Coupled Sensing of Hunger and Thirst Signals Balances Sugar and Water Consumption

Graphical Abstract

Highlights

- Four interoceptive neurons oppositely regulate sugar and water consumption
- These neurons detect a peptide hormone that signals nutrient deprivation
- These neurons directly sense changes in osmolality through an ion channel
- These neurons report homeostatic changes and drive consumption to restore balance

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In Brief
The same set of neurons responds to internal hunger and thirst states, with activation promoting eating over drinking and inactivation promoting the inverse effect, suggesting that they weigh competing needs.

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Coupled Sensing of Hunger and Thirst Signals Balances Sugar and Water Consumption

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SUMMARY

Hunger and thirst are ancient homeostatic drives for food and water consumption. Although molecular and neural mechanisms underlying these drives are currently being uncovered, less is known about how hunger and thirst interact. Here, we use molecular genetic, behavioral, and anatomical studies in Drosophila to identify four neurons that modulate food and water consumption. Activation of these neurons promotes sugar consumption and restricts water consumption, whereas inactivation promotes water consumption and restricts sugar consumption. By calcium imaging studies, we show that these neurons are directly regulated by a hormone signal of nutrient levels and by osmolality. Finally, we identify a hormone receptor and an osmolality-sensitive ion channel that underlie this regulation. Thus, a small population of neurons senses internal signals of nutrient and water availability to balance sugar and water consumption. Our results suggest an elegant mechanism by which interoceptive neurons oppositely regulate homeostatic drives to eat and drink.

INTRODUCTION

To achieve homeostasis, animals must regulate consumption of external nutrients based on internal metabolic needs. Remarkably, animals whose nervous systems differ dramatically in organization exhibit some of the same homeostatic consumption behaviors, such as orally consuming food or water in response to starvation or dehydration. While these similarities suggest conserved underlying mechanisms, how nervous systems regulate consumption in a manner that reflects internal state remains an open question.

A key requirement for homeostatic regulation is the ability to sense internal nutrient abundance and either promote consumption in nutrient-deprived states or inhibit consumption in nutrient-replete states. In mammals, a major site of internal nutrient sensing is the hypothalamus, a conserved forebrain region that senses nutrients and coordinates behavioral responses to changes in their abundance (Sternson, 2013). Neurons in the hypothalamus include direct sensors of circulating sugars like glucose, as well as sensors of metabolic cues, like insulin, ghrelin, and glucagon. The hypothalamus also contains neurons whose activity is regulated by extracellular osmolality and are therefore sensitive to internal water abundance (Bourque, 2008). However, how the nervous system uses information encoded by hypothalamic sensors to regulate consumption of food and water remains unresolved.

Like mammals, the fruit fly Drosophila melanogaster regulates consumption of food and water depending on internal metabolic state. Although flies lack a direct homolog of the hypothalamus, neural populations in the Drosophila brain function as internal nutrient sensors, including glucose, fructose, and amino acid sensors that regulate feeding decisions (Dus et al., 2015; Bjordal et al., 2014; Miyamoto et al., 2012). Flies also regulate water consumption based on internal water abundance (Dethier, 1976), although internal sensors underlying this behavior have not previously been characterized. Thus, mammals and insects both regulate food and water consumption based on internal metabolic state, and in many instances, accomplish this regulation by similar mechanisms. However, the neurons and molecules that regulate homeostatic consumption remain incompletely understood. In particular, mechanisms that coordinate the consumption of different essential nutrients, such as sugar or water, have been largely unexplored.

Here, we report findings from two behavioral screens for neurons that regulate food or water consumption in Drosophila. Surprisingly, these screens independently identified the same four neurons as regulators of both food and water consumption. The neurons are located in the subesophageal zone (SEZ), a key relay for feeding regulation in the fly brain, and we name them interoceptive SEZ neurons (ISNs). Using genetic tools, behavioral assays, and calcium imaging, we show that ISNs are sensitive both to an internal signal of nutrient deprivation, the glucagon-like peptide adipokinetic hormone (AKH), and an internal signal of water abundance, extracellular osmolality. We identify the G protein coupled receptor, adipokinetic hormone receptor (AKHR), and a conserved TRPV channel, Nanchung (Nan), as underlying responses to AKH and osmolality, respectively. Finally, we show that ISNs oppositely regulate sugar and water consumption, suggesting that they function to restore internal homeostasis. The convergence of internal signals of nutrient and water availability onto interoceptive neurons
suggests an unexpected principle by which the nervous system might coordinate homeostatic behaviors.

**RESULTS**

**A Behavioral Screen for Neurons that Regulate Feeding**

To identify neurons that regulate feeding, we transiently activated candidate neurons with the heat-activated cation channel, dTRPA1, and determined the effect on feeding in adult flies. Consumption was monitored by scoring the amount of blue dye in the abdomens of flies with access to 200 mM sucrose containing blue dye for 30 min. Approximately 600 Gal4 lines from the InSite collection (Gohl et al., 2011) with expression in the CNS were crossed to flies carrying the UAS-dTRPA1 transgene (Hamada et al., 2008), allowing for Gal4-dependent neural activation. Fed flies were tested for sucrose consumption during heat-induced depolarization of Gal4-expressing neurons (Figure 1A; Table S1 reports transgenic flies used in this study). Four lines exhibited dramatically increased feeding, with the 954-Gal4 line showing the strongest consumption.

**Four Neurons in the 954-Gal4 Line Promote Ingestive Behaviors to Sucrose**

To identify neurons causal for increased feeding in the 954-Gal4 line, we began by characterizing its expression pattern. In the central brain, 954-Gal4 drove UAS-mCD8::GFP expression in neurons of the pars intercerebralis, dorsal lateral protocerebrum, subesophageal zone (SEZ), and ventral nerve cord (VNC) (Figure 1B). To identify which neurons contribute to the feeding phenotype of the 954-Gal4 line, we employed an intersectional approach using the Gal4 inhibitor Gal80 to restrict Gal4 expression to smaller neural populations. The Tshirt-Gal80 transgene (Clyne and Miesenböck, 2008) blocked Gal4 expression in the VNC but did not eliminate increased consumption upon dTRPA1 activation of 954-Gal4 neurons, demonstrating that VNC neurons are not required (Figure S1A). Next, we identified one line, 149-Gal80 (M.D. Gordon, personal communication), that eliminated SEZ GFP expression without affecting pars intercerebralis or dorsal lateral protocerebrum expression (Figure S1B). This transgene...
eliminated the increased feeding phenotype in the 954-Gal4 line, arguing that 954-Gal4 SEZ neurons are necessary for increased consumption.

