars). The absorbance value and feeding preference was more than double at 50mM concentration in case of high salt fed flies (Figure 5b and supplementary figure 5a). Since, high salt (200mM) is an aversive cue, we also tested other bitter compound caffeine (10mM) in our feeding assay. We didn't observe any change in caffeine feeding responses when *Gr5aGAL4>UAS-TNT* flies were compared between normal media and high salt fed conditions (Supplementary figure 5d). Both parental controls (*Gr5aGAL4/+* and *UAS-TNT/+*; Figure 5c and d; supplementary figure 5b and c) behaved as wildtype flies (Figure 3b, c and d) and showed increase feeding preferences and absorbance at 630nm in case of 200mM high salt fed flies but not at 50mM.

Next, we looked at the absorbance values and feeding behavior of flies expressing other sweet taste receptors including *Gr43a* (Figure 5f and supplementary figure 5e), *Gr64a*, *Gr64e* and *Gr64f* (Figure 5h, j and l; Supplementary figure 5g, i and k) after knocking their neuronal activity down by expressing tetanus toxin. Unlike *Gr5a* which expresses only in labellum neurons, each of these sweet taste receptors express in LSO pharyngeal neurons (Figure 5e; some have additional expression at the periphery or in other regions of pharynx)^{23,24}. It has been shown that *Gr43a* and *Gr64e* co-express with other members of the sweet clade in LSO²⁵. Silencing the neuronal activity of all these sweet receptor neurons didn't show any change in feeding preferences when 50 and 200mM NaCl concentrations were provided to normal media and high NaCl media fed flies (Figure 5f, h, j and l; Supplementary figure 5e, g, i and k). Knock down of neuronal activity in these sweet LSO neurons reduce high salt intake and brings down the feeding preferences back to normal as seen in case of normal media fed flies. Our results suggest that intact and active LSO pharyngeal neurons are sufficient and required under starvation as seen in case of *Gr5a>TNT* or wildtype flies (Figure 3b-d, 5b and supplementary figure 5a) that regulate high NaCl intake. The parental control flies (*UAS-TNT/+*, *Gr43aGAL4/+*, *Gr64aGAL4/+*, *Gr64eGAL4/+*, *Gr64fGAL4/+*; Figure 5d, g, i, k and m; Supplementary figure 5c, f, h, j and l) responded as wildtype flies (Figure 3b, c and d) and showed increased feeding preferences and absorbance values at 630nm at 200mM concentration in high salt fed flies but not at 50mM between normal and high NaCl fed flies (grey and black bars).

Active and functional bitter LSO neurons of pharynx modulate feeding preferences after starvation in high NaCl fed flies

It has been shown ² that high-salt recruits two primary aversive taste pathways in mice by activating the sour and bitter taste-sensing cells. Genetic silencing of sour and bitter pathways eliminates behavioral aversion to concentrated salt, without impairing salt attraction. Mice lacking salt-aversion pathways exhibit unrestricted, continuous attraction even to tremendously high concentrations of NaCl. To test if the same is true in insects as well, we looked at the role of *Gr33a*+ve bitter receptor neurons already known to detect bitter compound caffeine. It has already been shown that *Gr33a* does not express in LSO pharyngeal neurons ^{24,26}. Hence, next we determined if silencing the activity of bitter neurons at the periphery (Figure 6a) where LSO pharyngeal neurons are intact would result in high NaCl feeding as seen in case of *Gr5a>TNT* flies. We silenced the activity of bitter neurons (*Gr33a-GAL4>UAS-TNT*) by expressing tetanus toxin (Figure 6b and supplementary figure 6a). Even in this case, we found high absorbance values in case of high NaCl fed flies compared to normal media flies but only at 200mM concentration of NaCl in our spectrophotometry analysis and increased feeding preference in feeding assays (Figure 6b and supplementary figure 6a). The parental control flies (*Gr33aGAL4/+* and *UAS-TNT/+*; Figure 6c and d; Supplementary figure 6b and c) showed absorbance values and feeding responses as wildtype flies (Figure 3b, c and d) where we observed increase in absorbance and feeding preferences only at 200mM in high salt fed flies but not at 50mM between normal and high salt fed flies (grey and black bars).

Next we looked at the feeding behavior of flies for some more bitter receptor neurons including *Gr93a* that does not express in LSO ²⁴ (Figure 6e and f) and *Gr66a* which

expresses in LSO²⁴ (Figure 6h and i; Supplementary figure 6d and f) after knocking down their neuronal activity by expressing tetanus toxin (*Gr93a-GAL4>UAS-TNT* and *Gr66a-GAL4>UAS-TNT*). Since, *Gr66a* expresses in LSO pharyngeal neurons silencing the neuronal activity of *Gr66a* receptor neurons didn't show any change in feeding preferences when 50 and 200mM NaCl concentrations were provided to control (normal media fed) and test flies (high NaCl media fed; Figure 6i and supplementary figure 6f). But in case of *Gr93a* (*Gr93a-GAL4>UAS-TNT*), we observed enhanced feeding preference (Figure 6f and supplementary figure 6d) at 200mM concentration as seen in case of *Gr33a* emphasizing again the role of active LSO neurons (bitter in this case) in regulating high salt intake in high NaCl fed flies. The parental control flies (*Gr93aGAL4/+*, *Gr66aGAL4/+* and *UAS-TNT/+*; Figure 6d, g and j; Supplementary figure 6c, e, g) showed absorbance values and feeding responses as wildtype flies (Figure 3b, c and d).

