Supporting information

SI Materials and Methods

Fly stocks

The *w[*]; P{w[+mC]=GAL4-elav.L}3, y[1] w[*]; P{w[+mC]=NPF-GAL4.1}1, w[1118]; P{y[+t7.7] w[+mC]=GMR64B05-GAL4}attP2, w[*]; P{w[+mC]=ple-GAL4.F}3, w[1118]; P{y[+t7.7] w[+mC]=GMR64F05-GAL4}attP*2 and *w[1118]; P{y[+t7.7] w[+mC]=GMR64C11-GAL4}attP2* driver lines, the *w[*]; P{w[+mC]=UAS-Hsap\KCNJ2.EGFP}7, w[*]; P{y[+t7.7] w[+mC]=UAS-TrpA1(B).K}attP16* and *w[*]; P{y[+t7.7] w[+mC]=UAS-ReaChR}attP40* neural manipulation lines, as well as the *y1 v1; P{y[+t7.7] v[+t1.8]=TRiP.JF01338}attP2* and CCAP-R RNAi line (RNAi) (tested for functionality with elav-Gal4 (SI Appendix Fig. S4A)), were received from the Bloomington Stock Center. The *y, w; P{attP, y+, w3, KK113043}VIE-260B CCAP-R RNAi* line (RNAi2) (tested for functionality with *elav-GAL4* (Fig. S4A)), was received from the Vienna Drosophila RNAi Centre (VDRC). $CCAP^{exc7}$ null flies were a generous gift from Dr. John Ewer (1). All flies were crossed into the same w^{1118} genetic background before any experiments were performed.

Immunohistochemistry

For all staining related to peptide expression under starvation conditions, starvation was started so that flies were collected within 3 hours of lights on, to avoid any changes in peptide expression due to circadian rhythm. Male flies were beheaded and kept in 1X PBS for 15

minutes. The heads were then transferred to 1ml of 1X fix buffer and 4% formaldehyde overnight at 4 ^{o}C. After fixation the heads were transferred to silicon padded petri dish and brains were dissected in 1X PBS buffer under dissection microscope using fine forceps. Brain tissue was fixed in 4% PFA and 1X PBS for 2 hours. It was then blocked with 400 ul of 1% goat serum in PBL (Phosphate Buffer with Lysine) overnight and again with 5% goat serum for 1 hour. The brains were then treated with primary antibodies, anti-CCAP rabbit polyclonal (1:1000 dilution) (2), anti-NPF rabbit polyclonal (1:1000 dilution) (Biorbyt, Cambridge, United Kingdom) anti-GFP chicken polyclonal (1:200 dilution) (Sigma-Aldrich, Stockholm, Sweden) in 0.1M PBL containing 0.5% Triton and incubated on rotation at 4^{o}C. Following 18hr of incubation the brains were washed three times with 1% goat serum and then treated with secondary antibodies (ThermoFisher Scientific, MA, USA, goat anti-chicken Alexa Fluor 488 and goat anti-rabbit Alexa Fluor 594), diluted 1:2000 in 0.1M PBL, overnight. The tubes were covered with aluminum foil during the whole process. Tissues were washed with 1X PBS containing 0.5% Triton for three hours and then treated with DAPI for 15 minutes. After washing the DAPI with 1X PBS twice, the brains were mounted in Vectashield (Vector Laboratories, Peterborough, UK). Images were captured on a Zeiss LSM 510 confocal microscope and visualized using ImageJ.

CAFE Assay

A vial, 9 cm by 2 cm (height X diameter), containing 1% agarose (5cm high) to provide moisture and humidity for the flies, was used for this assay (3). A calibrated capillary glass tube (5 µl, VWR International) was filled with liquid food and 0.5% food-coloring dye. Mineral oil was used to prevent the liquid food from evaporating. The vial was covered with paraffin; a capillary

tube was inserted from the top through the paraffin. The experimental set up was kept at 25 ^{o}C and the initial and final food level in the capillary tube was marked to determine total food intake. Number of feeding bouts per fly was counted from the recording; average meal size was calculated by dividing the total food intake by the number of feeding bouts. Five 5-7 day old males per vial were used for this assay.