We screened existing Gal4 collections (Jenett et al., 2012; B.J. Dickson, personal communication) to identify lines that exhibit Gal4 expression in these SEZ neurons and identified two Gal4 lines, R34G02 and VT011155, that labeled neurons that resembled the 954-Gal4 SEZ cluster. The R34G02 line also drove expression in a pair of VNC neurons and abdominal ganglia projections (Figure 1C). Remarkably, the VT011155 line exclusively labeled the SEZ neurons (Figure 1D). Both lines exhibited the feeding phenotype identified in 954-Gal4 (Figures 1E–1G). In addition, activation of 954-Gal4, R34G02, or VT011155 neurons with UAS-dTRPA1 increased rates of proboscis extension to sugar stimuli, a non-ingestive behavior that flies exhibit to an appetitive taste stimulus (Figure S1C). We generated a R34G02-LexA line and performed double labeling experiments with the Gal4 lines to test for co-expression and found that 954-Gal4, VT011155, and R34G02-Gal4 are all co-expressed in the same two SEZ neurons per hemisphere, with 954-Gal4 also showing expression in two additional SEZ neurons per hemisphere (Figures S1D and S1E). These findings demonstrate that activation of four neurons in the SEZ causes increased feeding and promotes proboscis extension in well-fed animals. We name these interoceptive SEZ neurons (ISNs) and the VT011155 line that specifically labels them ISN-Gal4.

ISNs Directly Respond to AKH and Are Indirectly Inhibited by Insulin

Pre- and post-synaptic sites on ISNs overlap in the dorsal SEZ (Figure S1F). ISNs might therefore be components of feeding sensorimotor circuits, or they may modulate activity in these circuits in response to internal cues. To distinguish between these models, we tested whether ISNs respond to sensory detection of taste compounds. Tastants were applied to the proboscis while monitoring activity in ISNs by GCaMP5G or GCaMP6s calcium imaging in live flies (Akerboom et al., 2012; Harris et al., 2015). Although stimulation with sucrose, water, or the bitter compound denatonium triggered sensory neuron responses, no activation was seen in ISNs (Figure S2), indicating that the ISNs are unlikely to report detection of taste compounds.

An alternative hypothesis is that these neurons encode information about hunger state. As hormones often signal metabolic status, we tested whether existing hormone receptor Gal4 lines marked the ISNs. One line, adipokinetic hormone receptor (AKHR)-Gal4 (Bharucha et al., 2008) exhibited strong labeling of ISNs, confirmed by double labeling with R34G02-LexA, suggesting these neurons may be regulated by AKH (Figure 2A).

AKH is a peptide hormone that is synthesized exclusively by neurosecretory cells in the corpus cardiacum and secreted into the circulating hemolymph, where it acts in a similar manner to mammalian glucagon. AKH secretion is stimulated under low nutrient conditions, which in turn leads to lipolysis, glycogenolysis, and release of sugar and lipid nutrients into the hemolymph from the fat body, the primary site of nutrient storage in Drosophila (Kim and Rulifson, 2004; Lee and Park, 2004). The endocrine role of AKH in regulation of insect metabolism is established, and this hormone is well positioned to signal nutrient status to the brain (Bharucha et al., 2008).

To directly test whether AKH modulates ISNs, calcium levels in these neurons were monitored by GCaMP5G fluorescence upon AKH perfusion in a dissected brain preparation (Figure 2B). Brief pulses of AKH produced rapid, robust, and dose-dependent GCaMP5G fluorescence increases with picomolar to nanomolar AKH concentrations, the physiological range of AKH measured in locust hemolymph (Candy, 2002). The ISNs of animals lacking AKHR did not respond to AKH, verifying that the AKH-induced responses were a result of activation of AKHR (Figure 2C).

In principle, the AKH-induced response might be cell-autonomous (AKH might directly bind to AKHR on ISNs to increase calcium) or non-autonomous (AKH might bind a receptor on other neurons that increase ISN activity via synaptic transmission). To test whether AKH directly modulates ISN activity, we applied the voltage-gated sodium channel blocker tetrodotoxin (TTX) to inhibit action potentials. ISNs responded to AKH even in the presence of TTX (Figure 2C), arguing that AKH directly activates ISNs.

AKH plays a role analogous to glucagon in signaling nutrient depletion and promoting release of stored nutrients. Insulin plays an opposing role, signaling nutrient abundance and promoting storage of circulating nutrients. Given the opposing endocrine roles of insulin and AKH, we tested whether insulin might regulate ISNs in a manner opposite to AKH. Insulin application alone did not induce a calcium response in ISNs (not shown). To test whether insulin affects the ability of ISNs to respond to AKH, dissected brains were perfused with two spaced pulses of AKH, separated by a 3-min perfusion with insulin (Figure 2D). Insulin reduced the response of ISNs to AKH in a concentration-dependent manner. To test whether insulin acts cell-autonomously, TTX was applied to eliminate action potentials and non-autonomous effects. We found that insulin no longer reduced AKH responses in the presence of TTX (Figure 2D). This argues that ISNs do not directly sense insulin, but receive synaptic input from other neurons that provide an inhibitory drive onto ISNs. These studies demonstrate that ISNs are capable of receiving and integrating inputs reflecting both low and high nutrient levels, directly responding to AKH and receiving inhibition from an insulin-sensitive pathway.

To directly test whether ISNs report physiological need, we reasoned that the activity in ISNs would likely be different in starved versus fed animals. We monitored ISN activity by cell-attached electrophysiological recordings in the living fly under fed or starved conditions. ISN activity decreased in the fed state and increased in the starved state, and this state-dependent activity of ISNs was absent in the AKHR mutant (Figure 2E). These experiments show that the ISNs are modulated in the living animal based on nutritional state and that this requires AKHR.

