Supplemental Figures and Tables

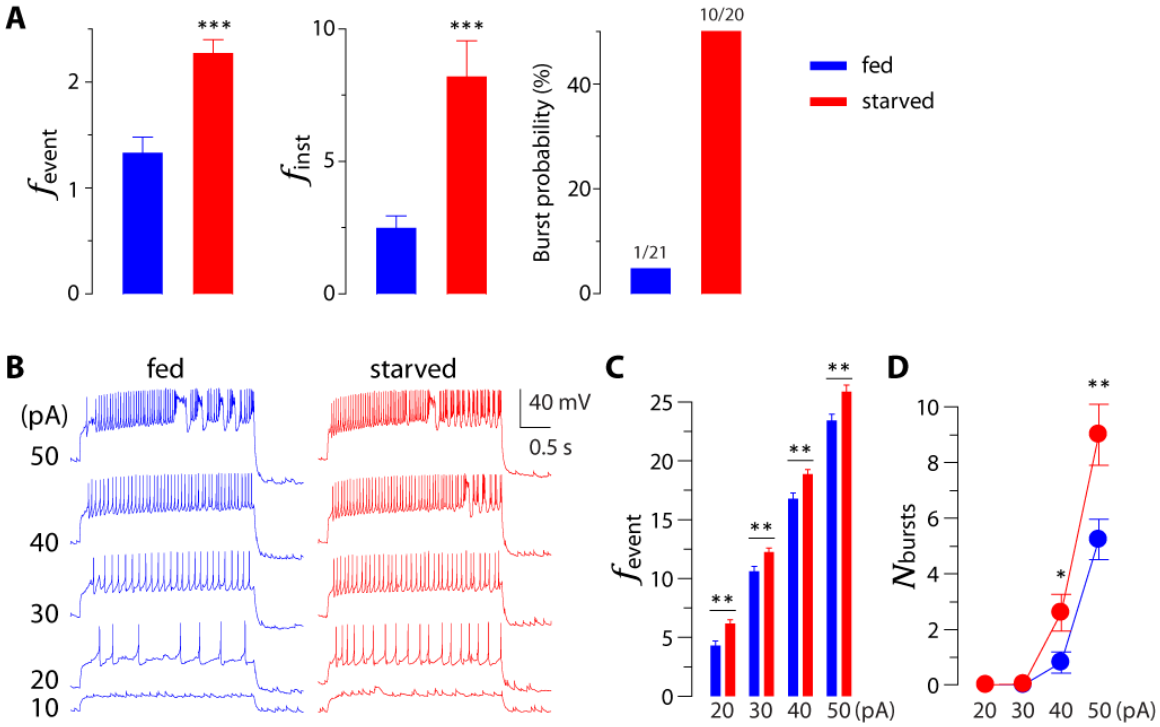

Figure S1. Starvation enhances the excitability of SLC5A11 neurons, related to Figure 1.

(A) Quantification of action potential firing frequency and bursting probability in SLC5A11 neurons from isolated brains of fed and starved flies described in Fig. 1a. The number of bursting neurons out of the total number of recorded neurons is indicated above the bars (n = 20-21). **(B-D)** Responses to step current injections of 10, 20, 30, 40, and 50 pA from a holding potential of -65 mV. **(B)** Representative traces; **(C)** quantification of firing frequency; **(D)** number of bursting events during step depolarization in SLC5A11 neurons of fed and starved flies (n = 25-27). f_{event}, event frequency. f_{inst}, instantaneous frequency. N_{burst}, number of burst firing event. *P < 0.05, **P < 0.01, ***P<0.001; error bars indicate SEM.