RNA purification, cDNA synthesis and qRT-PCR:

RNA

The phenol-chloroform method was used for RNA extraction from tissue samples. Fifty fly heads were homogenized with 800 µl TRIzol (Invitrogen, USA), 200 µl Chloroform (Sigma-Aldrich) was added and samples were centrifuged at 12000 rpm for 15 minutes at 4°C. The aqueous layer, which contained RNA, was separated and 500 µl isopropanol (Solvaco AB, Sweden) was added. The RNA was precipitated by storing the samples at -32°C for 2 hours. Samples were centrifuged at 12000 rpm for 10 minutes at 4°C, to collect the RNA pellets, which were then washed with 75% ethanol (Solvaco AB, Sweden) to remove the organic impurities. Samples were allowed to air dry to remove any traces of ethanol. Dried RNA pellets were dissolved in 21.4 µl of RNAse free water (Qiagen GmBH, Germany) and 2.6 µl of DNAse incubation buffer (Roche GmBH, Germany). The samples were incubated at 75°C for 15 minutes to ensure complete dissolution of RNA-pellets. 2 µl of DNAse I (10 U/µl, Roche GmBH, Germany) was added to each sample, and incubated at 37°C for 3 hr to remove DNA contamination. DNAse was deactivated by incubating the samples at 75°C for 15 minutes. Removal of DNA was confirmed by PCR using Taq polymerase (5 U/µl, Biotools B & M Labs,

Spain), followed by agarose gel electrophoresis. The RNA concentration was measured using a nanodrop ND 1000 spectrophotometer (Saveen Werner).

cDNA synthesis:

cDNA was synthesized from RNA template using 20 mM dNTP (Fermentas Life Science), random hexamer primers and M-MLV Reverse Transcriptase (200 U/µl, Invitrogen, USA) by following manufactures instructions. cDNA synthesis was confirmed by PCR followed by agarose gel electrophoresis.

qRT-PCR:

Relative expression levels of three housekeeping genes (Rp49 & RpL11) and of the genes of interest were determined with quantitative RT-PCR (qPCR). Each reaction, with a total volume of 20 µl, contained 20 mM Tris/HCl pH 9.0, 50 mM KCl, 4 mM MgCl2, 0.2 mM dNTP, DMSO (1:20) and SYBR Green (1:50000). Template concentration was 5 ng/µl and the concentration of each primer was 2 pmol/µl. Primers were designed with Beacon Designer (Premier Biosoft) using the SYBR Green settings. All qPCR experiments were performed in duplicates; for each primer pair a negative control with water and a positive control with 5 ng/µl of genomic DNA were included on each plate. Amplifications were performed with 0.02 µg/ml Taq DNA polymerase (Biotools, Sweden) under the following conditions: initial denaturation at 95°C for 3 min, 50 cycles of denaturing at 95°C for 15 sec, annealing at 52.8–60.1°C for 15 sec and extension at 72°C for 30 sec. Analysis of qPCR data was performed using MyIQ 1.0 software (Bio-Rad) as previously reported. Primer efficiencies were calculated using LinRegPCR (4) and

samples were corrected for differences in primer efficiencies. The GeNorm protocol described by Vandesompele et al. (5) was used to calculate normalization factors from the expression levels of the housekeeping genes. Differences in gene expression between groups were analyzed with ANOVA followed by Fisher's PLSD test where appropriate. $P<0.05$ was used as the criterion of statistical significance. The following primers (ThermoFisher Scientific, Germany) were used: Rp49 F 5´-CACACCAAATCTTACAAAATGTGTGA-3´, R 5´-AATCCGGCCTTGCACATG-3´; RpL11 F 5´-CCATCGGTATCTATGGTCTGGA-3´, R 5´-CATCGTATTTCTGCTGGAACCA-3´, CCAP F 5´- TCGCTGGAAAGGGAGAACAAC-3´, R 5´-TCGTCCACAGCCTGTAAATGC-3´; CCAP-R F 5´-ATGAGACCGAACAGTTTG-3´, R 5´-ACATCACGAACAGAACGA-3´; NPF F 5´-ATTATATGCTCCTCTTACTTC-3´, R 5´-CAGTGAATGTTACCGTAA-3´

Sigmoidal fitting of data and statistics for PER sugar sensitivity.

