Supplemental Information

A Small Potassium Current in AgRP/NPY Neurons
Regulates Feeding Behavior and Energy Metabolism

Yanlin He, Gang Shu, Yongjie Yang, Pingwen Xu, Yan Xia, Chunmei Wang, Kenji Saito, Antentor Hinton, Jr., Xiaofeng Yan, Chen Liu, Qi Wu, Qingchun Tong, and Yong Xu
A small potassium current in AgRP/NPY neurons regulates feeding behavior and energy metabolism

**Figure S1.** Related to Figure 1. (A) Dual immunofluorescence for GFP and SK3 in male NPY-GFP mice that were fed ad libitum or fasted for 24 hours. Arrow heads point to very few AgRP/NPY neurons co-express SK3 at fasted condition. The scale bar=50 µm. 3V, 3rd ventricle; ARH, arcuate nucleus of the hypothalamus; ME, median eminence. (B) Quantification showing percentage of AgRP/NPY neurons expressing SK3 at the fed versus fasted conditions. N=4 per condition. Results are shown as mean ± SEM. ***, P<0.001 in t-tests. (C) Recording from a TOMATO-labelled AgRP/NPY neuron from AgRP-Cre/Rosa26-tdTOMATO mouse; the lucifer yellow dye was injected into the recorded neuron for post hoc verification. Scale bars=10 µm. (D) A voltage clamp protocol to induce SK-mediated currents. (E) Representative traces for SK-mediated currents recorded in AgRP/NPY neurons from fed or fasted AgRP-Cre/Rosa26-tdTOMATO mice. Blue traces were baseline SK-mediated currents and red traces were SK-mediated currents after acute apamin perfusion (100 nM, 6 minutes). (F) Quantification showing the amplitude of SK-mediated currents from fed or fasted mice. N=38 neurons per condition. Results are shown as mean ± SEM. ***, P<0.001 in t-tests.
Figure S2. Related to Figure 1. (A) Immunofluorescence for SK3 in the paraventricular nucleus of the hypothalamus (PVH) male mice that were fed ad libitum or fasted. The scale bar=50 μm. 3V, 3rd ventricle.
**Figure S3.** Related to Figure 1. (A) Three continuous action potentials in AgRP/NPY neurons from fasted mice, from fed mice, or from fed mice and incubated with apamin (100 nM, 2 hours); values at the bottom of each trace are amplitudes of the AHP. (B) Quantification showing the amplitude of the AHP. N=9-21 neurons per condition. Results are shown as mean ± SEM. ***, P<0.001 in one way ANOVA analyses followed by post hoc Sidak tests. (C) Representative current clamp traces in AgRP/NPY neurons from fasted mice, from fed mice, or from fed mice and incubated with apamin (100 nM, 2 hours). (D-E) Quantification showing the firing rate (D) and resting membrane potential (E). N=16-23 neurons per condition. Results are shown as mean ± SEM. ***, P<0.001 in one way ANOVA analyses followed by post hoc Sidak tests.
**Figure S4.** Related to Figure 1. (A) Dual staining for TOMATO (red fluorescence) and SK3 (green immunofluorescence) in Kcnn3<sup>fl/fl</sup>/AgRP-CreERT2/Rosa26-tdTOMATO mice. Note that strong SK3 immunoreactivity was observed in non-TOMATO neurons in the ARH, and weak SK3 immunoreactivity was also detected in TOMATO-labelled neurons.
**Figure S5.** Related to Figure 1. (A) The amplitude of SK-mediated currents recorded in AgRP/NPY neurons from ad libitum fed control or AgRP-SK3-KO mice that had been fed on chow or on HFD for 4 weeks. N=18-26 neurons per condition. Results are shown as mean ± SEM. ###, P<0.001 between control vs. AgRP-SK3-KO at the same feeding condition in two way ANOVA analyses followed by post hoc Sidak tests. (B) The amplitude of the NMDA-induced currents in AgRP/NPY neurons from ad libitum fed control or AgRP-SK3-KO mice that had been fed on chow or on HFD for 4 weeks. N=14-22 neurons per condition. Results are shown as mean ± SEM. ##, P<0.01 between control vs. AgRP-SK3-KO at the same feeding condition in two way ANOVA analyses followed by post hoc Sidak tests. (C-D) The firing rate (C) and resting membrane potential (D) in AgRP/NPY neurons from ad libitum fed control or AgRP-SK3-KO mice that had been fed on chow or on HFD for 4 weeks. N=18-32 neurons per condition. Results are shown as mean ± SEM. #, P<0.05 between control vs. AgRP-SK3-KO at the same feeding condition in two way ANOVA analyses followed by post hoc Sidak tests.
Figure S6. Related to Figure 4. (A) Absolute body weight curves of male control and AgRP-SK3-KO littermates before and after tamoxifen induction. (B) Body weight gain of these mice since they received tamoxifen injections. (C) Cumulative chow intake after tamoxifen inductions. N=7 mice per group. Results are shown as mean ± SEM. *, P<0.05 in t-tests for each time point. (D-E) Temporal changes in body temperature (D) and physical activity (E) in chow-fed control and AgRP-SK3-KO littermates during a 24-hour ad libitum period. N=4 mice per group. Results are shown as mean ± SEM. *, P<0.05 in t-tests for each time point.
**Supplemental Table 1: Primer sequence. Related to Figure 4.**