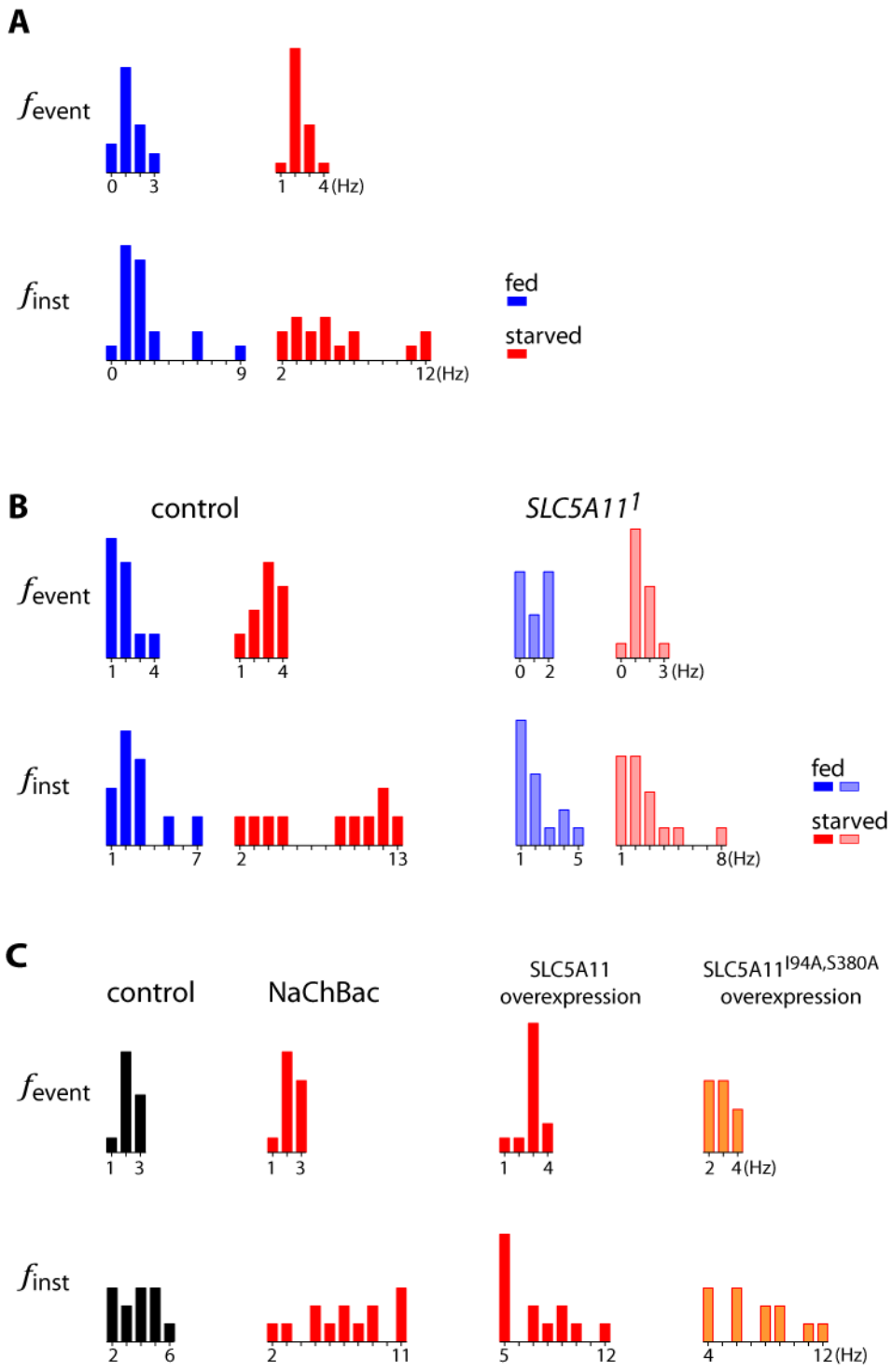

Figure S2. Distribution of firing frequencies, related to Figure 1, 2, and 5.

Histogram representations of the event and instantaneous firing frequencies of SLC5A11 neurons. Histogram representations of the data **(A)** in Figure 1A; **(B)** in Figure 1D; **(C)** in Figure 2B and Figure 5C.