To fit the data in a sigmoidal curve, sigmoid interpolation was performed. The sigmoid curves were defined as in (6). In brief:

$$F_S = 1/1 + e(-\alpha_s log2 * \frac{S_{con}}{S_{50}})$$

Where

F_S : Fraction of flies showing the PER

S_{con} : Concentration of sucrose

S_{50}: Sucrose concentration where 50% of flies show the PER

α_S : slope of the sigmoid curve

Based on the experimentally measured quantities (S_{con} and F_S), S_{50} and α_S were chosen to best fit the data. For all experimental data, fitting based on nonlinear regression was calculated with Matlab. Goodness-of-fit was tested by two-way ANOVA between the sigmoidal curve and the actual PER response curve, which indicated a good fit for all cases ($p<0.05$. two-way ANOVA) Since we were interested in sugar sensitivity, we used S_{50} for data analysis.

References

1. Lahr EC, Dean D, & Ewer J (2012) Genetic analysis of ecdysis behavior in Drosophila reveals partially overlapping functions of two unrelated neuropeptides. *J Neurosci* 32(20):6819-6829.
2. Moris-Sanz M, Estacio-Gomez A, Alvarez-Rivero J, & Diaz-Benjumea FJ (2014) Specification of neuronal subtypes by different levels of Hunchback. *Development* 141(22):4366-4374.
3. Ja WW, *et al.* (2007) Prandiology of Drosophila and the CAFE assay. *Proceedings of the National Academy of Sciences* 104(20):8253-8256.
4. Ramakers GJ, *et al.* (2011) Dysregulation of Rho GTPases in the {alpha}Pix/Arhgef6 mouse model of X-linked intellectual disability is paralleled by impaired structural and synaptic plasticity and cognitive deficits. *Hum Mol Genet*.
5. Vandesompele J, *et al.* (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome biology* 3(7).
6. Inagaki HK, Panse KM, & Anderson DJ (2014) Independent, reciprocal neuromodulatory control of sweet and bitter taste sensitivity during starvation in Drosophila. *Neuron* 84(4):806-820.

Figures and Figure Legends

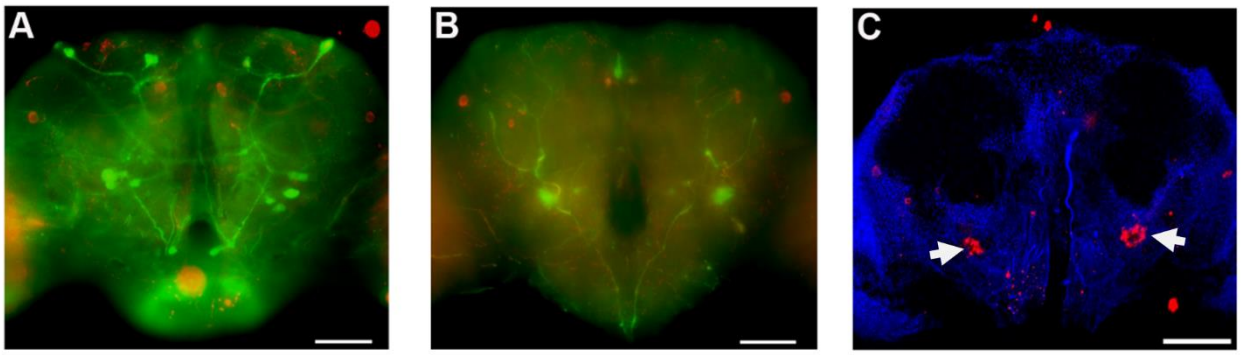

Fig. S1 Dissected (A) *R64C11-GAL4;UAS-GFP* or (B) *R64B05-GAL4;UAS-GFP* male adult brains (5-9 days post-eclosion), were co-stained with anti-GFP (green) and anti-NPF (magenta). Scale bar denotes 50 µm. Pictures are a representative Z-stack, which includes 40 (2 µm) slices. (C) Dissected *CCAP*^{exc7} null male adult brains (10-12 days post-eclosion), stained for CCAP. Expression (white arrows) is most likely background staining. Scale bar = 50 µm. Pictures are a representative Z-stack, which includes 40 (2 µm) slices.