| Target gene | Primer sequences                       | PCR products | GenBank accession |
|-------------|----------------------------------------|--------------|------------------|
| Cyclophilin | CYCLO F: TGGAGAGCACCAAGACAGACA         | 66bp         | NM_011149        |
|             | CYCLO R: TGGCGGAGTGGAACATAGAT          |              |                  |
| UCP1        | UCP1F: GAGGTGTCAGTGGTCTATTG            | 59bp         | NM_009463        |
|             | UCP1R: GGTTCGATGTCGACGTTCA            |              |                  |
| PGC-1α      | PGC-1α F: AACCACACCCACAGGATCAGA       | 73bp         | NM_008904.2      |
|             | PGC-1α R: TCTTCGCTTTTATGGCTCATGA      |              |                  |
| PPAR γ      | PPAR γ F: CGTACGGCAATGGCTTTATC         | 55bp         | NM_011144.6      |
|             | PPAR γ R: AACGGCTTCCTCAGGTCTTT         |              |                  |
| UCP3        | UCP3 F: TTCTGACCCTGGAGCTT              | 63bp         | NM_009464.3      |
|             | UCP3 R: GCCCTCTTCAGGTCTCAT             |              |                  |
| ADRB3       | ADRB3 F: GACTACAGACCATAACACAGTG        | 82bp         | NM_013462        |
|             | ADRB3 R: CCTGGTCCATTACGAGGA            |              |                  |
| PRDM16      | PRDM16 F: CCACCAAGGAGACCTTCAC         | 107bp        | NM_027504        |
|             | PRDM16 R: GGAGGACTCTCGTAGCTCAGA       |              |                  |
Supplemental Methods

Validation of genomic deletion of Kcnn3 in AgRP/NPY cells

Kcnn3\textsuperscript{fl/fl} and Kcnn3\textsuperscript{fl/fl}/AgRP-Cre\textsubscript{ERT2} littermates received tamoxifen inductions, as described above. These mice were anesthetized with inhaled isoflurane, and sacrificed. Various tissues, including the ARH, cortex, brain stem, pituitary, adipose tissue, liver and muscle were collected. Genomic DNAs were extracted using the REDExtract-N-Amp Tissue PCR Kit (#XNATS; Sigma-Aldrich, St Louis, MO), followed by PCR amplification of the floxed or recombined alleles. For the Kcnn3 floxed allele, we used primers: forward- AGGAGAGGGCTGATTCTCAAG and reverse- GTATCGGTGACTGCTTCATCC. The floxed Kcnn3 allele was recognized as a 643 bp band. For the recombined Kcnn3 allele, we use primers: forward- CTTGCCATATAACAGTGTCAAG and reverse- GTATCGGTGACTGCTTCATCC. The recombined Kcnn3 allele was recognized as a 550 bp band.