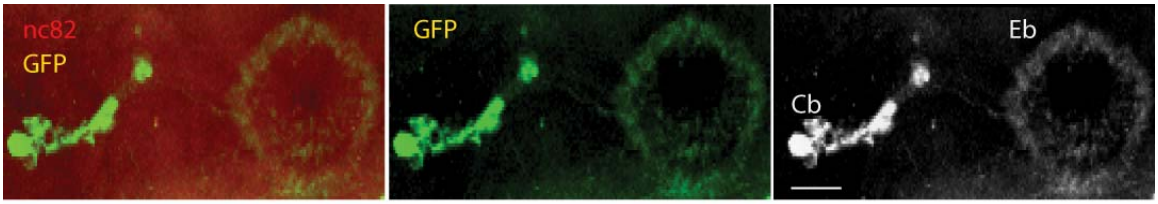

Figure S3. High magnification images of the Ellipsoid Body R4 neurons that show upregulated CaLexA-mediated GFP reporter expression when starved, related to Figure 1.

Cb, cell body; Eb, ellipsoid body. Scale bar, 20 μm.

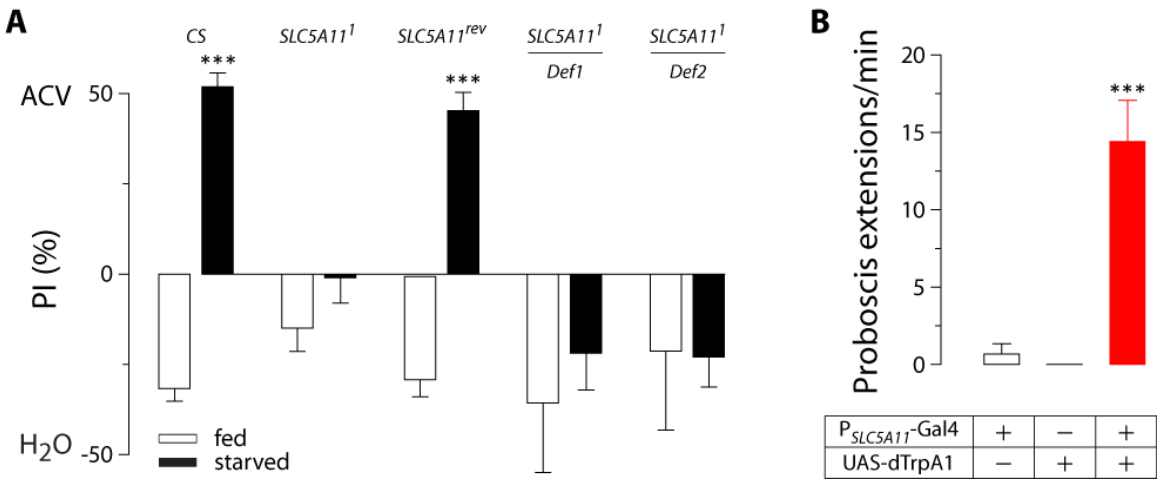

Figure S4. *SLC5A11* is necessary for hunger-driven odor preference and artificial activation of SLC5A11 neuron is sufficient for promoting PER responses, related to Figure 2.

(A) Behavior responses of different *SLC5A11* allelic combinations and controls that were 5-hr (fed) or 22-hr (starved) food deprived were given a choice between a tube containing 10ul of 1% ACV and another containing 10ul of water in a T-maze (n = 6-20; ***P < 0.001 vs. fed in the same genotype). **(B)** Robust PER responses mediated by acute activation of SLC5A11 neurons using dTrpA1 (n = 12-19; ***P < 0.001 vs. control). Flies harboring either $P_{SLC5A11}$-GAL4 or UAS-dTrpA1 alone were used as controls. PER responses were measured for three consecutive minutes. Error bars indicate SEM.