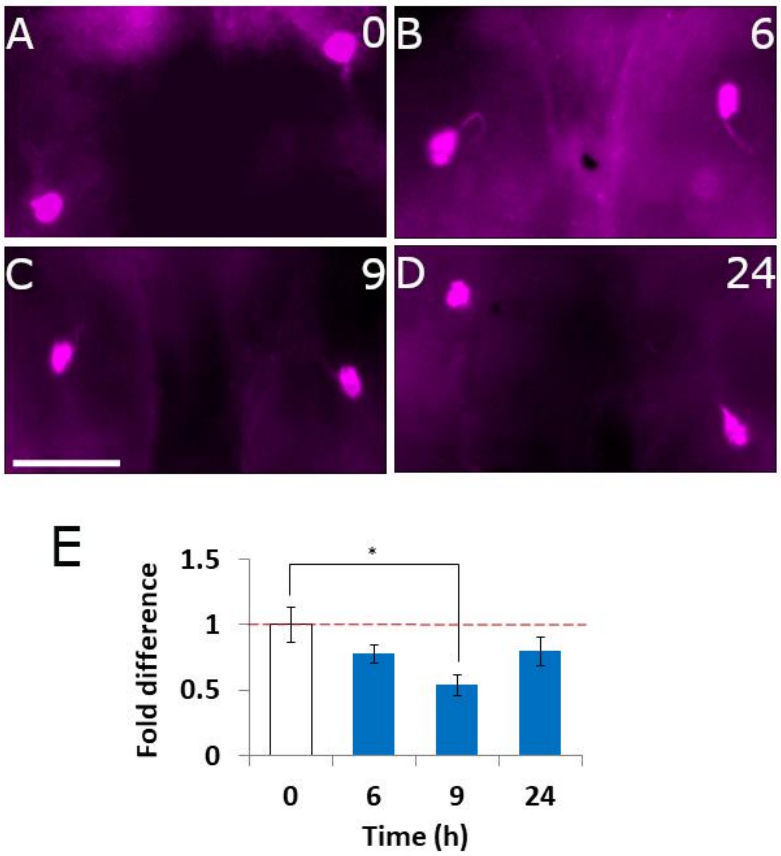

Fig. S2. (A-D) Close up of CCAP neurons in whole Drosophila male brains, 5-7 days post-eclosion, visualizing CCAP expression after various times of starvation. Size bar = 50 µm. (E) Relative CCAP immunofluorescence expression levels in fed flies and after various times of starvation. CCAP neurons of 10–12 brains for each condition were investigated. (* $P < 0.05$, Initially, separate a Kolmogorov-Smirnov Test of Normality was performed, followed by One-way ANOVA with Tukey's comparison). Error bars = SEM.

