SUPPLEMENTAL INFORMATION

A Postsynaptic AMPK→p21-Activated Kinase Pathway Drives Fasting-Induced Synaptic Plasticity in AgRP Neurons

Dong Kong, Yossi Dagon, John N. Campbell, Yikun Guo, Zongfang Yang, Xinchi Yi, Pratik Aryal, Kerry Wellenstein, Barbara B. Kahn, Bernardo L. Sabatini, Bradford B. Lowell

Inventory of Supplemental Information:

Figure S1: related to Figure 2. It provides evidence that expression of DN-AMPKα2 mutant in hypothalamic neurons does not cause cell death.

Supplemental Experimental Procedures: Due to the length limitation of the main text, the detailed information on the reagents and experimental methods is provided in this section.

SUPPLEMENTAL FIGURE

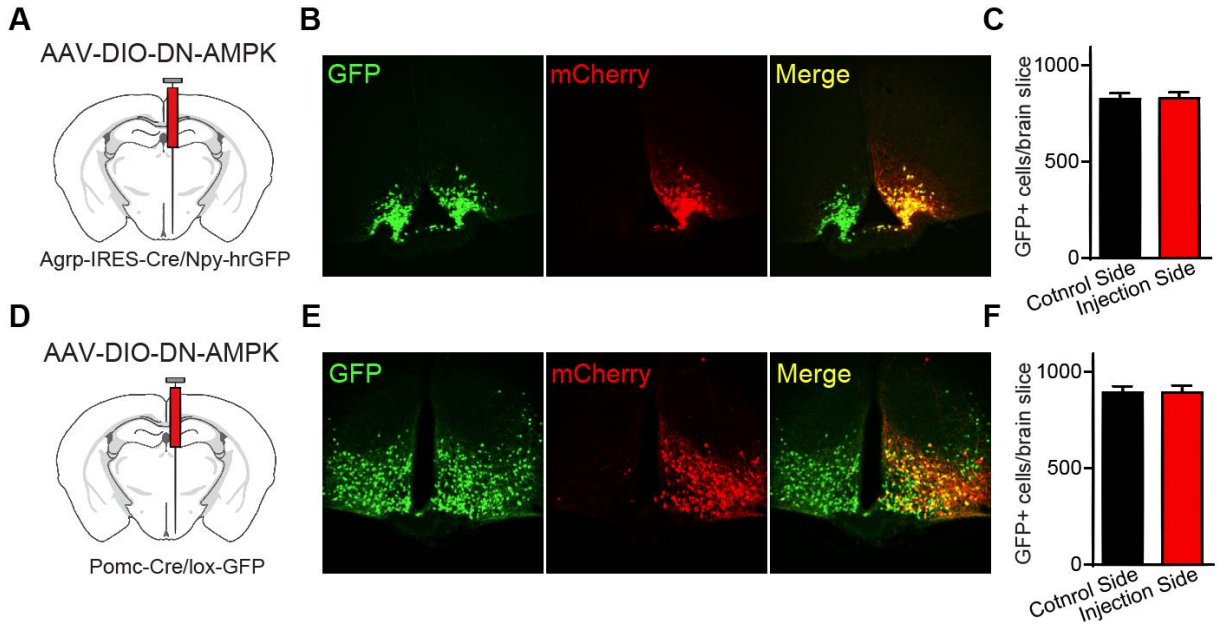

Figure S1, related to Figure 2. Toxicity test of DN-AMPKα2 mutant in hypothalamic AgRP and POMC neurons.

(A-C) AgRP neurons, (D-F) POMC neurons.

(A, D) Schematic of unilateral stereotaxic injection, (B, E) immunofluorescence, and (C, F) cell counting of GFP^+ cells from control non-virus infected side (left side of each panel in B and E) and from virus-injected side (right side of each panel in B and E). GFP^+ neurons were counted on each continuous brain slice containing the arcuate nucleus and the averaged number per slice per side are indicated (mean ± SEM; n=14 brain slices from 3 mice of each genotype).

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Animals

All animal care and experimental procedures were approved by the Beth Israel Deaconess Medical Center Institutional Animal Care and Use Committee and by the Harvard Standing Committee on Animal Care, in accordance with NIH guidelines. Mice were housed at 22–24 °C with a 12 h light: 12 h dark cycle with standard mouse chow (Teklad F6 Rodent Diet 8664; 4.05 kcal g^{-1}, 3.3 kcal g^{-1} metabolizable energy, 12.5% kcal from fat; Harlan Teklad) and water provided ad libitum. All diets were provided as pellets. Mice were euthanized by CO_2 narcosis. *Agrp-IRES-Cre* knockin mouse, *Pomc-Cre* and *Npy-hrGFP* transgenic mice were described previously (Balthasar et al., 2004; Tong et al., 2008; van den Pol et al., 2009) and are available from the Jackson Laboratory.