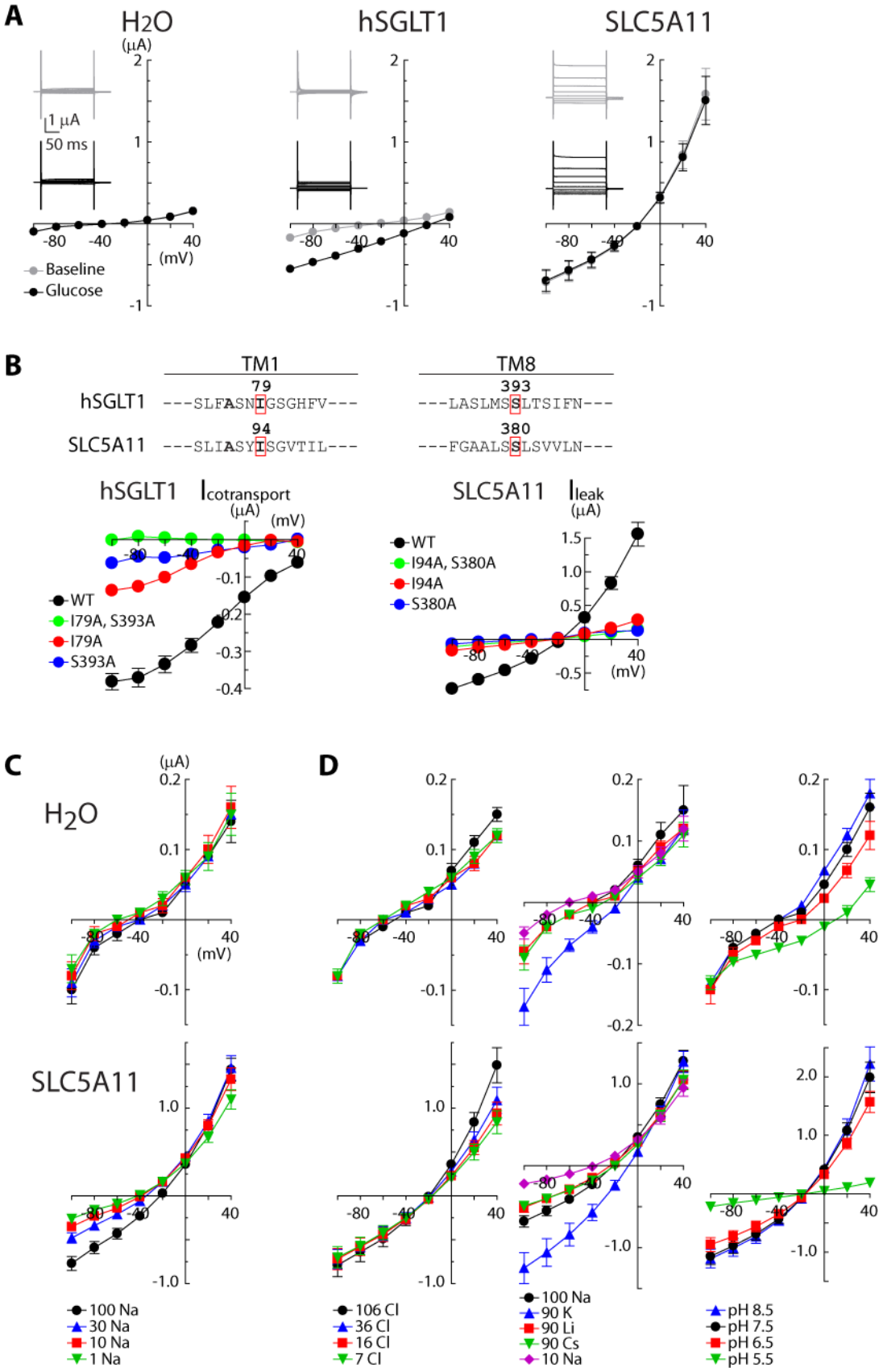
A
H2O
hSGLT1
SLC5A11
(μA)
1 μA
50 ms
(mV)
Baseline
Glucose
B
TM1
TM8
79
393
hSGLT1 ---SLFASNIGSGHFV--- ---LASLMSSLTSIFN---
94
380
SLC5A11 ---SLIASYISGVTIL--- ---FGAALSSLSVVLN---
hSGLT1 Icotransport
SLC5A11 Ileak
WT
I79A, S393A
I79A
S393A
I94A, S380A
I94A
S380A
C
H2O
SLC5A11
100 Na
30 Na
10 Na
1 Na
D
106 Cl
36 Cl
16 Cl
7 Cl
100 Na
90 K
90 Li
90 Cs
10 Na
pH 8.5
pH 7.5
pH 6.5
pH 5.5

Figure S5. SLC5A11 generates leak current, which requires sodium binding sites, related to Figure 4.