A Behavioral Screen for Molecules that Regulate Water Consumption

Our studies indicate that ISNs respond to signals of internal energy state and that their activity is sufficient to promote feeding, separating metabolic and neural functions of AKH. In a different behavioral screen to identify molecules that regulate water consumption, we ultimately identified additional molecules that are...
expressed in ISNs, regulate their activity, and provide unexpected insight into their function.

With the goal of identifying molecules that might signal thirst or water satiety in Drosophila, we screened a panel of 94 candidate ion channels and neuropeptide receptors for water consumption defects following RNAi knockdown with the pan-neuronal nSynaptobrevin-Gal4 (nSyb-Gal4) line. Flies were placed in <20% relative humidity for 2 hr, which specifically increased water consumption (Lin et al., 2014) (Figure 3A). Upon pan-neuronal RNAi expression, the majority of RNAi lines showed behavior similar to GFP RNAi controls (109% control consumption). As expected, RNAi to PPK28, an ion channel expressed in sensory neurons and essential for water taste detection (Cameron et al., 2010; Chen et al., 2010), reduced water consumption (12% control consumption). In addition, RNAi against a number of genes reproducibly increased or decreased water consumption compared to sibling controls. These included the insulin-like receptor (5% control consumption) and the follicle stimulating hormone receptor (315% control consumption), which have been implicated in water homeostasis in insects (Liu et al., 2015; Palluzzi et al., 2014; Sellami et al., 2011).

Among the candidates with no known role in water consumption behavior was the TRPV family member, Nan (184% and 197% control consumption, independent RNAi lines). Nan is a non-selective cation channel that participates in Drosophila proprioception, hearing, and hygrosensation (Gong et al., 2004; Kim et al., 2003; Liu et al., 2007; Zhang et al., 2013). Interestingly, TRPV channels function as osmosensors in C. elegans and mammals and likely play a role in water consumption regulation in mice (Colbert et al., 1997; Liedtke and Friedman, 2003; Liedtke et al., 2003). We therefore chose to further investigate the role of Nan in Drosophila water consumption.

**nanchung Is Required to Restrict Water Consumption**

To examine whether nan-expressing neurons regulate water consumption, we tested the behavioral response of nan

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mutants as well as animals expressing nan RNAi specifically in nan-Gal4 neurons, using described lines (Kim et al., 2003). Water consumption time was measured in single flies following 2 hr of acute desiccation (Figure 3B). Both nan36a and nan dy5 mutants as well as nan-Gal4, UAS-nan RNAi flies consumed significantly more water than controls, demonstrating that nanchung is necessary to restrict water consumption.

To examine how activity in nanchung neurons influences water consumption, we inducibly activated these neurons with dTRPA1. Flies expressing UAS-dTRPA1 in nan-Gal4 neurons reduced water consumption upon dTRPA1 activation. Transient activation of nan neurons was also sufficient to rescue elevated water consumption observed in nan56 heterozygotes (Figure 3C), suggesting that nanchung neurons function to restrict water consumption.

### Nanchung Neurons in the SEZ Are Osmosensitive

The expression pattern of nanchung has been described previously using nan-Gal4 (Kim et al., 2003; Liu et al., 2007) (Figure 3D). These studies found expression in chordotonal organs of the legs and antennae, as well as in a small number of putative interneurons in the higher brain. Because nan has been proposed to detect water vapor in antennal neurons, we tested whether antennae were required for dTRPA1-mediated activation of nan neurons to reduce water consumption in nan56 heterozygotes and found no role for antennae (not shown).

An alternative possibility is that Nanchung functions in central brain neurons labeled by nan-Gal4 to regulate water consumption. Nan belongs to a class of mechanosensitive TRPV channels and is sufficient to confer osmosensitivity to cultured mammalian cells (Kim et al., 2003). Moreover, TRPV channels have been proposed to confer osmosensitivity to central neurons in the mammalian brain (Bourque, 2008; Liedtke and Friedman, 2003). We therefore tested whether central neurons labeled by the nan-Gal4 line were directly osmosensitive.

nan-Gal4, UAS-GCaMP6s brains were perfused with artificial hemolymph (AHL) of different osmolalities while GCaMP6s
Fluorescence was monitored (Chen et al., 2013). Decreasing osmolality caused robust calcium responses from bilaterally symmetric nan-Gal4 neurons in the ventrolateral SEZ (Figure 4A). These responses were dose-dependent and observed following osmolality decreases but not increases (Figures 4B and 4C). To test whether these responses are specific to nan SEZ neurons, brains expressing GCaMP6s with the pan-neuronal driver nSyb-LexA were perfused with low osmolality solution. Again, strong calcium responses were observed only in the SEZ neurons labeled by nan-Gal4 (Figures 4D and S3). Moreover, Nan is necessary for the osmolality responses, as calcium responses to low osmolality were significantly decreased in a nan36a mutant background and were rescued with a UAS-nan transgene (Kim et al., 2003) (Figures 4E and 4F).

See also Figure S3 showing quantification of whole brain responses.

**Figure 4. Nanchung SEZ Neurons Respond to Osmolality**

(A) nan-Gal4, UAS-GCaMP6s expression in brain (left) and example ΔF/F heat-map of the same brain (right) upon an extracellular osmolality decrease of 200 mOsm/kg. Scale bar, 50 μm.

(B) ΔF/F traces (mean ± SEM) for osmolality decreases of 25, 50, 100, and 200 mOsm/kg (n = 9–12 brains/condition).

(C) Maximum ΔF/F responses (mean ± SEM) for osmolality changes. n = 7–12 brains/condition, one-way ANOVA, Dunnet’s post hoc to mock (0 mOsm change) ***p < 0.01, **p < 0.001, data from (B) plus additional osmolalities.

(D) Top, left: nSyb-LexA, lexAop-GCaMP6s expression in brain. Top, right: ΔF/F heat-map to an osmolality decrease of 200 mOsm/kg in the same brain. Bottom, left: ΔF/F response (green) overlaid on nSyb-LexA, lexAop-GCaMP6s expression (gray). Bottom, right: ΔF/F response (green) overlaid on nSyb-LexA, lexAop-GCaMP6s (gray) and nan-Gal4 expression (red) in same brain. Scale bar, 50 μm.