In case of *Gr66a>TNT* flies, the feeding preference and absorbance values at 200mM were found higher and the aversion to 200mM was weakened (compare normal media fed flies grey bars only, Figure 6i and supplementary figure 6f) suggesting a possible role of *Gr66a*+ve LSO bitter neurons in regulating high salt intake even under normal media condition.

Genetic suppression of LSO pharyngeal neurons in high NaCl fed flies inhibit excessive salt intake

To again confirm and probe the role of LSO pharyngeal neurons in regulating the high salt intake, next we silenced pharyngeal LSO neurons specifically. We used

Gr2a-GAL4 and *Poxn-GAL4* that show expression in the LSO region only (Supplementary figure 7a and b, white arrow in a and b right side images). As expected, silencing pharyngeal LSO neurons by expressing active form of tetanus toxin (*Gr2a-GAL4>UAS-TNT* and *Poxn-GAL4>UAS-TNT*) showed no significant change in feeding behavior between flies fed on high salt media and normal media (Supplementary figure 7c and e left graphs). The results were no different for both low and high salt concentrations of NaCl even in the case of spectrophotometry analysis (Supplementary figure 7d and f left graphs) at both the concentrations. The parental control flies (*Gr2a-GAL4/+*, *poxn-GAL4/+* and *UAS-TNT/+*; Supplementary figure 7c, d, e, f right hand side graphs; Figure 5d and supplementary figure 5c) showed absorbance values and feeding preferences as wildtype flies (Figure 3b, c and d).

Next we tested *Drosophila Ir76b* which is required for both, high and low salt taste ^{6, 12,16}. We observed expression pattern of *Ir76b* in many LSO neurons (Supplementary figure 8a, white arrow). After silencing the neuronal activity of *Ir76b* neurons by tetanus toxin (*UAS-TNT*), we found no difference in feeding preferences for *Ir76b GAL4>UAS-TNT* flies between flies fed on high salt media and normal media (Supplementary figure 8b) when tested for 50mM and 200mM NaCl concentrations. In fact, *Ir76b GAL4>UAS-TNT* flies showed lower feeding responses at both 50 and 200mM concentration compared to parental flies (compare grey bars in Supplementary figure 8b and c; Supplementary figure 5c). *Ir76b GAL4/+* flies behaved liked wildtype flies and showed increased feeding responses at 200mM when high salt fed flies were compared to normal media fed flies. Taken together, our behavioral results support that the active LSO pharyngeal neurons are required

and necessary to regulate high salt intake in flies pre-exposed to high salt under starvation condition. In the absence of activity in LSO neurons, flies show no difference in feeding behavior between normal media and high NaCl fed conditions.

Role of dopamine and activity in regulating high salt consumption

Recent studies have shown that both starvation and L-dopa increase behavioral sensitivity to sucrose^{27,28}. We wanted to check whether the activity of LSO pharyngeal neurons in high NaCl fed flies is also modulated via dopamine signaling. We administered 3-(3,4-dihydroxyphenyl-2,5,6- d3)-L-alanine (L-Dopa) via food and measured feeding preferences. Consistent with the results of starved flies, we found that high NaCl fed test flies (*Gr5a-GAL4>UAS-TNT*) showed increased feeding preferences at 200mM and 50mM NaCl concentrations (Figure 7a, black bars) after L-Dopa feeding (L-Dopa + food). Feeding preferences were found more than double at 50mM NaCl concentration (Figure 7a, black bars). Both parental controls (*Gr5aGAL4/+* and *UAS-TNT/+*; Figure 7b and c) behaved as wildtype flies (Figure 3b). Under normal media fed condition (no feeding on L-Dopa), we didn't see any significant difference between normal media and high salt fed flies (*Gr5a-GAL4>UAS-TNT*, *Gr5aGAL4/+* and *UAS-TNT/+*) at 50 and 200mM (Figure 7d-f).

Similarly, in case of *Gr33a-GAL4>UAS-TNT* (Figure 7g) and *Gr93a-GAL4>UAS-TNT* (Figure 7i) flies, we found high feeding preferences in high NaCl fed flies than normal media flies at 200mM (not at 50mM) concentration after feeding on L-Dopa. The parental control flies (*Gr33aGAL4/+*, *Gr93aGAL4/+* and *UAS-TNT/+*; Figure 7c, h, j) showed feeding preferences as wildtype flies (Figure 3b) where we observed increase in feeding preferences only at 200mM NaCl concentration (not at 50mM)

between normal and high salt fed flies (grey and black bars). Under normal media fed condition (no feeding on L-Dopa), we didn't see any significant difference between normal media and high salt fed flies (*Gr33a-GAL4>UAS-TNT*, *Gr93a-GAL4>UAS-TNT*, *Gr33aGAL4/+*, *Gr93aGAL4/+* and *UAS-TNT/+*) at 50 and 200mM (Figure 7f, k-n). Taken together, our results indicate modulation of salt taste behavior by both starvation or via dopamine in the presence of neuronal activity in LSO neurons.

Discussion

Apart from peripheral taste cells, there are distinct internal taste organs present in adult flies' pharynx namely LSO, VCSO (ventral cibarial sense organ) and DCSO (dorsal cibarial sense organ). After the food intake is initiated, pharynx controls ingestion of food, and encourages only intake of appetitive food in flies. A recent receptor-to-neuron map of pharyngeal taste organs describes distinctive functional groupings of pharyngeal neurons²⁴. In recent years' attention has been paid on the peripheral salt coding in flies but what remains unclear is the role of pharyngeal neurons in regulating salt intake.

