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 tastant 10 times on the proboscis and forelegs, and consumption time was recorded. Consumption time correlates with consumption volume.

RNAi 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 tastant 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 $CaSO_4$ (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 RNAi

For *nan* RNAi experiments, RNAi-only controls were performed subsequent to Gal4-only controls and experimental genotypes. Data from the same +/*nan RNAi* (BSC# 31925) controls is shown in Figure 3B, 7C, and S7D-E. Data from the same +/*nan RNAi* (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 for in 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. $\%\Delta F/F = 100\%*((F_t-F_0)/F_0)$, where F_0 is the mean fluorescence of 5-15 time points prior to stimulus onset and F_t is the fluorescence at each timepoint. Max $\Delta F/F = F_{max} - F_0$, where F_{max} is the maximum $\Delta F/F$ observed during the stimulus. For Arclight responses following 30 mOsm/Kg decreases or GCaMP6s responses following 30 mOsm/Kg increases, minimum $\Delta F/F$ was calculated as $F_{min} - F_0$, where F_{min} is the minimum $\Delta F/F$ observed during the stimulus. For ArcLight responses following osmolality decreases that occur subsequent to a prior increase (figure 4G, H), minimum $\Delta F/F$ was calculated as $F_{min} - F_0$, where F_{min} is the minimum $\Delta F/F$ 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 GAGGCTCTTTATGATCCCGTGGACG and CGACGACACTCGCCACAACCCAAAG primers, and recombined into the pBPLexA::p65Uw plasmid (Pfeiffer et al., 2010).

Table S1: Transgenic flies used in this study, related to Figure 1

Figure	Genotypes Used
1A	*Clandinin-Gal4 x UAS-dTRPA1, UAS-dTRPA1*
1B	*954-Gal4/+; UAS-mCD8::GFP/+; +/+*
1C	*954-Gal4/+; UAS-dTRPA1/+; UAS-dTRPA1/+* (*isoD*^{1} background) *UAS-dTRPA1/+; UAS-dTRPA1/+* (*isoD*^{1} background)
1D	*+/UAS-mCD8::GFP; R34G02/+*
1E	*+/UAS-dTRPA1; R34G02/UAS-dTRPA1* (w^{1118} background) *UAS-dTRPA1/+; UAS-dTRPA1/+* (w^{1118} background)
1F	*+/UAS-mCD8::GFP; VT011155/+*
1G	*+/UAS-dTRPA1; VT011155/UAS-dTRPA1* (w^{1118} background) *UAS-dTRPA1/+; UAS-dTRPA1/+* (w^{1118} background)
2A (left panel)	*+/UAS-mCD8::GFP; +/AKHR-Gal4*
2A (other panels)	*+/lexAop-mCD8::GFP, UAS-mCD8::RFP; R34G02-LexA/+; AKHR-Gal4/+*
2B, C	*954-Gal4;+/+; UAS-GCaMP5G*
2D, E	AKH response in AKHR mutant: *954-Gal4; AKHR*^{null}*/AKHR*^{null}*; UAS-GCaMP5G* Other panels: *954-Gal4;+/+; UAS-GCaMP5G*
2F, G	*954-Gal4;+/+; UAS-GCaMP5G*
2H, I	*954-Gal4/+; UAS-mCD8::GFP/+*
3A	*nSyb-Gal4/+; UAS-RNAi/UAS-dcr* (experimental for each RNAi candidate) *+/+; UAS-RNAi/UAS-dcr* (control for each RNAi candidate)
3B	WT: w^{1118} Otherwise as shown in figure
3C	As shown in figure
3D	*nan-Gal4/UAS-mCD8::GFP; +/+*
4A-C	*nan-Gal4; UAS-GCaMP6s/+*
4D	*nan-Gal4, UAS-mCD8::tdTomato/+, nSyb-LexA, lexAop-GCaMP6s/+*
4E	*nan*^{WT}: *nan-Gal4, UAS-GCaMP6s/+; +/+* *nan*^{36a}: *nan-Gal4, UAS-GCaMP6s/+; nan*^{36a}
4F	*nan*^{WT}: *nan-Gal4, UAS-GCaMP6s/+;* *nan*^{36a}: *nan-Gal4, UAS-GCaMP6s/+; nan*^{36a} *UAS-nan*: *nan-Gal4, UAS-GCaMP6s/UAS-nan; nan*^{36a}
4G-H	GCaMP: *+/+; VT011155, UAS-GCaMP6s/+* Arc.: *UAS-ArcLight/+; VT011155/+*
5A	*nan-Gal4, UAS-mCD8::tdTomato/R34G02-LexA (BSC#54138); lexAop-mCD2::GFP/+*
5B	Left: *+/+; VT011155, UAS-GCaMP6s/+* Right: *nan-Gal4, UAS-GCaMP6s/+; +/+*
5C	Left: *+/+; VT011155, UAS-GCaMP6s/+* Middle: *nan-Gal4, UAS-GCaMP6s/+; +/+* *nan-Gal4, UAS-GCaMP6s/+; nan*^{36a} Right: *954-Gal4; +/+; UAS-GCaMP5G* *954-Gal4; AKHR*^{null}*; UAS-GCaMP5G*
5D-E	*+/+; VT011155, UAS-GCaMP6s/+*
6B, C	*CantonS*
6D, E	*UAS-cd8tdTomato/+; VT011155,UAS-ArcLight/+* *UAS-cd8tdTomato/+; VT011155, UAS-GCaMP6s/+*
7A-D	As shown in figure
7E	*CantonS*
S1A (top two panels)	*954-Gal4/+*; *UAS-dTRPA1/Tsh-Gal80*; *UAS-dTRPA1/ UAS-mCD8::GFP*
S1A	*UAS-dTRPA1/+; UAS-dTRPA1/UAS-mCD8::GFP* (*isoD*^{1} background)