(E) ΔF/F traces upon osmolality decreases of 200 mOsm/Kg in nan36a mutant or wild-type (WT) flies. n = 28–33. Data are mean ± SEM.

(F) ΔF/F graphs for WT, mutant, and rescue flies (UAS-nan; nan36a). n = 33, 28, and 12. One-way ANOVA, Tukey post hoc, *p < 0.05, **p < 0.01, data from (E) plus rescue. Data are mean ± SEM.

(G) ΔF/F traces to 200 mOsm/Kg osmolality decreases in flies expressing UAS-GCaMP6s (green, n = 9 brains) or UAS-ArcLight (magenta, n = 11 brains) in ISNs. Data are mean ± SEM.

(H) ΔF/F graphs for GCaMP6s and ArcLight from (F). One-sample t tests for difference from theoretical mean of 0.0, *p < 0.05, ***p < 0.001. Data are mean ± SEM.
These data indicate that nan SEZ neurons are uniquely capable of responding to low extracellular osmolality with calcium increases. Because GCaMP6s preferentially reports calcium increases over decreases, we used the genetically encoded voltage sensor ArcLight (Cao et al., 2013) to test whether nan SEZ neuron activity is bidirectionally regulated by osmolality changes. Consistent with GCaMP6s imaging, decreasing extracellular osmolality decreased ArcLight fluorescence, indicating depolarization. In addition, we found that increasing extracellular osmolality significantly increased ArcLight fluorescence in these neurons, indicating hyperpolarization (Figures 4G and 4H).

Taken together, these data suggest that nan SEZ neurons report bidirectional extracellular osmolality changes in the brain. The ISNs Express nanchung and AKHR and Respond to Osmolality and AKH

During the course of these studies, we noted that the osmo-sensitive nan SEZ neurons had a similar spatial location and morphology as the ISNs that express AKHR, suggesting that they might be the same neurons. We tested this by examining overlap between nan-Gal4 neurons and the ISNs, marked by R34G02-LexA, and indeed found nan-Gal4 labels the ISNs as well as two additional SEZ neurons per hemisphere (Figure 5A). Consistent with this overlap, the ISNs responded to low osmolality and to AKH (Figures 5B and 5C). Responses were detected in neurites and cell bodies (Figures S3C and S3D). The AKHR-negative, Nan-positive neurons did not respond to osmolality or contribute to consumption behavior (Figure S4), arguing that the ISNs are a unique class of neurons that respond to osmolality and AKH. Thus, two screens for neurons regulating different homeostatic behaviors independently identified the ISNs.

ISNs are responsive to both osmolality and the hormone AKH. To examine how ISNs might integrate these two signals, we first asked whether Nan might be required for ISNs to sense AKH and whether AKHR might be required for ISNs to sense osmolality. We therefore monitored osmolality responses in AKHR mutants and AKH responses in nan mutants (Figure 5C). By calcium imaging, loss of AKHR did not affect the ability of ISNs to respond to osmolality, nor did loss of Nan affect the AKH response. All imaging experiments were performed under controlled osmolality and AKH conditions, as monitoring GCaMP activity by necessity requires removing cuticle and exposing the brain to artificial hemolymph. Thus, these experiments indicate that each input increases ISNs activity via an independent molecular mechanism, but do not directly monitor interactions between ISN inputs.

We therefore asked whether ISN activity resulting from changes in one input, osmolality, might affect the ability of ISNs to respond to another input, AKH. To test this, we monitored calcium responses to AKH in ISNs of brains perfused with high or low extracellular osmolality. We found that high extracellular osmolality significantly reduced AKH responses by an average of 70% (Figures 5D and 5E). These results argue that ISNs sense extracellular AKH and osmolality via independent molecular mechanisms, AKHR and Nanchung, but that these two inputs regulate a common output, ISN activity.

Starvation Reduces Drosophila Hemolymph Osmolality

AKH levels increase with starvation and activate ISNs, consistent with its action as a hunger signal that drives feeding. Low extracellular osmolality also activates ISNs, suggesting that low osmolality might also act as an internal signal of nutrient deprivation. Flies starved for 1 day have ~75% lower hemolymph sugar
levels (Na et al., 2013), and we hypothesized that this reduction in sugar levels might reduce hemolymph osmolality. To test this, we measured the hemolymph osmolality of single fed or starved flies with a temperature gradient osmometer (Arav and Rubinsky, 1994) (Figure 6A).

To confirm that we could detect physiological changes in osmolality, we measured hemolymph osmolality of single flies placed in dry (<20% relative humidity [RH]) or humid (>80% RH) environments for 6–8 hr. Consistent with previous studies (Albers and Bradley, 2004), desiccation increased hemolymph osmolality by an average of 55 mOsm/kg. In addition, hemolymph osmolality of desiccated flies returned to control levels 5 min after water consumption (Figure 6B).

To test the effect of starvation on hemolymph osmolality, hemolymph was collected from single flies that were either fed or starved for 24 hr with access to water. We found that hemolymph osmolality of starved flies was lower than that of well-fed animals by ∼30 mOsm/kg (Figure 6C). Importantly, physiological changes in osmolality of the magnitude observed following starvation were sufficient to elicit responses from ISNs in imaging preparations (Figures 6D and 6E). Thus, both low extracellular osmolality and increased AKH abundance may be starvation signals that increase ISN activity and promote feeding.

ISNs Oppositely Regulate Sugar and Water Consumption

Taken together, our data suggest a model in which ISNs are sensitive to internal signals for both water and sugar abundance and are sufficient to modulate both water and sugar consumption. To directly ask how ISN activity impacts sugar and water consumption, we drove dTRPA1 expression with ISN-Gal4, which exclusively labels ISNs. Consistent with our previous observations, ISN-Gal4, UAS-dTRPA1 flies avidly consumed sucrose upon TRP activation at 32°C, but not at 23°C. In contrast, these flies exhibited markedly reduced water consumption at 32°C but not at 23°C (Figure 7A).