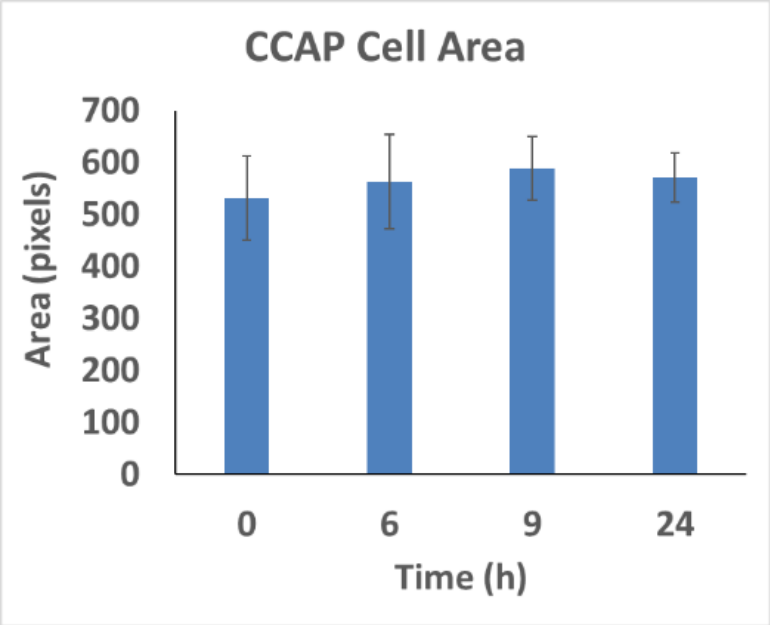

Fig. S3. For each condition, CCAP neuron size was measured in whole *Drosophila* male brains, 5-7 days post-eclosion. All cell sizes were measured using ImageJ. The graph shows there were no significant change in cell size when comparing the different starvation time points. Initially, a Kolmogorov-Smirnov Test of Normality was performed for each time-point, followed by a One-way ANOVA with Tukey's comparison between all time-points). Error bars = SEM.

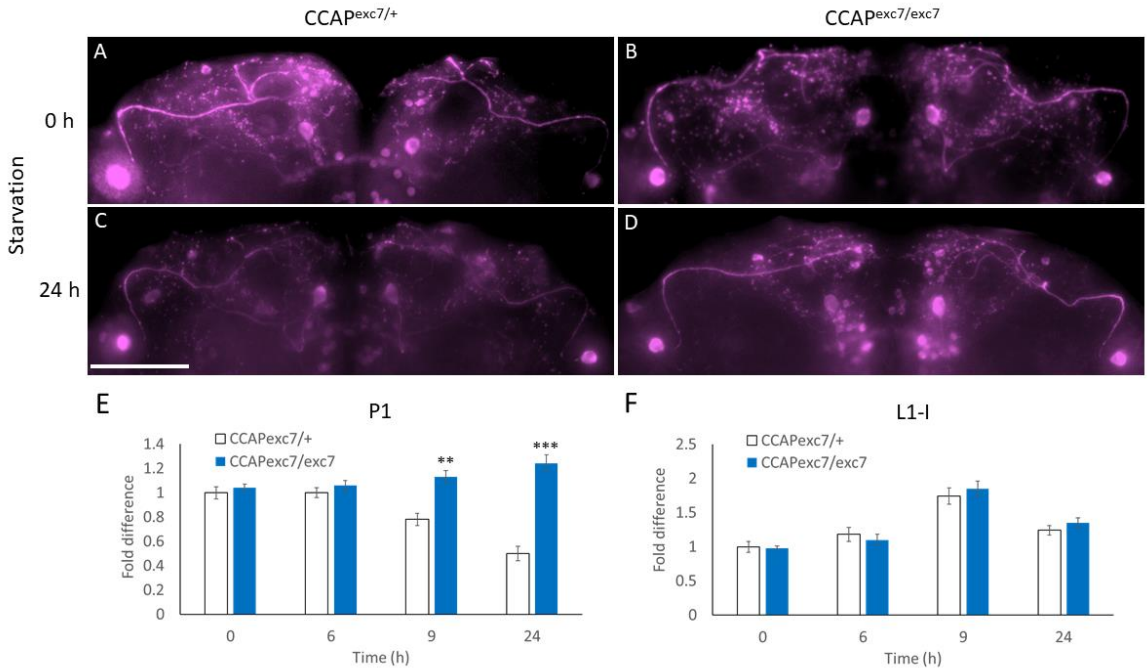

Fig. S4. (A-D) Close up of NPF neurons in whole *Drosophila* male brains, 5-7 days post-eclosion, visualizing NPF expression (A, B) Adult male flies fed *ab libitum* (A) $CCAP^{exc7}$ > w^{1118} controls (B) $CCAP^{exc7}$ > $CCAP^{exc7}$ (C, D) Adult male flies maintained starved for 24-hours (C) $CCAP^{exc7}$ > w^{1118} controls (D) $CCAP^{exc7}$ > $CCAP^{exc7}$. Size bar = 50 µm. (E) Relative NPF immunofluorescence expression levels in both dorsal median P1 and (F) dorsal lateral L1-I NPF neurons. NPF neurons of 10–12 brains for each condition were investigated. (** $P < 0.01$, *** $P < 0.005$, Initially, separate a Kolmogorov-Smirnov Test of Normality was performed, followed by a One-way ANOVA with Tukey's comparison). Error bars = SEM.