(A) Current-voltage analysis of hSGLT1 and SLC5A11 expressed in oocytes (n = 12-13). Pre-glucose treatment (gray); post-glucose treatment (black). Inset, representative current traces. **(B)** Sequence alignment of *hSGLT1* and *SLC5A11* shows conserved sodium-binding sites (upper). Current-voltage analysis carried out using oocytes injected with WT or sodium-binding site mutants (lower): *hSGLT1* (left) or *SLC5A11* (right) (n = 9-26). **(C,D)** Current-voltage analysis carried out using oocytes injected with water or *SLC5A11* cRNA in bathing solutions containing different sodium concentrations **(C)** and different concentrations of Cl^-, different cation compositions, or pH **(D)** (n = 10-11).

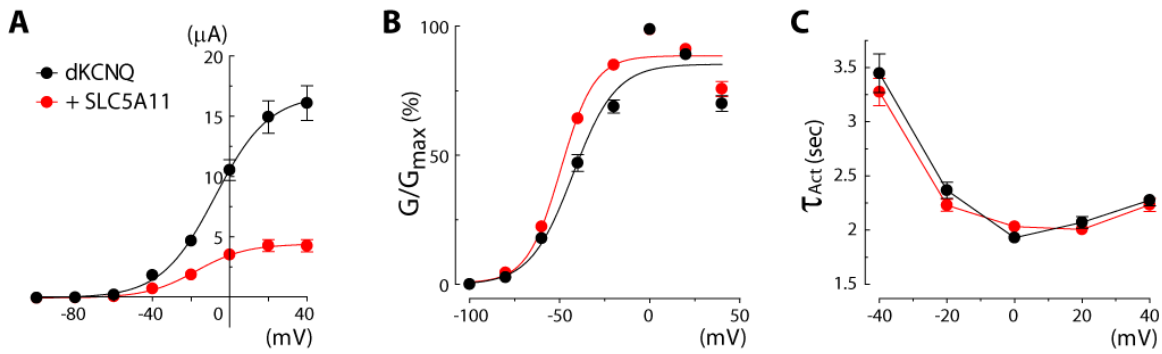

Figure S6. Electrophysiological properties of oocytes expressing dKCNQ with or without the expression of SLC5A11, related to Figure 6.

(A) Current voltage relationships of oocytes injected with *dKCNQ* only and *dKCNQ* + *SLC5A11* (n = 13-14). **(B)** Mean normalized G/G_{max}. Currents were analyzed at the beginning of -30 mV tail pulse. dKCNQ only, V_{50} = -43 ± 1 mV; dKCNQ + SLC5A11, V_{50} = -49 ± 2 mV; n = 39-40. **(C)** Activation time constants (n = 40-49).

Table S1. Fly strains used in the figures.