To test the effect of inhibiting ISN activity on water and sugar consumption, we used ISN-Gal4 to selectively drive expression of RNAi against nSynaptobrevin (nSyb), which is required for synaptic transmission. Flies expressing nSyb RNAi in ISNs increased water consumption relative to controls by almost 2-fold. In contrast, expression of nSyb RNAi in ISNs decreased sucrose intake by an average of 44% (Figure 7B). Thus, neural activity in ISNs oppositely regulates sugar and water consumption behaviors: increased ISN activity both promotes sugar and restricts water consumption, whereas decreased activity promotes water and restricts sugar consumption.

To ask whether nanchung and AKHR are important for the ability of ISNs to regulate food and water intake, we expressed nan RNAi or AKHR RNAi selectively in ISNs and examined the effect
on water and sucrose consumption. Flies expressing either of two independent nan RNAi constructs in ISNs increased water consumption and decreased sucrose consumption relative to controls, consistent with phenotypes observed when silencing ISNs (Figures 7C and S5A–S5C). Flies expressing nan RNAi under the control of nSyb-Gal4 and nan-Gal4 drivers produced the same reciprocal effects on water and sucrose consumption (Figures S5D and S5E). Like flies expressing nan RNAi, flies expressing AKHR RNAi in ISNs also increased water intake and decreased sucrose intake relative to controls (Figure 7D). The nan and AKHR RNAi data are consistent with the notion that both AKHR and osmolality contribute to the activation of ISNs. The loss of either signal decreased activity in ISNs, leading to loss-of-function phenotypes similar to those observed when silencing ISNs with nSyb RNAi.

The finding that the ISNs sense signals of hunger and thirst, AKH, and osmolality, and oppositely regulate sugar and water consumption, suggests that hunger and thirst may be competing drives under some conditions. To test this, we examined whether there were consumption differences in flies with competing needs. Under conditions when flies were not thirsty, there was no drive to consume water and this was not affected by starvation state (Figure 7E). However, under conditions when flies were mildly starved (2 hr), we found that sucrose consumption was reduced in thirsty flies compared to water-sated flies (Figure 7E). These data argue that under conditions of competing needs, there is a balance between water and sucrose consumption.

As the relative weight of these needs changes, the balance would be predicted to change as well.

**DISCUSSION**

In this study, we uncover a neural mechanism that coordinates two essential homeostatic behaviors: sugar and water consumption. This coordination is achieved by two neurons per SEZ hemisphere of the Drosophila brain, the ISNs, which are sensitive to internal signals for both hunger and thirst and whose activity oppositely regulates sugar and water consumption (Figure 7F). The antagonistic manner in which ISNs couple these behaviors suggests a regulatory principle by which animal nervous systems might promote internal osmotic and metabolic homeostasis.

**Four Neurons Oppositely Regulate Sugar and Water Consumption in Drosophila**

Low internal osmolality and high AKH are signals of water satiety and hunger, respectively. ISN activity increases both in the presence of low extracellular osmolality and AKH. We find that...
increasing ISN activity promotes sugar consumption and reduces water consumption. Conversely, high internal osmolality and low AKH are signals of thirst and food satiety. ISN activity decreases and AKH responses are reduced in the presence of high extracellular osmolality or insulin. Decreasing ISN activity increases water consumption and reduces sugar consumption.

How do ISNs achieve opposite regulation of a single behavior, consumption, in a manner that depends on the substance being consumed? One possibility is that the downstream targets of ISNs include interneurons involved in the behavioral response to water and sugar taste. This model predicts that increased ISN activity promotes the ability of sugar taste interneurons to drive consumption while inhibiting the ability of water taste interneurons to do so. It may be possible to test hypotheses about the neural circuits in which ISNs participate through the use of large-scale calcium imaging.

Molecules for Sensing Internal Hunger and Thirst Cues

ISNs regulate sugar and water consumption in a manner that appropriately reflects internal hunger and thirst states. Here, we show that two genes, AKHR and nanchung, are expressed in ISNs and function to confer sensitivity to these states.

AKHR is a G protein coupled receptor expressed in the fat body and the brain that has been well characterized in the context of insect metabolic regulation (Bharucha et al., 2008; Candy, 2002). The ligand for this receptor, AKH, is secreted into the hemolymph by specialized neurosecretory cells in the corpus cardium (Kim and Ruifilson, 2004), where it acts under conditions of food deprivation. Here, we identify a role for AKH in regulating the activity of four interneurons in the SEZ, the ISNs, and we show that this activity promotes sugar consumption. AKH abundance in the hemolymph therefore promotes feeding via the ISNs. Manipulating AKHR exclusively in the ISNs provided a means to separate the metabolic and neural effects of AKH, uncovering a role for AKH in the nervous system.

Sensors for internal hemolymph osmolality have not previously been described. Here, we find that the non-selective cation channel Nanchung is expressed in ISNs and is required for their responses to low osmolality. Although we do not know if Nan is the direct osmosensor in ISNs, previous studies found that Nan confers low osmolality responses when expressed in heterologous cells (Kim et al., 2003), consistent with this notion. Nan family members of the TRPV4 family have been shown to participate in osmosensation in insect and mammalian cells (Colbert et al., 1997; Liedtke and Friedman, 2003; Liedtke et al., 2003), suggesting an ancient and conserved function. Nanchung participates in sensory detection of mechanical stimuli in Drosophila, including proprioception, audition, and low humidity sensing (Gong et al., 2004; Kim et al., 2003; Liu et al., 2007; Zhang et al., 2013). It is interesting that the same molecule that is involved in external sensory detection of mechanical stimuli also participates in internal detection of osmolality, a mechanical stimulus. Similar molecular re-tooling has recently been described for the GR43a gustatory receptor, which acts as a sensory receptor to monitor fructose in the environment and as an internal sensor monitoring circulating fructose levels in brain hemolymph (Miyamoto et al., 2012).

In the mammalian brain, osmosensitive neurons are generally found in areas that lack a blood-brain barrier. The blood-brain barrier of Drosophila expresses multiple aquaporins and may potentially regulate hemolymph osmolality (Limmer et al., 2014). Whether changes in hemolymph osmolality are regulated by the blood-brain barrier to impact ISN activity is an interesting question for future study.