Co-localization of SK3 in AgRP/NPY neurons

Male NPY-GFP mice (8 weeks) were fed ad libitum or were fasted for 24 hours (4 mice per condition). At 9 am, these mice were anesthetized with inhaled isoflurane and perfused with saline followed by 10% Formalin. Brain sections (25 \textmu m in thickness) were collected and then subjected to dual immunofluorescence for SK3 and GFP. Briefly, brain sections were incubated overnight with rabbit anti-SK3 antibody (1:1000 dilution; #APC-025, Alomone Labs) followed by biotinylated goat anti-rabbit IgG (1:500, Jackson ImmunoResearch Laboratories) for 1.5 hours. The complex was visualized using avidin-biotin conjugated to DyLight 594 (1:500, Jackson ImmunoResearch Laboratories). After
thorough washing, the sections were then incubated overnight with chicken anti-GFP antibody (1:5000; #GFP-1020, Aves Labs Inc.), followed by incubation in goat anti-chicken IgG conjugated to Alexa Fluor 488 (1:250, Invitrogen) for 1.5 hours. All procedure was performed at room temperature. Sections were mounted on slides and cover-slipped with DAPI mounting medium. Fluorescence images were taken using the Leica 5500 fluorescence microscope with OptiGrid structured illumination. Neurons double labelled by SK3 and GFP were counted and averaged in at least 4 consecutive coronal brain sections containing the ARH from each mouse and this data was treated from one biological sample. Data from 4 different mice were used in statistical analyses. We also examined the SK3-labelled neurons in the PVH using these same mice.

**CLAMS metabolic chambers**

As described before (Xu et al., 2015), physical activity and energy expenditure were monitored using Comprehensive Lab Animal Monitoring System (CLAMS, Columbus Instruments). Briefly, a cohort of male HFD-fed AgRP-SK3-KO and control mice were housed individually at room temperature (22-24°C) under an alternating 12:12-h light-dark cycle. After adaptation for 5 days, mice were then subjected to a 3-day ad libitum→fast→refeed paradigm. Physical activity was determined using a multi-dimensional infrared light beam system with beams installed on cage bottom and cage top levels. Ambulatory movement was defined as breaks of any two different light beams at cage bottom level, while rearing was recorded when the mouse broke any light beam at the top levels. Simultaneously, heat production was measured to determine the energy expenditure. O2 consumption and CO2 production were also measured and the ratio
(CO2/O2) was calculated as respiratory exchange rate (RER). To avoid the possible confounding effects from diverged body weight and lean mass on the energy expenditure (Butler and Kozak, 2010), we selected mice with matched body weight, fat mass and lean mass, and energy expenditure was normalized by lean mass (Butler and Kozak, 2010).

**E-mitter study**
A separate cohort of chow-fed female control and AgRP-SK3-KO littermates were anesthetized, and telemetric Mini Mitter probes ER-4000 (E-mitter; Respironics Inc., Murrysville, PA) were implanted in the abdominal cavity. After a 7-day recovery and acclimation period, mouse cages were put on top of the ER-4000 Receivers (Respironics Inc., Murrysville, PA) to measure physical activity and body temperature, similarly as we did before (Xu et al., 2015).

**Long-term metabolic characterization**
A cohort of male control and AgRP-SK3-KO littermates were weaned on the regular chow and singly housed. These mice received tamoxifen inductions at 7 weeks of age as described above. The mice were maintained on the regular chow till 96 days of age. Body weight and food intake were monitored every 3 days.

Another cohort of male control and AgRP-SK3-KO littermates were weaned on the regular chow and received tamoxifen inductions at 7 weeks of age. At week 9, these mice were then singly housed and fed with a high fat-diet (HFD; 65% fat, #D12492, Research Diets, New Brunswick, NJ). Body weight and food intake were monitored weekly for 5
consecutive weeks. Body composition was determined using quantitative magnetic resonance before and after the HFD feeding, and fat/lean mass gain was calculated. These mice were then deeply anesthetized and decapitated during the morning after a 2-hour fast (to empty the stomach). The brown adipose tissue, the gonadal white adipose tissue and the inguinal white adipose tissue were isolated and weighted. BAT pads were quickly stored at -80°C. As described previously (Xu et al., 2010), total mRNA was isolated from the BAT using TRIzol Reagent (Invitrogen) according to the manufacturer's protocol and reverse transcription reactions were performed from 2 µg of total mRNA using a High-Capacity cDNA Reverse Transcription Kits (Invitrogen). Expression of various genes was quantified with real-time PCR with primers as listed in Supplemental Table 1.
Reference:

Xu, P., Cao, X., He, Y., Zhu, L., Yang, Y., Saito, K., Wang, C., Yan, X., Hinton, A.O., Jr., Zou, F., et al. (2015). Estrogen receptor-alpha in medial amygdala neurons regulates body weight. J Clin Invest 125, 2861-2876.

Xu, Y., Hill, J.W., Fukuda, M., Gautron, L., Sohn, J.W., Kim, K.W., Lee, C.E., Choi, M.J., Lauzon, D.A., Dhillon, H., et al. (2010). PI3K signaling in the ventromedial hypothalamic nucleus is required for normal energy homeostasis. Cell Metab 12, 88-95.