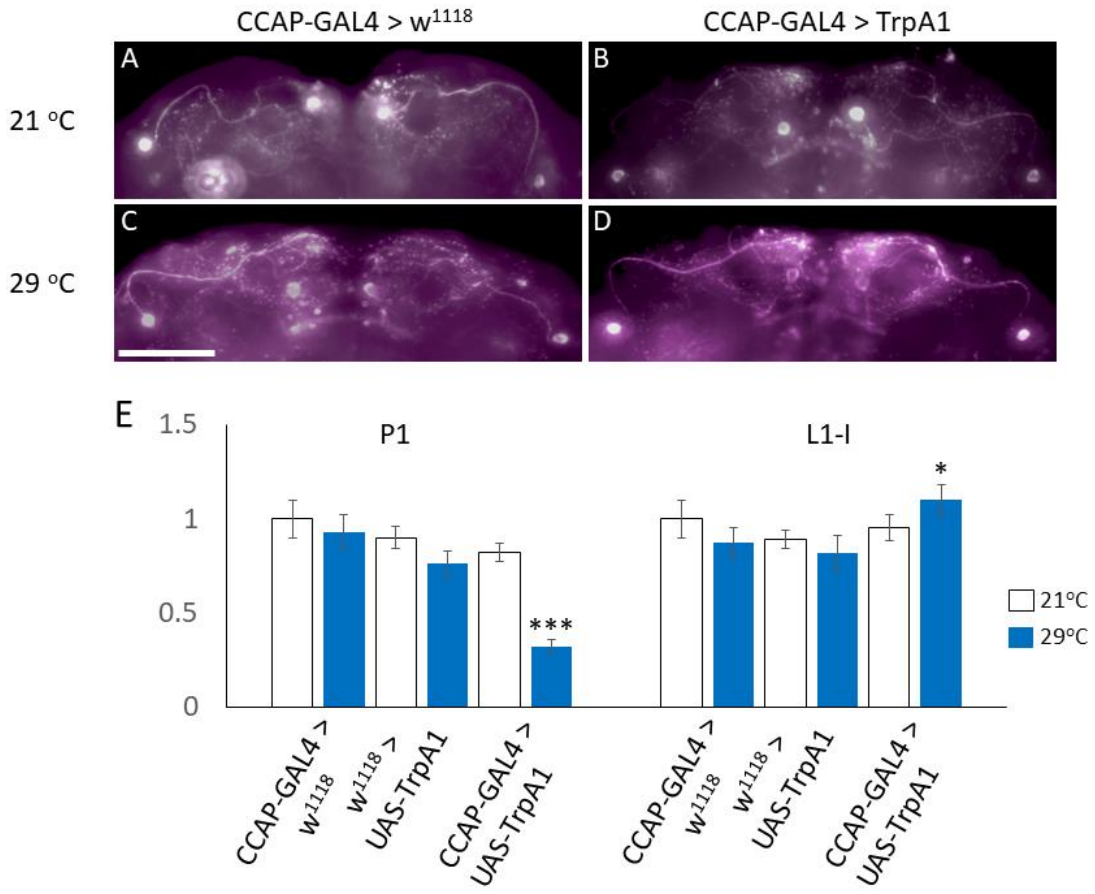

Fig. S5. (A-D) Close up of NPF neurons in whole *Drosophila* male brains, 5-7 days post-eclosion, visualizing NPF expression (A, B) Adult male flies maintained at 21 °C. (A) *CCAP-GAL4 > w^{1118}* controls (B) *CCAP-GAL4 > UAS-TrpA1* (C, D) Adult male flies maintained at 29 oC (C) *CCAP-GAL4 > w^{1118}* controls (D) *CCAP-GAL4 > UAS-TrpA1* 24 hours of starvation. Size bar = 50 µm. (E) Relative NPF immunofluorescence expression levels in both Central and Peripheral NPF neurons. NPF neurons of 10–12 brains for each condition were investigated. (* $P < 0.05$, *** $P < 0.005$, Initially, separate a Kolmogorov-Smirnov Test of Normality was performed, followed by a One-way ANOVA with Tukey's comparison). Error bars = SEM.