Coupling of Sugar and Water Consumption Behaviors as a Mechanism for Homeostasis

ISNs oppositely regulate the behavioral responses to hunger and thirst states. How might this type of coordination be adaptive? One possibility is suggested by the fact that sugar and water consumption perturb internal osmotic homeostasis in opposite directions. In Drosophila and mammals, sugar consumption leads to increased blood-sugar levels and increased blood osmolality. Conversely, water consumption leads to lowered blood osmolality. Our studies show that ISNs are sensitive to extracellular osmolality and that they oppositely regulate sugar and water consumption. Under high osmotic conditions, decreased ISN activity promotes water consumption, reducing internal osmolality. Under low osmotic conditions, increased ISN activity promotes sucrose consumption, increasing internal osmolality. Thus, ISNs may monitor internal osmolality to reciprocally regulate sugar and water consumption to restore homeostasis.

Reciprocal regulation of food and water consumption has been reported in both classical and recent rodent studies. For example, increasing blood osmolality promotes water consumption and inhibits food consumption in rats, whereas decreasing osmolality has the opposite effect (Gutman and Krausz, 1969). In addition, ghrelin, a key internal signal for hunger in mammals, is sufficient not only to promote feeding but also to inhibit water consumption in rats (Mietlicki et al., 2009). Thus, vertebrates and invertebrates may share mechanisms for coupling water and sugar consumption in a manner that promotes homeostasis. In Drosophila, the convergence of internal signals onto the ISNs provides a mechanism to weigh homeostatic deviations and drive consumption to restore balance.

Other neurons in the Drosophila brain process homeostatic needs for water and sugar separately. For example, water reward and sugar reward are processed by different subsets of mushroom body input neurons, likely independent of gustatory sensory activation (Burke et al., 2012; Lin et al., 2014; Liu et al., 2012). Neuropeptide F, small Neuropeptide F, and dopamine are all signals of nutrient deprivation that promote nutrient intake (Hergarden et al., 2012; Inagaki et al., 2012, 2014; Lee et al., 2004; Marella et al., 2012). Circulating glucose and fructose in the hemolymph also report the nutritional state and alter feeding behavior by direct activation of a few central neurons (Miyamoto et al., 2012; Dus et al., 2015). The ISNs are unique in that they detect multiple internal state signals and use this information to weigh competing needs. In addition to parallel, independent pathways for eating and drinking, this study demonstrates the existence of a pathway that couples these drives.

**EXPERIMENTAL PROCEDURES**

Additional details are available in the Supplemental Experimental Procedures.
Blue Dye Consumption Experiments
Flies (15–20/vial) were transferred from food to filter paper soaked in 200 mM sucrose and blue dye. After feeding animals were scored a 0 (no dye in abdomen), 1 (less than half of the abdomen was blue), or 2 (half or more of the abdomen contained dye). The scores of all flies in one vial were averaged and considered one trial.

Proboscis Extension Response Assays
Proboscis extension response (PER) was performed as described (Marella et al., 2012), except that each animal was considered a data point and was categorized as responding 0, 1, 2, or 3 times.

Temporal Consumption Assays
Assays were performed as described (Pool et al., 2014). Animals were presented with a taste stimulus ten times and total consumption time was monitored. To generate thirsty flies, flies were placed in a sealed chamber with water taste in Drosophila melanogaster during dehydration and rehydration. J. Exp. Biol. 207, 2313–2321.

Hemolymph Osmolality Measurements
An osmometer was assembled and used as described (Arav and Rubinsky, 1994), with minor modifications detailed in the Supplemental Experimental Procedures.

Electrophysiology
Extracellular recordings were performed in live animals as described (Pool et al., 2014).

Statistical Analyses
Student’s t-test, with Sidak correction for multiple comparisons, was used to compare two groups. ANOVA followed by Tukey’s post hoc test was used to compare three or more groups. ANOVA followed by Dunnet’s post hoc test was used to compare multiple responses of varying stimuli.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Supplemental Experimental Procedures, five figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2016.06.046.

REFERENCES


Figure S1. Neurons that Promote Sucrose Consumption, Related to Figure 1
(A) Tshirt-Gal80 (Tsh-Gal80) eliminated Gal4 expression in the ventral nerve cord but did not eliminate increased sucrose consumption upon dTRPA1 activation of 954-Gal4 neurons. n = 5-6 vials, 12-20 flies/vial, mean ± SEM, one-way ANOVA, Tukey’s Post Hoc, ***p < 0.001.
(B) 149-Gal80 eliminated Gal4 expression in SEZ neurons and eliminated increased sucrose consumption upon dTRPA1 activation of 954-Gal4 neurons. n = 5-6 vials, 12-20 flies/vial, mean ± SEM, one-way ANOVA, Tukey’s Post Hoc, ***p < 0.001.
(C) Activation of 954-Gal4, R34G02, or VT011155 neurons upon dTRPA1-mediated depolarization at 30°C increased proboscis extension to sucrose concentrations. n = 39-48, mean ± SEM, ***p < 0.001 t test (22 versus 30°C), Holm-Sidak correction. WT = isoD1 (top), w1118 (others).
(D) 954-Gal4, AKHR-Gal4, and VT011155-Gal4 nuclear expression in the SEZ. There are four neurons in each 954-Gal4 cluster, two neurons in each AKHR-Gal4 cluster, and two neurons in each VT011155-Gal4 cluster.
(E) (top) 954-Gal4, UAS-mCD8::RFP and R34G02-LexA, lexAop-mCD8::GFP flies showed co-expression of reporters in ISNs. (middle) R34G02-Gal4, UAS-mCD8::tdTomato and R34G02-LexA, lexAop-mCD2::GFP showed co-expression of reporters in ISNs. (bottom) VT011155, UAS-mCD8::tdTomato and R34G02-LexA, lexAop-mCD2::GFP flies showed co-expression of reporters in ISNs. Scale 50 μm.
(F) DenMark and Synaptotagmin expression in ISN neurites, showing overlapping pre- and post-synaptic sites in the dorsal SEZ. Scale 20 μm.
Figure S2. ISNs Are Not Taste Responsive, Related to Figure 2

(A) (left) Expression of mCD8:tdTomato in ISNs and ppk28 positive water-taste projections that also express GCaMP6s. (middle) Example ΔF/F heat-map of GCaMP6s increase in brain from left panel upon stimulation of the proboscis with water. Solid ROI: water taste projections. Dashed ROI: ISN cell bodies. (right) ΔF/F traces (mean ± SEM) for water taste stimuli for water taste projections (solid line) or ISN cell bodies (dashed line). n = 6 brains. Scale 50 μm. One sample t tests for difference from theoretical mean of 0.0, water sensory **p < 0.01, ISNs ns.