Calcium imaging experiments in the past suggested *Gr5a-Gal4* sweet neurons mediate low salt attraction in insects⁸ and also label additional non-sweet GRNs outside the sweet class²⁹. Other groups proposed the role of Ionotropic receptor *Ir76b*^{6,12} and *Gr66a* GRNs^{8,30} mediating salt responses. Another level of complexity of salt coding in flies has been shown recently suggesting role of *Gr64f* and *Ir94e* in mediating attraction towards low salt and *Gr66a* and *Ppk23^{glut}* drive avoidance to high salt concentrations¹⁶. In all the above-mentioned studies, internal organs

including pharyngeal areas were not taken into consideration for salt behavioral responses. Later functional and behavioral studies done on pharyngeal neurons in *Poxn* mutants suggested avoidance of many aversive compounds including high salt¹⁹ suggesting role of pharyngeal neurons in regulating salt intake. But this study didn't show how modulation of salt taste behavior by various taste pharyngeal neurons in flies.

Our results present the role of LSO pharyngeal neurons in regulating high salt intake and modulation of salt taste behavior. Our study suggests that high dietary salt modulates and reshapes salt and sweet taste curves to promote over consumption of food in flies through LSO pharyngeal neurons. Our data suggest a role of activity together with state dependent modulation of salt behavior in flies by LSO neurons. We found that dopamine signaling also plays a role in this kind of modulation. Multiple taste receptor neurons and pathways are independently involved in LSO neurons contributing to one output and when one of them is inhibited, we observed partial reduction in increased salt responses. Our data suggest genetic suppression of LSO neurons inhibits excessive salt intake, demonstrating acute regulation of salt feeding behavior by this neuronal population.

Reducing activity specifically in LSO neurons as seen with *Gr2a-GAL4* and *poxn-GAL4* strengthens our data that the sufficiency comes from LSO neurons (Supplementary figure 7). As suggested in a recent study that salt taste is encoded by the combined activity of most of all GRN classes at the periphery¹⁶. Our results also suggest that under starvation LSO region of pharynx controls and regulate high salt ingestion via many channels instead of one dedicated line. In our study, it remains unknown whether pre-exposure to high salt directly modulates LSO neurons

or the higher brain areas as the identity of high salt structures in the brain is still elusive (Figure 8).

Fruit flies, moths and locust abate food avoidance to certain bitter foods after prolonged exposure ³¹. It has been shown that the dietary exposure to the unappealing but safe additive like camphor causes decline in the camphor rejection. The long term feeding on camphor diet has been also been shown to result in reversible down regulation of TRPL (transient receptor potential-like) levels in the proboscis ³². A significant modulation of salt taste behavior has been recently examined in flies by salt deprivation ¹⁶. The state dependent modulation of salt taste behavior mediated by *Ppk23^{glut}* was shown under salt fed (3 day feeding with food containing 10mM NaCl) and salt deprivation condition ¹⁶. Salt depletion in humans' cause increase in salt palatability too ³³. It has been suggested that the hunger signal overcomes aversive behavior to unappetizing foods ¹³. Our study suggests a state dependent role of internal pharyngeal taste neurons in modulation of salt taste behavior (high and unappetizing concentration) which is least explored. We used a paradigm where 200mM NaCl (high and aversive concentration) was mixed with the normal food to pre-expose the flies to high salt condition for three continuous days which is a different concentration what Jaeger et al ¹⁶ used. They used a concentration which is generally present in yeast added to normal fly media.

Mating modifies the feeding behavior in female *Drosophila* ³⁴ and induces a salt appetite by increasing gustatory response to sodium ³⁵. We also found that mated females show higher feeding preference for salt (Figure 1d). Interestingly, dNPF and sNPF neuropeptides modulate multiple feeding related behaviors, including control

of food intake during starvation in flies³⁶⁻⁴⁰. During energy deficit conditions, animals become less selective in their food choices by enhancing their sensitivity to nutritious resources, such as sugar^{17,27,41-44} suggesting role of neuromodulatory cascades as key mediators of state-dependent control⁴⁵⁻⁴⁸. Although we found dopamine dependent modulation of salt taste behavior (Figure 7), but the role of dNPF/sNPF signaling via LSO neurons in such a modulation calls for future investigation (Figure 8). Our data also suggest high salt concentration (pre-feeding on high salt media) activates distinct taste pathways modulated by hunger state suggested by Devineni et al⁴⁹ and drive opposing behaviors.

The role of activity in modulating any taste so far has not been studied. It has been shown that acute alteration of ventromedial hypothalamus (VMH) steroidogenic factor 1 (SF1) neurons in mice alters food intake⁵⁰ via changes in appetite and feeding related behaviors. The study also found that SF1 neuron activity is sensitive to energy status. In our study, we observed changes in valence for NaCl taste in the presence of LSO neuronal activity under starvation condition or via dopamine in high salt fed flies. Our study represents an attractive population of neurons for regulating a key aspect of feeding behavior in decision making and acute salt intake. How reduced neuronal activity in LSO neurons is connected to central circuits that control various aspects of feeding behavior under different internal state is yet to determine. Our data suggest interesting roles of LSO pharyngeal neurons (sweet, bitter, salt and *poxn*) where neuronal activity and internal state play an important role in regulating salt intake. Such an analysis was missed in a study done by Chen et al¹⁹. Identifying a key part of feeding neuronal circuitry controlling both ingestive behavior and related affective states as well as characterization of post synaptic high salt

population will greatly enhance our understanding of how internal states modify perception and behavior towards unappetizing salt concentrations (Figure 8).