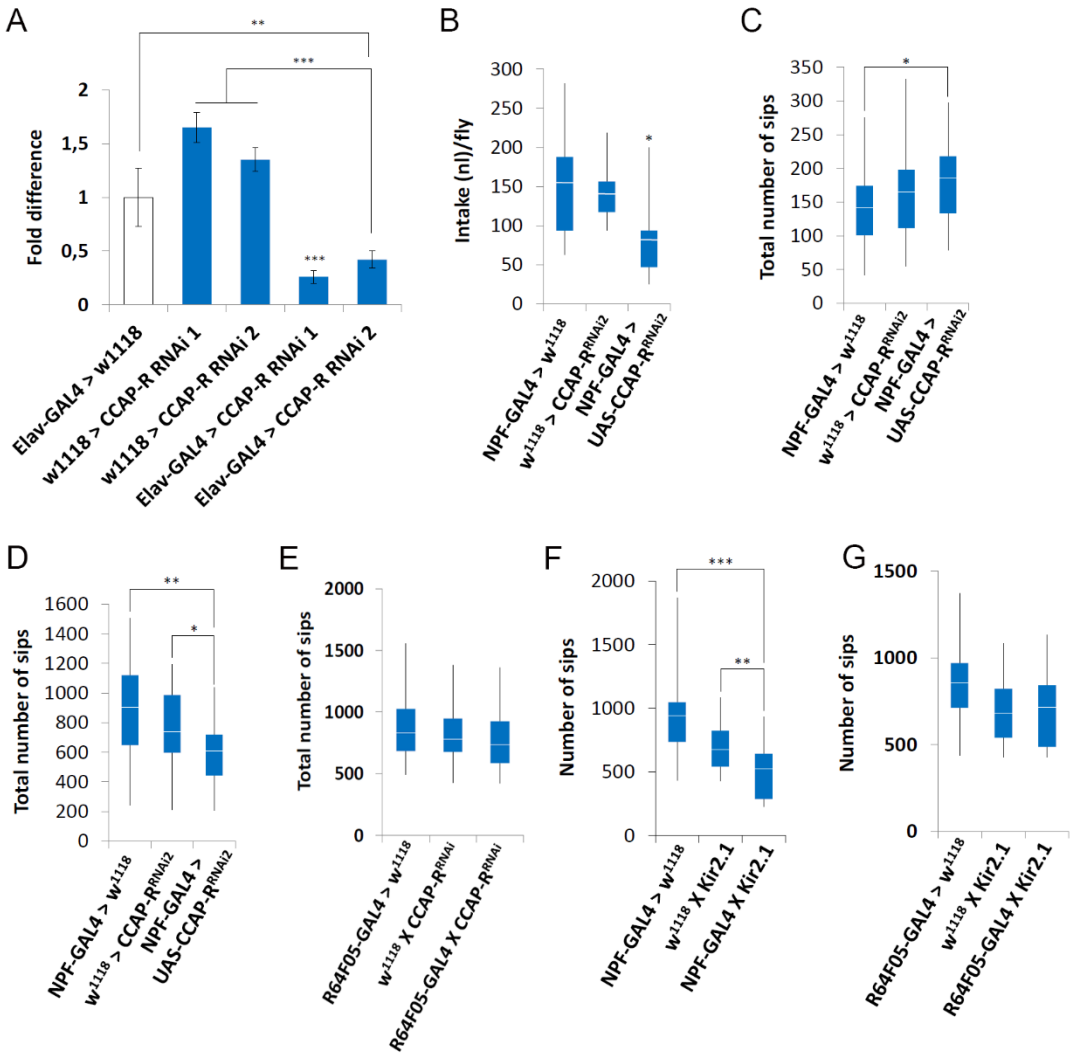

Fig. S6. (A) Relative expression level of CCAP-R in 5–7 day old control males or males where CCAP-R was knocked down in the entire nervous system throughout development (*elav-GAL4* > *CCAP-R*^{RNAi}). This assay was repeated at least 7 times. (n = 25 males per treatment; *** P<0.005 compared with controls, one-way ANOVA with Tukey's post hoc test for multiple comparisons). Error bars = SEM. (B) A CAFE assay was used to assess total food intake over a 24-hour period in 5–9 day old adult males. Five males were used for each replicate and the assay was repeated at least 10 times for each genotype. The food source was 150 mM sucrose. A non-parametric