(B) (left) Expression of GCaMP5G in ISNs and Gr64f positive sugar-taste projections. (middle) Example ΔF/F heat-map of fluorescence increase in brain from left panel upon stimulation of the proboscis with 1M sucrose. Solid ROI: sugar taste projections. Dashed ROI: ISN cell bodies. (right) ΔF/F traces (mean ± SEM) for sugar taste stimuli for sugar taste projections (solid line) or ISN cell bodies (dashed line). n = 6 brains. One sample t tests for difference from theoretical mean of 0.0, sugar sensory ***p < 0.001, ISNs ns.

(C) (left) Expression of GCaMP5G in ISNs and Gr66a positive bitter-taste projections. (middle) Example ΔF/F heat-map of fluorescence increase in brain from left panel upon stimulation of the proboscis with 10 mM dentonium. Solid ROI: bitter taste projections. Dashed ROI: ISN cell bodies. (right) ΔF/F traces (mean ± SEM) for bitter taste stimuli for bitter taste projections (solid line) or ISN cell bodies (dashed line). n = 6 brains. One sample t tests for difference from theoretical mean of 0.0, bitter sensory **p < 0.01, ISNs ns.
Figure S3. Quantification of Responses to Osmolality Decreases, Related to Figure 4

(A) Example response to 200 mOsm/kg decrease in nSyb-LexA, lexAop-GCaMP6s; nan-Gal4, UAS-cd8tdTomato Brain. (Top) brain tiled with 7,225 ROIs color coded by the number of standard deviations each ROI’s ΔF/F value is from the mean of all ΔF/F values (z-score). (Middle) Raw GCaMP fluorescence at each time point. (Bottom) Raw cd8tdTomato fluorescence at each time point, showing only the left nan+ SEZ cluster is present in this brain. One or both ISN clusters are often lost during dissection because ISN cell bodies sit in a nerve that needs to be cut to separate the brain from the cuticle. Perfusion channels were switched at t = 0. In these experiments, 6 s were required to replace high osmolality with low osmolality solution inside the imaging chamber. Cd8tdTomato expression was used to trace nan+ SEZ cluster outline (solid line), which includes two ISNs and two additional cells (see Figure S4). Scale 100 μm.

(B) Maximum z-scores for each of 7,225 ROIs in four replicate brains. Cd8tdTomato expression was used to trace ISN outline (indicated by red outline). Brain regions were traced using neuropil morphology. Brain regions correspond to the pars intercerebralis (a), right SEZ (b), left SEZ (c), right antennal lobe (d), left antennal lobe (e), and higher brain (f–i). One way ANOVAs, tukey’s post hoc, ***p < 0.0001. Brain #1 is the brain in (A), above. Brain #4 is the brain in Figure 4D.

(C) (Left) GCaMP6s expression in ISNs, with ROI drawn around left neurite. (Middle) Example ΔF/F heat-map of GCaMP6s increase in cell body and neurite of ISNs in response to 200 mOsm/kg decrease. Figure 5B is a zoomed-in image of the left ISN in this image.

(D) ΔF/F trace (mean ± SEM) for neurite responses to 200 mOsm/kg decrease (n = 14 brains).
Figure S4. Non-ISN, nan+ Neurons Do Not Respond to Osmolality or Affect Consumption Behavior, Related to Figure 5

(A) Left: nuclear expression of nan-Gal4. Scale 50 um. Middle: Zoom of left panel showing four nan-Gal4 nuclei in a single SEZ cluster. Scale 20 um. Right: Gal80 excludes expression of nan-Gal4 in ISNs. Scale 20 μm.

(B) ΔF/F traces (mean ± SEM) to 200 mOsm/Kg osmolality decreases in flies expressing UAS-GCaMP6s in nan positive SEZ neurons in the presence (R34G02LexA, nan-Gal4) or absence (R34G02LexA, nan-Gal4; LexAop-Gal80) of ISNs.

(C) Maximum ΔF/F (mean ± SEM) responses of traces in (C). n = 7 for each condition. t test *p < 0.05.

(D) ISNs are required for water consumption phenotype of nan-Gal4>UAS-nanRNAi flies. n = 49–63, one-way ANOVA, Tukey’s Post Hoc, ***p < 0.001.

(E) ISNs are required for sugar consumption phenotype of nan-Gal4>UAS-nanRNAi flies. n = 49–59, one-way ANOVA, Tukey’s Post Hoc, ***p < 0.001.
Figure S5. Behavioral Phenotypes with nan RNAi, Related to Figure 7

(A) nan-RNAi (BSC# 31674) phenotypes with nSyb-Gal4 driver. Desiccated (des.), hydrated (hyd.). n = 46-65 flies, one-way ANOVA, Tukey’s Post Hoc, **p < 0.01, ***p < 0.001.

(B) nan-RNAi (BSC# 31674) phenotypes with nan-Gal4 driver. n = 46-65 flies, one-way ANOVA, Tukey’s Post Hoc, **p < 0.01, ***p < 0.001.

(C) nan-RNAi (BSC# 31674) phenotypes with ISN-Gal4 driver. n = 37-73 flies, one-way ANOVA, Tukey’s Post Hoc, ***p < 0.001.

(D) nan-RNAi (BSC# 31925) phenotypes with nSyb-Gal4 driver. n = 48-67 flies, one-way ANOVA, Tukey’s Post Hoc, *p < 0.05, **p < 0.01, ***p < 0.001.

(E) nan-RNAi (BSC# 31925) phenotypes with nan-Gal4 driver. n = 48-73 flies, one-way ANOVA, Tukey’s Post Hoc, **p < 0.01, ***p < 0.001.
Supplemental Information

Coupled Sensing of Hunger and Thirst Signals
Balances Sugar and Water Consumption

Nicholas Jourjine, Brendan C. Mullaney, Kevin Mann, and Kristin Scott
Supplemental Information

Extended Experimental Procedures

Blue Dye Consumption experiments
15-20 female flies were transferred to vials with 2.5cm filter paper (Whatman) soaked with 300ml of 200mM sucrose with 0.25mg/mL blue dye (Erioglaucine, Sigma). The cotton flug was soaked with water. In the initial screen, flies were allowed to feed for 30 min, and the sucrose soaked filter paper was at the vial bottom. For subsequent experiments, flies were allowed to feed for 2 hrs in inverted vials with the sucrose paper at the vial top. For 30°C experiments, vials were pre-warmed 20 min at 30°C.