Our results suggest that pharyngeal taste organs like LSO play an important role in sodium homeostasis. Neurons like LSO in the pharynx present a regulatory system that has evolved to fine tune calorie intake with energy metabolism. Interestingly, in many species, including humans, consuming sodium can drive the desire to eat even more. Examination of molecular and behavioral impact of appetitive cues like sugar or low salt/high salt on the brain's reward circuitry⁵¹ that leads to overeating and metabolic issues is required to study the underlying mechanisms that drive changes in the neural activity. Answering this question may open up avenues to help people with health issues related to high salt consumption to eat less sodium in their diets.

EXPERIMENTAL PROCEDURES:

Fly stocks

CsBz and *w¹¹¹⁸* (from NCBS, Bangalore), *UAS-TNT* (BL 28838), *Gr5a-GAL4* (BL 57592), *Gr33a-GAL4* (BL 57623), *Ir76b-GAL4* (BL 51311), *Gr43a-GAL4* (BL 57636), *Gr64f-GAL4* (BL 57669), *Gr64a-GAL4* (BL 57661), *Gr64e-GAL4* (BL 57667), *Gr66a-GAL4* (BL 57670), *Gr93a-GAL4* (BL 57679), *Gr2a-GAL4* (BL 57590), *UAS-mcd8GFP* (BL 5137) and *poxn-GAL4* (BL 66685) were obtained from the *Drosophila* Bloomington Stock Center. *Drosophila* stocks were reared on standard cornmeal dextrose medium at 25⁰ C, unless specified otherwise.

The *Drosophila* media composition used was (for 1 litre of media) - corn flour (80g), D-glucose (20g, SRL-cas no.50-99-7), Sugar (40g), Agar (8g, SRL- cas.no. 9002-18-0), Yeast powder (15g, SRL-REF-34266), propionic acid (4ml, SRL, cas no. 79-09-

4), TEGO (1.25 g in 3ml of ethanol, fisher scientific, cas no. 99-76-3), and Orthophosphoric acid (600ul, SRL, cas no.7664-38-2).

Feeding behavior assays

For feeding assays, flies of the required genotype were raised from eggs to adults at 25⁰ C. Flies were sorted in vials of 10 males and 10 females (20 flies/vial) upon eclosion and maintained at 25⁰ C for 3 days on fresh media (normal fly food or high NaCl media), after which flies were starved for 24 hrs at 25⁰ C. Flies were tested as described previously^{28,52}, and abdominal coloration was scored as positive if there was any pink or red eating (red or pink- feeding on salt or taste compound; white- no feeding; blue- feeding on agar and water; purple- feeding on both red and blue). Purple was not scored in any of the experiments. 60X15mm feeding plates from Tarsons were used for the assay.

% flies feeding on taste compound was calculated as follows: First % flies feeding on red or blue (Supplementary figure 1a) for each plate was calculated. Mean of % flies feeding for 6 or more plates was taken as a final value.

Proboscis extension reflex (PER) assay (tarsal and labellum)

For PER experiments, flies were tested as described previously²⁸. Flies were collected after 2-3 days of eclosion and kept on standard food for 2 days. Both male and female flies were used for the PER assay. The flies were starved for 24 hrs in vials with water-saturated (4ml) tissue papers. Prior to the PER experiment, flies were immobilized by cooling on ice for at least 15 minutes and then mounted using

nail polish, vertical aspect up, on glass slides (76mm X 26mm X 1mm from Borosil). Flies were allowed to recover in a moist chamber (plastic box with wet tissues) for at least 2 hrs prior to testing. Tastant solutions prepared in water were applied to tarsi or labellum via a drop extruded using 2ul pipette. Before testing the taste solutions, flies were allowed to drink water *ad libitum*. Flies not responding to water were excluded before the assay. Flies satiated with water were then tested with NaCl or other tastant solutions. Ingestion of any tastant solutions was not permitted, and, following each tastant application, flies were retested with water as a negative control. Each fly was tested five times with each tastant solution stimulus. The interval between consecutive tastant solution applications was at least 2-3 min to minimize adaptation. Flies showing three or more proboscis extensions were considered responders.

For all PER experiments, more than 50 flies were tested in batches, and the percentage of responders was calculated for each set. Graphs depict mean responses, and error bars indicate SEM (Standard error of the mean).

Chemicals

Sugars used were all obtained from Sigma Aldrich- Sucrose (57-50-1), Fructose (57-48-7), Trehalose (6138-23-4), Sucralose (56038-13-2), Galactose (59-23-4), L-(-) Glucose (Sigma -921-60-8) and D-(+)-Glucose (50-99-7). The other compounds used in the study- NaCl salt (Fisher Scientific- 7647-14-5) which was of 99.9% purity; Caffeine (Sigma-Aldrich # 58-08-2); Blue dye- Indigo carmine (Sigma: 860-22-0); Red dye-Sulforhodamine B (Sigma- 3520-42-1); Potassium Di Hydrogen orthophosphate (Fisher Scientific -7778-77-0); Magnesium Chloride (Fisher

Scientific- 7791-18-6); Di Sodium Hydrogen O-phosphate (Fisher Scientific- 7558-79-4); Sodium Hydrogen carbonate (Fisher Scientific- 144-55-8); Potassium Chloride (Fisher Scientific- 7447-40-7) and Calcium chloride (Fisher Scientific- 10043-52-4).

Fly weight analysis

After eclosion, 3-day old wildtype flies were sorted separately as males and females into batches i.e. males (30 flies each) X6 vials and females (30 flies each) X6 vials on normal media which served as a control. Similarly, 3 day old flies were separated as males and females to put them on different salt concentrations (10, 50, 100, 200 and 500mM NaCl) mixed with normal fly food. Flies were kept on these media conditions for 3 days as we did for feeding assays. For the weight analysis: weight of the flies (male and females separately) was measured before keeping them on different media conditions and after allowing them to feed on different media conditions for 3 days.