(bottom panel, left to right)	*954-Gal4/+; UAS-dTRPA1/+; UAS-dTRPA1/+* (*isoD*^{1} background) *Tsh-Gal80/UAS-dTRPA1; UAS-mCD8::GFP/UAS-dTRPA1* *954-Gal4/+*; *UAS-dTRPA1/Tsh-Gal80*; *UAS-dTRPA1/UAS-mCD8::GFP*
S1B (top two panels)	*954-Gal4/+; UAS-mCD8::GFP/+; 149-Gal80/+*
S1B (bottom panel, left to right)	*UAS-dTRPA1/+*; *UAS-dTRPA1/+* (*isoD*^{1} background) *954-Gal4/+; UAS-dTRPA1/+; UAS-dTRPA1/+* (*isoD*^{1} background) *UAS-dTRPA1/+; UAS-dTRPA1/149-Gal80* *954-Gal4/+; UAS-dTRPA1/+; UAS-dTRPA1/149-Gal80*
S1C (left to right)	*954-Gal4/+; +/+; UAS-redStinger/+* *R34G02-Gal4/+; UAS-redStinger/+* *+/+; VT011155-Gal4/UAS-redStinger*
S1D	Top three panels: *954-Gal4/lexAop-mCD8::GFP, UAS-mCD8::RFP; R34G02-LexA/+; +/+* Middle three panels: *+/lexAop-mCD8::GFP, UAS-mCD8::RFP; R34G02-LexA/R34G02-Gal4; +/+* Bottom three panels: *UAS-mCD8::tdTomato/R34G02-LexA (BSC#54138); VT011155/lexAop-mCD2::GFP*
S1E (top panel)	WT x *dTRPA1*: *UAS-dTRPA1/+*; *UAS-dTRPA1/+* (*isoD*^{1} background) *954-Gal4* x *dTRPA1*: *954-Gal4; UAS-dTRPA1/+; UAS-dTRPA1/+* (*isoD*^{1} background)
S1E (middle panel)	WT x *dTRPA1*: *UAS-dTRPA1/+*; *UAS-dTRPA1/+* (*w*^{1118} background) *R34G02* x *dTRPA1*: *UAS-dTRPA1/+; UAS-dTRPA1/R34G02* (*w*^{1118} background)
S1E (bottom panel)	WT x *dTRPA1*: *UAS-dTRPA1/+*; *UAS-dTRPA1/+* (*w*^{1118} background) *VT011155* x *dTRPA1*: *UAS-dTRPA1/+; UAS-dTRPA1/VT011155* (*w*^{1118} background)
S1F	*+/UAS-DenMark, UAS-eGFP::synaptotagmin*; *VT011155/ UAS-DenMark, UAS-eGFP::synaptotagmin*
S2A	*UAS-GCaMP6s, UAS-mCD8::tdTomato/+; ppk28-Gal4/VT011155*
S2B	*954-Gal4/+; Gr64f-Gal4/+; UAS-GCaMP5G/+*
S2C	*954-Gal4/+; Gr66a-Gal4/+; UAS-GCaMP5G/+*
S3A, B	*+/+*; *VT011155, UAS-GCaMP6s*
S3C-E	*nan-Gal4, UAS-mCD8::tdTomato/+, nSyb-LexA, lexAop-GCaMP6s/+*
S4A	*nan-Gal4, R34G02-LexA(BSC#54138)/+; +/UAS-RedStinger* *nan-Gal4, R34G02-LexA(BSC#54138)/+; LexAop-Gal80/UAS-RedStinger*
S4B, C	*nan-Gal4, R34G02-LexA(BSC#54138)/+; +/UAS-GCaMP6s* *nan-Gal4, R34G02-LexA(BSC#54138)/+; LexAop-Gal80/UAS-GCaMP6s*
S4D, E	As shown in figure.
S5	As shown in figure.