Temporal Consumption Assays
Flies were mounted on glass slides with nail polish, then placed in a humidified chamber for 2 hrs. Each animal was presented a tasta tant 10 times on the proboscis and forelegs, and consumption time was recorded. Consumption time correlates with consumption volume.

RNAsi crosses used for temporal consumption assays were maintained at 25°C, 65% relative humidity (RH). Flies were aged 2-7 days (initial RNAi screen) or 4-7 days (other assays). Prior to testing, flies were provided water and allowed to drink to satiety, unless the experimental tasta tant was water. However, in tests for a reduction in sucrose consumption, flies were not provided water prior to testing to exclude interactions between sucrose consumption and water consumption.

For temporal consumption assays using thirsty flies, flies were mounted as described above and placed in a sealed chamber with ~250g CaSO₄ (Drierite, stock# 23001) for 2 hours, unless otherwise noted. Each animal was provided distilled water 10 times and cumulative drinking time was recorded.

For dTRPA1 experiments, crosses to UAS-dTRPA1; UAS-dTRPA1/TM2 flies were raised at 20°C, 60% RH. Flies with two copies of UAS-dTRPA1 were aged 2-4 or 4-7 days. Flies were heated on a Peltier device for five min before testing.

Behavioral experiments using RNAsi
For nan RNAsi experiments, RNAsi-only controls were performed subsequent to Gal4-only controls and experimental genotypes. Data from the same +/-nan RNAsi (BSC# 31925) controls is shown in Figure 3B, 7C, and S7D-E. Data from the same +/-nan RNAsi (BSC# 31674) control is shown in Figure S7A-C.

Immunohistochemistry
The primary antibodies were rabbit anti-GFP (Invitrogen, 1:1000), mouse anti-GFP (Sigma 1:1000), rabbit anti-RFP (Biovision, 1:500), mouse anti-Bruchpilot (nc82) (Developmental Studies Hybridoma Bank, 1:500). For double labeling experiments, R34G02-LexA (BSC#54138) and R34G02-LexA (made for this study) were used. R34G02-LexA (BSC#54138) was used in Figure 5A, S1C (VT011155 double label), S5A, and S5B. The R34G02-LexA made for this study was used in for Figure 2A and the 954-Gal4 double label in Figure S1C.

Calcium and voltage imaging
For experiments involving AKH, AHL with hormone or KCl was applied as indicated. For serial applications, the protocol was 0-300 sec AHL, 300-320 sec 316pM AKH in AHL, 320-620 sec drug treatment (TTX, insulin, or insulin + TTX), 620-640 sec 316pM AKH in AHL, 640-900 sec AHL. Single-plane imaging was performed with a 20x water immersion objective on a Zeiss PASCAL confocal microscope, with an open pinhole. Images were collected at 1 Hz.

For GCaMP6s imaging of osmolality responses, brains were dissected, pinned, and perfused as above. Brains were imaged on a 3i spinning disc confocal microscope with a 20x water immersion objective at ~0.3 Hz. For ArcLight experiments, imaging rate was ~0.8 Hz, analysis was performed on max-z projections of 10 sections per time point, 0.8-1.4 mm sections. For GCaMP6s imaging, analysis was done on max-z projections of 17 imaging sections, 0.8-1.4 mm sections. %ΔF/F = 100%*((Fₐ-F₀)/F₀), where F₀ is the mean fluorescence of 5-15 time points prior to stimulus onset and Fₐ is the fluorescence at each time point. Max ΔF/F = F₉₅ – F₀, where F₉₅ is the maximum ΔF/F observed during the stimulus. For ArcLight responses following osmolality decreases that occur subsequent to a prior increase (figure 4G, H), minimum ΔF/F was calculated as F₉₅ – F₀, where F₉₅ is the minimum ΔF/F observed in a window beginning 6 s following osmolality decrease and ending 38 s thereafter.

Hemolymph osmolality measurements
The osmometer was placed in a glass-lidded box with desiccant (Drierite) to prevent condensation. Coolant was ethanol chilled to -60°C. Standards and samples were loaded into square capillaries (0.05mm x 0.05 mm, Vitro Dynamics). For each measurement, 4 standards were included: distilled water, 150mM, 210mM, and 269mM NaCl, as well as hemolymph from four individual flies. Osmolality measurements from three standards (water, 150mM NaCl and 269mM NaCl) were used to fit a second-order polynomial curve, to calculate osmolality of experimental
samples. The difference between known and measured osmolality of the fourth standard (210mM NaCl) was used to estimate error. An Olympus SZX16 microscope with a mounted camera (Point Grey Firefly MV) captured images of the ice-liquid interface. The distance from the water standard to each NaCl standard and experimental sample was measured using Adobe Photoshop.

To collect hemolymph of single flies, non-anesthetized flies were loaded into a pipette tip and positive pressure was applied, such that the head protruded. Removing head cuticle including the antenna caused hemolymph to protrude; this was collected in a capillary.

Flies were desiccated or hydrated in the same manner as in temporal consumption assays, except that treatment time was 6 hours instead of 2 hours. Also, flies desiccated or hydrated for hemolymph collection were allowed to walk freely in an empty vial capped with mesh cloth. Flies were starved in vials with wet flugs containing kimwipes soaked in water. In Figure 6C, flies were rehydrated by presentation of a water droplet in the same manner as in temporal consumption assays.

**Generation of R34G02-LexA**

A 1,066-bp genomic DNA fragment, containing the R34G02 tile from the FlyLight collection (Pfeiffer et al., 2012), was amplified using GAGGCTCTTTATGATCCCGTGGAGC and CGACGACACTCGCCACAACCCAAAG primers, and recombined into the pBPLexA::p65Uw plasmid (Pfeiffer et al., 2010).
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