Spectrophotometry analysis

After eclosion, 3 days old flies were sorted (10 males and 10 females) into batches on normal media which served as a control (X 6 vials). Similarly, 3 days old flies were separated as 10 males and 10 females on different salt concentrations (10, 50, 100, 200 and 500mM NaCl) mixed with normal fly food (X 6 vials). Flies were kept on these media conditions for 3 days as we did for feeding assay in Figure 3B.

For spectrophotometry analysis: after feeding assays, total 120 flies were divided into two sets (2 sets X 60 flies each) for each concentration. Later they were put in

2ml Eppendorf in 70% ethanol (60 flies in one Eppendorf). Flies were first crushed in 150ul of 70% ethanol and then 150ul 70% ethanol was added to crush them more. After crushing, 200 ul of double distilled water (to get the content sticking to pestle and the wall of the Eppendorf tube) was added in the same soup and centrifugation was done at 3000 rpm for 15 minutes. After centrifugation, pellet was discarded and supernatant was transferred in the fresh Eppendorf's. To do the spectrophotometry analysis, supernatant was further diluted with 350 ul double distilled water to make up the total final volume of 600ul in the cuvette. Spectrophotometry analysis was done at 630 nm wavelength. One reading was taken for each sample and only the mean values were plotted. Spectrophotometer used was Perkin Elmer, lambda 35 UV/VIS Spectrometer.

Immunohistochemistry

Immunohistochemistry for labellum was performed as mentioned before ²⁸. After anesthetizing flies on ice, labellum was dissected in chilled 1X PBS and kept for fixing (30 min) in 4% paraformaldehyde (0.3% Triton X-100) at room temperature. After washes with PBST (PBS with 0.3% TritonX-100), samples were blocked with 5% normal goat serum in PBST. Samples were incubated in appropriate primary antibody solutions for overnight nights at 4°C. Primary antibody used was rabbit anti-GFP (1:1000, Invitrogen, catalog no. A11122) and secondary antibody used was Alexa Fluor 488 goat anti-rabbit immunoglobulin G (IgG) (1:200, Invitrogen).

L-Dopa feeding experiments

For experiments in Figure 7, L-Dopa (Sigma-Aldrich, CAS no. - 53587-29-4) was first dissolved in water (5mg/ml) ²⁸. Then freshly prepared solution was spread on fly food. After this, flies were maintained on this L-Dopa food for 2 days in the dark at 25⁰ C incubator. The medium was changed once after 24 hr and freshly made L-Dopa was added to the media. Control flies were fed on normal fly food without any L-Dopa. No starvation was involved in these experiments.

Microscopy used for image analysis and video recording

GFP Imaging: Adult labellum were mounted in 70% glycerol in PBST after immunohistochemistry. Samples were analyzed and GFP fluorescence was visualized using a confocal microscope (Leica TCS SP5 II), and image stacks were generated acquired at 0.5 micron optical sections. Olympus SZX10 dual tube microscope was used for generating videos and Olympus SZ61 stereomicroscopes for doing the general fly pushing. Images were processed using ImageJ, Adobe Photoshop, and Illustrator software.

Statistical analyses

Unless otherwise stated, all the results from behavioral experiments were analyzed for statistical significance with parametric ANOVA followed by post hoc Tukey multiple comparisons test for obtaining the p- values.

Figure legends

Figure 1

Flies show attraction to low concentrations of salt (NaCl) and aversive behavior towards high salt concentrations. (a). Image of feeding assay plate. Feeding assay plate showing red spots having salt and agar. Blue represent control dots containing water and agar. (b) Dose response profile showing mean NaCl feeding preference of wild type flies (*CsBz*) for indicated concentrations of NaCl in the feeding assay. For each bar, n=12 trails of ~20 flies each for each concentration (10 males and 10 females). Dose response profile showing % mean NaCl feeding preference of wild type mated male (c) and mated female flies (d) separately for different concentrations of NaCl in the feeding assay. For each graph, each bar, n=6 trails of 20 flies each for each concentration. (e) Graphs showing mean absorbance values for wild type flies (*CsBz*) mated male and mated female flies (f) after spectrophotometry analysis (consumption of NaCl was measured-. NaCl was presented as blue dots in these feeding assays for measuring absorbance at 630 nm). N= 2 sets each concentration, 60 flies each set. For all graphs, error bars=SEM.

Figure 2

Flies show increased proboscis extension at low concentrations of NaCl. (a) Schematic of a fly showing tarsal proboscis extension reflex (PER) response in response to a positive stimulus. (b) Bright field images of flies showing extension of proboscis at an attractive NaCl concentration- 50mM NaCl (left side image) and no extension at higher concentration (200mM NaCl; right side image). Only small percentage of flies extended their proboscis at high NaCl concentration. (c) Dose response profile showing % mean positive PER responses of wild type flies (*CsBz*) for indicated concentrations of NaCl in the Tarsal and (d) Labellum PER assay. For

tarsal PER, n= 62 flies mounted on 8 slides. For labellum PER, n= 64 flies mounted on 6 slides. Dead or immobile flies were not considered or used for the assay. % Flies showing positive PER was calculated for 8 batches of flies in case of **c** and 6 batches in case of **d**. Mix of males and females were used in each case. For all graphs, error bars=SEM.