Strains	Figures
$P_{SLC5A11}$-Gal4,UAS-mCD8GFP	1A, S1A, S1B, S1C, S1D, S2A
$P_{SLC5A11}$-Gal4/ LexAop-CD8-GFP-2A-CD8-GFP; UAS-mLexA-VP16-NFAT, LexAop-CD2-GFP/+	1B, S3
$P_{SLC5A11}$-Gal4/UAS-tdTomato	1C, 1D, 1E, 3A, 3B, S2B
$P_{SLC5A11}$-Gal4,*SLC5A11*^{1}/UAS-tdTomato,*SLC5A11*^{1}	1C, 1D, 1E, S2B
$P_{SLC5A11}$-Gal4,UAS-tdTomato/+	2A, 2B, 2C, 5B, 5C, S2C
$P_{SLC5A11}$-Gal4,UAS-tdTomato/+; UAS-NaChBac/+	2A, 2B, 2C, S2C
$P_{SLC5A11}$-Gal4/+	2C, 2D, 2E, 2F, 2G, 2H, 2I, 2J, 6F, S4B
UAS-NaChBac/+	2C, 2F, 2G, 2J
$P_{SLC5A11}$-Gal4/+; UAS-NaChBac/+	2C, 2F, 2G, 2J
UAS-dTrpA1/+	2D, S4B
$P_{SLC5A11}$-Gal4/UAS-dTrpA1	2D, S4B
UAS-Shi^{ts}/+	2E
$P_{SLC5A11}$-Gal4/+; UAS-Shi^{ts}/+	2E
UAS-Kir2.1,tub-Gal80^{ts}/+	2H, 2I
$P_{SLC5A11}$-Gal4/+; UAS-Kir2.1,tub-Gal80^{ts}/+	2H, 2I
P_{R38H02}-Gal4/+	6F
P_{R38H02}-Gal4/UAS-Kir2.1,tub-Gal80^{ts}	2I
P_{R38H02}-Gal4/UAS-NaChBac	2J
P_{R38H02}-Gal4/UAS-KCNQ-RNAi	6F
$P_{SLC5A11}$-Gal4/+; UAS-Kaede/+	3C, 3D
C42-Gal4/+; UAS-Kaede/+	3C, 3D
UAS-SLC5A11/+	5A
$P_{SLC5A11}$-Gal4,UAS-tdTomato/+; UAS-SLC5A11/+	5A, 5B, 5C, S2C
UAS-SLC5A11^{I94A,S380A}/+	5A
$P_{SLC5A11}$-Gal4,UAS-tdTomato/+; UAS-SLC5A11^{I94A,S380A}/+	5A, 5B, 5C, S2C
$P_{SLC5A11}$-Gal4, *SLC5A11*^{1}	5A
$P_{SLC5A11}$-Gal4, *SLC5A11*^{1}; UAS-SLC5A11	5A
$P_{SLC5A11}$-Gal4, *SLC5A11*^{1}; UAS-SLC5A11^{I94A,S380A}	5A
dKCNQ^{97}	6E
dKCNQ^{186}	6E

UAS-KCNQ-RNAi/+	6F
$P_{SLC5A11}$-Gal4/+; UAS-KCNQ-RNAi/+	6F
$P_{SLC5A11}$-Gal4/+,*SLC5A11*^{1}; UAS-KCNQ-RNAi/+	6F
Canton-S	S4A
SLC5A11^{1}	S4A
SLC5A11^{rev}	S4A
SLC5A11^{1}/*Def1*	S4A
SLC5A11^{1}/*Def2*	S4A

Supplemental Experimental Procedures

Fly strains. Flies were raised in standard cornmeal-molasses medium on the 12 h light/dark cycle at 25 °C. Flies used for TrpA1/Shi^{ts} experiments were grown at 22 °C. All experiments were performed using adult male flies between 5 and 10 days after eclosion. *SLC5A11*^{1} (CG8451, stock #22498), deficiencies (stock #9705 and #9706) uncovering the *SLC5A11* locus, UAS-dKCNQ-RNAi (stock #27252), UAS-mCD8GFP, and UAS-tdTomato were obtained from Indiana Bloomington stock center. *SLC5A11*^{revertant} flies were generated by mobilizing the P element with Δ2-3 transposase[S1]. UAS-Kir2.1 and tubulin-GAL80^{ts} were provided by David Anderson (California Institute of Technology). UAS-NaChBac was from Justin Blau (New York University). UAS-dTrpA1 was from Paul Garrity (Brandeis University). UAS-Shi^{ts} was from Nicholas Stavropoulos (New York University). UAS-Kaede was from Ann-Shyn Chiang (National Tsing Hua University). *dKCNQ* null mutant (dKCNQ^{186}) and control (dKCNQ^{97}) were provided by Rolf Bodmer (Sanford Burnham Medical Research Institute). The genotypes used in each figure are shown in Table S1.

Transgenic constructs and flies. UAS-SLC5A11 and UAS-SLC5A11^{I94A,S380A} were constructed by cloning the cDNA sequences into pUAST vector. Transgenic flies were generated by Bestgene, Inc.