Kruskal-Wallis ANOVA was performed with Dunn's post-hoc test for multiple comparisons, (* = $P < 0.05$). (C,D) The flyPAD was used to measure the total number of sips over an hour using males where CCAP-R was knocked down in NPF neurons (*NPF-GAL4 > UAS-CCAP-R*^{RNAi2}) that were either (C) fed *ad libitum* or (D) previously starved for 18 hours. N is between 32 and 64 and a non-parametric Kruskal-Wallis ANOVA was performed with Dunn's post-hoc test for multiple comparisons, (* = $P < 0.05$, ** = $P < 0.01$). (E) flyPAD was employed to examine the feeding behavior of males where CCAP-R was knocked down using the CCAP-R specific drive (*R64F05-GAL4 > CCAP-R*^{RNAi}). The cumulative number of sips over an hour was counted in 5-9 day old adult males starved for 18 hours. (F,G) flyPAD was employed to examine the feeding behavior of males where Kir2.1 was overexpressed using either (F) *NPF-GAL4* or (G) CCAP-R specific drive (*R64F05-GAL4 > Kir2.1*). The cumulative number of sips over an hour was counted in 5-9 day old adult males starved for 18-hours. The boxes and whiskers represent the quartile, minimum and maximum values. For all assays n is between 32 and 64 and a non-parametric Kruskal-Wallis ANOVA was performed with Dunn's post-hoc test for multiple comparisons. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$).

Video S1: Branches of CCAP neurons superimpose NPF neuron cell bodies Video is of a representative Z-stack, which includes 40 (2 µm) slices. CCAP (red) and GFP (green, NPF-GAL4;UAS-GFP) neurons in dissected male adult brains (10-12 days post-eclosion).

Video S2: Transfer projection showing branches of CCAP neurons superimpose NPF neuron cell bodies Transverse-projection of CCAP (red) and GFP (green, *NPF-GAL4;UAS-GFP*) neurons in dissected male adult brains (10-12 days post-eclosion).

Video S3: Dissected $CCAP^{exc7}$ null male adult brains (10-12 days post-eclosion), stained for CCAP. Video is of a representative Z-stack, which includes 40 (2 µm) slices. CCAP expression (Red) is observed in what appears to be the antenno-mechanosensory and motor center (AMMC), most likely indicating background staining.

Video S4: CCAP neurons sufficient to induce a Proboscis Extension Response (PER). Male *CCAP-GAL4>UAS-ReaChR* flies (7-9 days old) were photo-stimulated using a 620 nm LED, 10 pulses of 500 ms photo-stimulation at 0.2 Hz were delivered. The video shows one pulse train for one replicate (5 flies). Immediately after the first photo-stimulations some of the flies can be seem to extend their proboscis, this becomes even more evident after the second stimulation. The flies also become more hyperactive (leg movement) after each stimulation.

Video S5: NPF neurons insufficient to induce a Proboscis Extension Response (PER). Male *NPF-GAL4>UAS-ReaChR* flies (7-9 days old) were photo-stimulated using a 620 nm LED, 10 pulses of 500 ms photo-stimulation at 0.2 Hz were delivered. The video shows one pulse train for one replicate (5 flies) No flies were seen to react to any photo-stimulation. In this video, the male on the left who continually extends his proboscis is not counted.

Video S6: Dopaminergic (ple) neurons sufficient to induce a Proboscis Extension Response (PER). Male *ple-GAL4>UAS-ReaChR* flies (7-9 days old) (Pale (ple) is the fly homolog of tyrosine hydroxylase and expresses in all dopaminergic neurons) were photo-stimulated using a 620 nm LED, 10 pulses of 500 ms photo-stimulation at 0.2 Hz were delivered. The video shows one pulse train for one replicate (4 flies). Immediately after every photo-stimulation some of the flies can be seen to extend their proboscis. Unlike, similar to when CCAP neurons are optogentically activated, photo-stimulation of dopaminergic neurons induced hyperactivity (leg movement), which become more apparent with later photo-stimulations.