Figure 3

High NaCl fed flies show decline in high salt aversion under starvation. (a)

Schematic of feeding paradigm used to pre-expose wild type flies to high NaCl condition. **(b)** Dose response profile of *CsBz* wildtype flies showing % mean NaCl feeding preferences for indicated concentrations of NaCl in two different feeding backgrounds. Black bars showing preferences of flies pre-fed on high salt diet (200mM NaCl mixed with normal fly food) for 3 days compared against grey bars (flies fed on normal fly media for 3 days). After 24hrs of starvation, flies were tested to check their preferences for indicated concentrations of NaCl. For each bar, n=12-18 trails of 20 flies each (10 males and 10 females). **(c and d)** Mean absorbance values for *CsBz* and *w¹¹¹⁸* flies kept on normal media and high salt media. n= 2 sets each concentration, 60 flies each set. For all graphs, error bars=SEM. Statistical analysis was performed using ANOVA Tukey's multiple comparison test for obtaining P values: *p < 0.05, **p < 0.005 and ***p < 0.0005. For all graphs, error bars=SEM and NS is not significant.

Figure 4

High NaCl fed flies show preference for selective sugars. (a)

Absorbance values for different concentrations of sucrose (1, 5, 10, 15, 20 and 25mM) between high NaCl

fed and normal media fed wildtype flies (*CsBz*). **(b)** Absorbance values for different sugars (fructose, trehalose, galactose, D-glucose, sucralose and L-glucose) at 100mM concentration between high NaCl fed and normal media fed flies. For a and b, n= 2 sets each concentration, 60 flies each set. Statistical analysis was performed using ANOVA Tukey's multiple comparison test for obtaining P values: *p < 0.05, **p < 0.005 and ***p < 0.0005. For all graphs, error bars=SEM and NS is not significant.

Figure 5.

Flies with active sweet LSO pharyngeal neurons show higher feeding preferences for salt under starvation condition. **(a)** Cartoon showing expression of *Gr5a*. **(b-d)** Comparison of mean intensity of flies after silencing neuronal activity of *Gr5a* sweet neurons by genetically expressed active form of tetanus toxin (*Gr5a-GAL4>UAS-TNT*) with parental control flies (*Gr5a-GAL4/+* and *UAS-TNT/+*) after spectrophotometry analysis (consumption of NaCl mixed with blue dye in 2 hrs feeding assay) done at absorbance 630 nm. N= 2 sets each concentration, 60 flies each set. The graphs are presenting absorbance values for low (50 mM) and high (200 mM) concentrations of NaCl after 24hrs of starvation. In all graphs, black bars represent responses of flies pre- exposed to high NaCl diet for 3 days compared against grey bars- flies fed on normal fly media for 3 days. Asterisks showing significant differences between black vs grey for genotypes *Gr5a-GAL4>UAS-TNT*, *Gr5a-GAL4/+* and *UAS-TNT/+*. **(e)** Cartoon showing expression of *Gr43a*, *Gr64a*, *Gr64e*, and *Gr64f* in LSO. **(f-m)** Mean absorbance values of flies after genetically manipulating neuronal activity of other sweet taste receptor neurons by expressing tetanus toxin (*UAS-TNT*) using *Gr43a-GAL4*, *Gr64a-GAL4*, *Gr64e-GAL4*, and *Gr64f-GAL4* drivers and their parental control flies (*Gr43a-GAL4/+*, *Gr64a-GAL4/+*, *Gr64e-*

GAL4/+, and *Gr64f-GAL4/+*). N= 2 sets each concentration, 60 flies each set. Statistical analysis was performed using ANOVA Tukey's multiple comparison test for obtaining P values: *p < 0.05, **p < 0.005 and ***p < 0.0005. For all graphs, error bars=SEM and NS is not significant.

Figure 6

Flies with active bitter LSO pharyngeal neurons show increased feeding preferences for high salt concentration under starvation condition. (a) cartoon showing expression of *Gr33a* (b-d) Mean absorbance (spectrophotometry analysis) values of flies after silencing neuronal activity of bitter neurons by expressing active form of tetanus toxin and parental control flies (*Gr33a-GAL4>UAS-TNT*, *Gr33a-GAL4/+* and *UAS-TNT/+*) for indicated concentrations of NaCl after 24hrs of starvation. Asterisks showing significant differences between *Gr33a-GAL4>UAS-TNT* flies fed on normal (grey bars) and high salt media (black bars). (e-g) Spectrophotometry analysis of flies after silencing activity of other bitter neurons (*Gr93a*) by expressing *UAS-TNT* (f- *Gr93a-GAL4>UAS-TNT*) and parental control (g- *Gr93a-GAL4/+*). (h-j) Spectrophotometry analysis of flies after silencing activity of other bitter neurons (*Gr66a*) by expressing *UAS-TNT* (i- *Gr66a-GAL4>UAS-TNT*) and parental control (j- *Gr66a-GAL4/+*) between normal media and high NaCl media fed flies (grey vs black bars). N= 2 sets each concentration, 60 flies each set. Statistical analysis was performed using ANOVA Tukey's multiple comparison test for obtaining P values: *p < 0.05, **p < 0.005 and ***p < 0.0005. For all graphs, error bars=SEM and NS is not significant.