Two-choice assay. Approximately 40 male flies were food deprived in an empty vial with wet Kimwipe for 5 hours (fed) or 22 hours (starved), and then given a choice for 2 hours between two food substrates, each color-coded with tasteless food dye. The concentrations of sugars used in the two-choice assay were determined based on the behavioral responses of fed flies that showed little or no preference between the two food substrates. 50mM D-glucose and various concentrations of L-glucose ranging from 220mM to 300mM were used. Preference index (PI) was calculated as follows: PI = (# eaten food 1 + 0.5 X # eaten both) – (# eaten food 2 + 0.5 X # eaten both) / (total # flies eaten).

T-maze preference assay. 10 µl of 1% apple cider vinegar (ACV) or 10 µl of H_2O were applied onto small pieces of filter paper, placed at the bottom of a 15-ml tube, and then the two tubes were inserted into the T-maze apparatus. Flies were then given a choice between these two tubes for one minute before the number of flies in each tube was counted. The preference index was calculated as follows: PI = (# in ACV tube) – (# in H_2O tube) / (# in ACV tube + # in H_2O tube).

Measurement of food intake. 5 hr (fed) or 22 hr (starved) food deprived flies were anesthetized on ice, and then transferred to 50% standard medium containing 50 mM D-glucose and 0.5% food dye (FD&C Blue 1, Eiroglaucine). Feeding was interrupted after 30 minutes by freezing the vials on dry ice. 30 frozen flies were transferred to a 1.7-ml tube and homogenized with a motorized pestle in 1 ml of 1X PBS containing 1% Triton X-100. After centrifugation to clear the debris, the absorbance of the supernatant was measured at 630 nm and interpolated with standards.

Proboscis Extension Reflex. Each starved fly was gently trapped into a chopped pipette tip, and then each tip was placed perpendicularly onto a slide covered with clay. After the flies were incubated in a 30 °C room for 5 minutes, the numbers of PER responses were counted for 3 consecutive minutes.

Quantitative real-time PCR. Approximately 100 flies were transferred to a 15-ml tube chilled on dry ice/ethanol, decapitated by vigorously vortexing the tube containing the flies. The fly heads were then separated from the bodies and other parts by using metal sieves (pore size # 25 and # 40). After the total RNA from the fly heads was isolated with TRIzol, followed by cDNA synthesis using SuperScript III kit (Invitrogen), quantitative real-time PCR was performed with LightCycler 480 System using SYBR Green I Master mix (Roche) with standard cycling parameters (10 min at 95 °C and 45 cycles of 10 s at 95 °C, 15 s at 52

°C, and 10 s at 72 °C). GAPDH was used as a control for normalization (ΔCp value). The primers used were as follows: GAPDH (forward, GAAATCAAGGCTAAGGTCG; reverse, AATGGGTGTCGCTGAAGAAGTC) and SLC5A11 (forward, CTGGGCCAATACTGTAGGCA; reverse, GGACAGTCGGTACAGTGGAA). Relative standard curves were used to determine amplification efficiencies using serial dilutions of cDNAs. The calculated efficiencies for GAPDH and SLC5A11 primers were 95% and 91% respectively. The small difference in the efficiencies (<5%), however, does not affect the analysis of relative gene expression[S2].

Kaede measurement. To quantify the newly synthesized green Kaede expressed in SLC5A11 neurons or c42 neurons of intact flies, pre-existing Kaede was photo-converted to red fluorescence after the flies were UV irradiated. These flies were then separated into two groups: flies that are food deprived for 5 hours (fed) or 22 hours (starved). Then fly brains from these two groups were removed and imaged under a Zeiss LSM 700 confocal microscope. Using the fluorescent intensity of red Kaede as an internal control, the rate of green Kaede synthesis in starved condition compared to fed condition was obtained using the formula (ΔF) = ($F_{G/R\_starved}$) / (F_{G/R_fed}), where $F_{G/R\_starved}$ is the average ratio of intensities between green (G) and red (R) Kaede from the brain samples in starved condition and F_{G/R_fed} is the average ratio of intensities between green (G) and red (R) Kaede from the brain samples in fed condition.