Figure 7

Role of Dopamine in modulating salt taste behavior. (a-c). Comparison of feeding preferences of flies with silenced neuronal activity of *Gr5a* sweet neurons by expressing active form of tetanus toxin (*Gr5a-GAL4>UAS-TNT*) and parental control flies (*Gr5a-GAL4/+* and *UAS-TNT/+*) after L-Dopa feeding. In all graphs, black bars represent preferences of high NaCl fed flies compared with flies fed on normal fly media (grey bars). Asterisks showing significant differences between black vs grey for genotypes *Gr5a-GAL4>UAS-TNT*, *Gr5a-GAL4/+* and *UAS-TNT/+*. **(b-f)** Comparison of feeding preference of flies with silenced neuronal activity of *Gr5a* sweet neurons and parental control flies (*Gr5a-GAL4/+* and *UAS-TNT/+*) after feeding on normal food (no L-dopa treatment). **(g-n)** Mean feeding preference of flies after genetically manipulating the neuronal activity of bitter taste receptor neurons by expressing tetanus toxin (*UAS-TNT*) using *Gr33a-GAL4* and *Gr93a-GAL4* and their parental control flies (*Gr33a-GAL4/+* and *Gr93a-GAL4/+*) with **(g-j)** and without L-Dopa treatment (k-n). For each bar, n=6-12 trials of 20 flies each (10 males and 10 females). Statistical analysis was performed using ANOVA Tukey's multiple comparison test for obtaining P values: *p < 0.05, **p < 0.005 and ***p < 0.0005. For all graphs, error bars=SEM and NS is not significant.

Figure 8

Working model. Three distinct internal taste organs are present in adult fly pharynx: the labral sense organ (LSO), ventral cibarial sense organ (VCSO), and dorsal cibarial sense organ (DCSO). Our study suggests that pre-exposure to high concentration of NaCl enhances taste preference/sensitivity for selective sugars and

unappetizing NaCl concentrations. Flies with intact functional LSO neurons under starvation or via dopamine signaling show increased feeding preference (thick red arrow) for low and high NaCl, suggesting role of active pharyngeal LSO neurons in regulating high salt intake in flies pre-fed on high salt media and present a complex code in terms of neuronal activity at the level of internal taste organ. The observed high NaCl feeding preferences get suppressed to normal feeding preferences in flies with reduced neuronal activity in LSO region as well as in fed state (red arrow) irrespective of what receptor they express (*Gr43a*, *Gr64a*, *Gr64e*, *Gr64f*, *Gr66a*, *Gr2a*, *Ir76b* and *poxn* all express in LSO) suggesting multiple pathways are involved that regulate high salt feeding in the hungry flies pre-fed on high salt. The identity of salt metabolic sensor in the brain and the role of central salt neurons causing high salt taste modulation is an open area to explore.

Supplementary figures

Supplementary figure 1

(a) Graph showing mean feeding preference of flies after switching the dyes. Black bars represent feeding preferences of flies for different concentrations of NaCl after 24 hrs of starvation. In these experiments salt was added to blue dye instead of red dye. N= 18 plates, 20 flies each plate (10 males and 10 females). (b and c) Mean positive PER responses of flies tested for 100mM sucrose each time before application of NaCl and by the end of the experiment in our tarsal and labellum PER assays. N= 62 flies for tarsal and 64 flies for labellum PER. Error bars represent SEM.

Supplementary figure 2

Mean feeding preference of wildtype flies (CsBz) tested for various concentrations of NaCl on two different media conditions. **(a)** Flies pre- exposed to high salt diet (black bars) and normal media for 1 day (grey bars), **(b)** and 2 days followed by 24hrs starvation condition. For each bar, n=6 trails of 20 flies each (10 males and 10 females). **(c and d)** Graph showing mean weight of mated female and mated male flies before and after feeding on indicated concentrations of salt for 3 days (Black line). Feeding on normal fly media was considered as a control (grey lines). Each point, n= weight of 6 batches of 30 flies each concentration. Statistical analysis was performed using ANOVA Tukey's multiple comparison test for obtaining P values: *p < 0.05, **p < 0.005 and ***p < 0.0005. For all graphs, error bars=SEM and NS is not significant.

Supplementary figure 3

Flies show no difference in feeding preferences under fed state. **(a)** % mean feeding preferences of wildtype flies pre- exposed to high salt diet (black bars) and normal media for 3 days (grey bars) under no starvation conditions. For each bar, n=6 trails of 20 flies each (10 males and 10 females). **(b)** KCl (potassium chloride) dose response curve of normal media (grey bars) and high salt media fed flies (black bars). N=6-12 plates, 20 flies each plate (10 males and 10 females). Statistical analysis was performed using ANOVA Tukey's multiple comparison test for obtaining P values: *p < 0.05, **p < 0.005 and ***p < 0.0005. For all graphs, error bars=SEM and NS is not significant.

Supplementary figure 4

Flies show no change in feeding preferences for high concentration of sucrose and other salts. **(a)** Mean feeding preference of wildtype (*CsBz*) flies for high concentrations of sucrose (50,100 and 200mM). **(b)** Mean feeding preference of wildtype (*CsBz*) flies for indicated concentrations of other salts - sodium hydrogen carbonate (25 and 100mM), Di-sodium hydrogen O-phosphate (25 and 100mM), Magnesium Chloride (10mM and 100mM and potassium di hydrogen O-phosphate (10mM and 100mM). **(c)** Mean feeding preference of wildtype (*CsBz*) flies for caffeine (10mM). In all graphs, black bars represent responses of flies pre-exposed to high NaCl diet compared to grey bars (normal fly media fed flies). N=6 plates, 20 flies each plate (10 males and 10 females). Statistical analysis was performed using ANOVA Tukey's multiple comparison test for obtaining P values: *p < 0.05, **p < 0.005 and ***p < 0.0005. For all graphs, error bars=SEM and NS is not significant.

Supplementary figure 5

Flies with active sweet LSO pharyngeal neurons show increased feeding preferences for salt under starvation condition. **(a)** Mean feeding preference of flies (*Gr5a-GAL4>UAS-TNT*) after silencing neuronal activity of peripheral *Gr5a* sweet neurons by expressing active form of tetanus toxin for low (50mM) and high (200 mM) concentrations of NaCl after 24hrs of starvation. In all graphs, black bars represent responses of high NaCl fed flies (3 days) compared with grey bars- normal media fed flies (3 days). Asterisks showing significant differences between *Gr5a-GAL4>UAS-TNT* flies (black vs grey). **(b and c)** Mean feeding preference of parental flies (*Gr5a-GAL4/+* and *UAS-TNT/+*). **(d)** Mean feeding preference of *Gr5a-GAL4>UAS-TNT* flies (both normal media and salt pre-exposed) for caffeine (10mM). **(e, g, i, and k)** Mean feeding preference of flies after genetically

manipulating the neuronal activity of other sweet taste receptor neurons by expressing tetanus toxin (*UAS-TNT*) using *Gr43a-GAL4*, *Gr64a-GAL4*, *Gr64e-GAL4*, and *Gr64f-GAL4* drivers. (f, h, j and l) Mean feeding preference of parental flies (*Gr43a-GAL4/+*, *Gr64a-GAL4/+*, *Gr64e-GAL4/+* and *Gr64f-GAL4/+*) for 50 and 200 mM NaCl. For each graph, each bar, n=6-12 trials of 20 flies each for each concentration (10 males and 10 females). Statistical analysis was performed using ANOVA Tukey's multiple comparison test for obtaining P values: *p < 0.05, **p < 0.005 and ***p < 0.0005. For all graphs, error bars=SEM and NS is not significant.

Supplementary figure 6

Flies with active bitter LSO pharyngeal neurons show increased feeding preferences for high salt concentration under starvation condition. (a) Mean feeding preference of flies after genetically silencing activity of peripheral bitter neurons by expressing active form of tetanus toxin (*Gr33a-GAL4>UAS-TNT*) for indicated concentrations of NaCl after 24hrs of starvation. Asterisks showing significant differences between *Gr33a-GAL4>UAS-TNT* flies fed on normal (grey bars) and salt media (black bars). (b and c) Mean feeding preference of parental control flies (*Gr33a-GAL4/+* and *UAS-TNT/+*). (d and f) Mean feeding preference of flies after silencing activity of other bitter neurons (*Gr93a* and *Gr66a*) by expressing *UAS-TNT* (d, *Gr93a-GAL4>UAS-TNT* and e, *Gr66a-GAL4>UAS-TNT*) between normal media and high NaCl media exposed flies (grey vs black bars). (e and g) Mean feeding preference of parental control flies (*Gr93a-GAL4/+* and *Gr66a-GAL4/+*). Statistical analysis was performed using ANOVA Tukey's multiple comparison test for obtaining P values: *p < 0.05, **p < 0.005 and ***p < 0.0005. For all graphs, error bars=SEM and NS is not significant.

Supplementary figure 7

Silencing LSO pharyngeal neurons specifically show no increase in feeding preference for NaCl in high salt fed flies. (a) Expression pattern of *Gr2a-GAL4* driven by *UAS-mCD8GFP* in the LSO pharyngeal neurons (white arrow in right image). (b) Expression pattern of *poxn-GAL4* driven by *UAS-mCD8GFP* in the LSO pharyngeal neurons (white arrow in right image). (c) Mean feeding preference of flies after silencing neuronal activity in the *Gr2a* (*Gr2a-GAL4>UAS-TNT* and parental control *Gr2a-GAL4/+*) positive LSO neurons tested for 50mM and 200mM NaCl (between normal media verses high salt media fed flies; compare grey vs black). (d) Spectrophotometry analysis of normal media and high salt media fed *Gr2a-GAL4>UAS-TNT* and *Gr2a-GAL4/+* flies. (e) Feeding preference of flies after silencing neuronal activity in the *poxn* (*poxn-GAL4>UAS-TNT* and parental control *poxn-GAL4/+*) positive LSO neurons (compare grey vs black) tested for 50mM and 200mM NaCl. (f) Spectrophotometry analysis of normal media and high salt media *poxn-GAL4>UAS-TNT* and *poxn-GAL4/+* flies. For c and e, n=6-12 plates, 20 flies each plate. For spectrophotometry analysis (d and f), n= 2 sets each concentration, 60 flies each set. Statistical analysis was performed using ANOVA Tukey's multiple comparison test for obtaining P values: *p < 0.05, **p < 0.005 and ***p < 0.0005. For all graphs, error bars=SEM and NS is not significant.

Supplementary figure 8

Silencing *Ir76b* LSO pharyngeal neurons showed no increase in feeding preference for NaCl in high salt pre-exposed flies. (a) Expression pattern of

Ir76b-GAL4 driven by *UAS-mCD8GFP* in the LSO pharyngeal neurons (white arrow).

(b) Mean feeding response of flies *Ir76b*-GAL4>*UAS-TNT* compared between normal media verses high salt media fed flies. (c) Mean feeding preference of *Ir76b*-GAL4/+ parental flies. Statistical analysis was performed using ANOVA Tukey's multiple comparison test for obtaining P values: *p < 0.05, **p < 0.005 and ***p < 0.0005. For all graphs, error bars=SEM and NS is not significant.

[Supplementary movie 1 for figure 2.](#)

[Tarsal PER - Fly showing extension of proboscis when 50mM NaCl was presented](#)

[Supplementary movie 2 for figure 2.](#)

[Tarsal PER - Fly showing no extension of proboscis when 200mM NaCl was presented.](#)

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Ethics declarations

Competing interests

The authors declare no competing interests.

Data availability statement

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Activity and state dependent modulation of salt taste behavior in *Drosophila melanogaster*

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Figures

Figure 1

Figure 2

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Supplementary figure 1

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