Electrophysiological recordings of *Xenopus* oocytes. All procedures were approved under New York University Institutional Animal Care and Use Committee protocols. All used cDNAs were subcloned into the plasmid pGEM-HEA containing 5' and 3' untranslated regions of the *Xenopus* β-globin gene for stronger expression in oocytes. To generate sodium-binding site mutations, site-directed mutagenesis was performed using Q5 Site-Directed Mutagenesis Kit (NEB). Plasmid DNAs were linearized with AflII and used to transcribe RNA *in vitro* with T7 RNA polymerase (Ambion). To obtain the stage V-VI oocytes,

several ovary lobes were surgically removed from mature female *Xenopus laevis* (Xenopus Express), torn into small clusters of 5-10 oocytes in ND96 solution (in mM: 96 NaCl, 2.5 KCl, 1 $MgCl_2$, 5 HEPES, 1.8 $CaCl_2$, pH 7.6) containing 50 μg/ml gentamicin, and then treated with collagenase (Type IA, 2 mg/ml) for 30 min in Ca^{2+}-free OR2 solution (in mM: 82.5 NaCl, 2.5 KCl, 1 $MgCl_2$, 5 HEPES, pH 7.6). Next day, after the oocytes were injected, they were maintained at 17 °C with fresh ND96 solution. Three or four days after cRNA injection, inward currents were measured using a two-microelectrode voltage clamp amplifier (OC-725C, Warner Instruments) at room temperature with electrodes (0.5~2 MΩ) filled with 3 M KCl. The cotransport current was obtained by subtracting the baseline current from the current in the presence of glucose or other substrates. Sodium replacement experiments were performed with choline chloride or, when appropriate, with Li^+, Cs^+, or K^+. Chloride replacement was carried out with sodium cyclamate. Solutions of different pH were obtained by adding Tris to HEPES or MES buffered ND96 solution. To measure the activation time constant, dKCNQ current was fitted with single exponential. For the activation curve, normalized tail currents were plotted against prepulse voltages and fitted with a single Boltzmann function.

CaLexA/GFP reporter system. Male flies carrying $P_{SLC5A11}$-GAL4 and UAS-CaLexA/GFP system were collected within several hours after eclosion and maintained in fly food for 4 days. They were then divided in two groups: one group was fed ad libitum in regular fly food and another group was starved for 24-28 hours in presence of water. Dissected brains were stained with anti-GFP (green) and bruchpilot NC82 (red) antibodies according to the protocol of Chiang et al., (2011)[S3]. The GFP fluorescence of the brains was acquired using a Zeiss LSM 700 confocal microscope before immunostaining and quantified by ImageJ software[S4]. We quantified the GFP fluorescence intensity in cell bodies and axonal terminals of SLC5A11 neurons in fed and starved flies. GFP intensity (a.u.) = GFP fluorescence intensity in dotted areas - GFP fluorescence intensity outside the dotted areas.

Surface Expression of SLC5A11-EGFP. All images were obtained by using a Zeiss LSM 700 confocal microscope. The oocytes were imaged at constant filter, gain, pinhole settings, and at equatorial focal plane.

Co-immunoprecipitation. As previously reported[S5], co-IP experiment was performed using a co-IP kit (Pierce) following the manufacturer's protocol with samples from three independently prepared HEK 293 cell cultures co-transfected with dKCNQ-Flag and SLC5A11-EGFP. Total lysates were pulled down with anti-Flag (Sigma) or anti-GFP antibody (Roche). 5% of lysates were used as an input, and mouse IgG was used for a negative control for IP.

Statistical analysis. All statistical analyses were performed using GraphPad Prism. Data were analyzed by unpaired, two-tailed *t*-test or one-way ANOVA followed by Tukey-Kramer *post hoc* test for multiple comparisons.

Supplemental References

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S3. Chiang, A.S., Lin, C.Y., Chuang, C.C., Chang, H.M., Hsieh, C.H., Yeh, C.W., Shih, C.T., Wu, J.J., Wang, G.T., Chen, Y.C., et al. (2011). Three-dimensional reconstruction of brain-wide wiring networks in Drosophila at single-cell resolution. Current biology : CB *21*, 1-11.

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