AAV viral production

Cre dependent Adeno-associated virus (AAV) viral vectors were constructed based on pAAV-EF1a-DIO-hChR2(H134R)-EYFP-WPRE plasmid which was kindly provided by Dr. Karl Deisseroth's group at Stanford. Briefly, Flag-tagged-α2 AMPK (for AAV-DIO-Tag-α2AMPK), α2AMPK(K45R)-2A-mCherry (for AAV-DIO-DN-AMPK), γ1AMPK(H150R)-2A-mCherry (for AAV-DIO-CA-AMPK), and AID-PAK1-IRES-EGFP (for AAV-DIO-DN-PAK) fragments were amplified with high-fidelity PCR and ligated into the *NheI/AscI* restriction enzyme sites of pAAV-EF1a-DIO-hChR2(H134R)-EYFP-WPRE plasmid. *HA-tagged-α2 AMPK*, *α2AMPK(K45R)*, *γ1AMPK(H150R)* were

constructed and reported previously (Dagon et al., 2012; Minokoshi et al., 2004). *AID-PAK1* cDNA encoding amino acids 83-149 of mouse PAK1 protein was amplified from *C57Bl/6* mouse cDNA library with the following pair of primers: F: *CATACAATTCATGTTGGTTTTG*; R: *TGACTTATCTGTAAAACTCATG*. Non-cre dependent AAV-DN-AMPK vector was obtained by transformation of AAV-DIO-DN-AMPK plasmid into EL350 bacteria strain (NCI@Frederick), followed by arabinose-induced cre-mediated recombination.

The above AAV vectors were amplified in recombination-deficient bacteria (OneShot Stbl3, Invitrogen) and Serotype 8 AAVs were packaged by vector core facilities (Boston Children's Viral Core and University of North Carolina Vector Core) and the following titer was achieved (viral molecules/ml): AAV-DIO-Tag-α2AMPK: $3.15x10^{14}$, AAV-DIO-DN-AMPK:$1.0x10^{13}$, AAV-DIO-CA-AMPK: $3.2x10^{13}$, AAV-DN-AMPK: $3.6x10^{15}$ and AAV-DIO-DN-PAK: $6.4x10^{13}$. Adenovirus expressing CA-AMPK was reported previously(Minokoshi et al., 2004).

Stereotaxic injections

Stereotaxic injections were performed as previously described (Kong et al., 2012). Mice were anaesthetized with xylazine (5 mg/ kg) and ketamine (75 mg/ kg) diluted in saline and placed into a stereotaxic apparatus (KOPF Model 963). After exposing the skull via small incision, a small hole was drilled for injection. A pulled-glass pipette with 20–40 mm tip diameter was inserted into the brain and virus was injected by an air pressure system. A micromanipulator (Grass Technologies, Model S48 Stimulator) was used to

control injection speed at 25 nl min^{-1} and the pipette was withdrawn 5 min after injection. 100-200 nl virus was injected bilaterally or unilaterally into the acuate nucleus (Coordinates: bregma: AP:−1.50 mm, DV:−5.80 mm, LR: ±0.20 mm) of 3-4-week old male mice. For postoperative care, mice were injected intraperitoneally with meloxicam (0.5 mg/kg). All stereotaxic injection sites were verified under electrophysiological microscopy (for 2-photon imaging and/or electrophysiology-related studies) or by immunohistochemistry (for anatomy and *in vivo* studies). All 'misses' or 'partial hits' were excluded from data analyses. Animals were allowed for 1 week to recover from surgery. Experiments were performed 2-3 weeks after surgery to allow sufficient gene expression in targeted neurons.

Slice preparation

Coronal hypothalamus slices were prepared as described previously (Liu et al., 2012). Briefly, mice were anaesthetized by inhalation of isoflurane. 300 μm thick coronal sections were cut with a Leica VT1000S vibratome and then incubated in carbogen-saturated (95% O_2 / 5% CO_2) ACSF (in mM: 125 NaCl, 2.5 KCl, 1 MgCl2, 2 CaCl2, 1.25 NaH2PO4, 25 NaHCO3, 10 glucose) at 34 °C for 30–45 min before recording. All recordings were obtained within 4 hours of slicing at room temperature.

Electrophysiology

Whole-cell recordings were obtained from arcuate AgRP neurons visualized under infrared differential interference contrast (IR-DIC) using patch pipettes with pipette resistance of 2.5–4.5 MΩ. To identify infected AgRP neurons, mCherry or GFP

fluorescence, or both were detected using epifluorescence illumination. For miniature excitatory postsynaptic current (mEPSC) recordings, the internal solution contained (in mM) 135 $CsMeSO_3$, 10 HEPES, 1 EGTA, 3.3 QX-314 (Cl^- salt), 4 Mg-ATP, 0.3 Na-GTP, 8 Na_2-phosphocreatine (pH 7.3 adjusted with CsOH; 295 $mOsm \cdot kg^{-1}$). For current-clamp recordings, the internal solution consisted of (in mM) 135 $KMeSO_3$, 3 KCl, 10 HEPES, 1 EGTA, 0.1 $CaCl_2$, 4 Mg-ATP, 0.3 Na-GTP, 8 Na_2-phosphocreatine (pH 7.3 adjusted with KOH; 295 $mOsm \cdot kg^{-1}$). Alexa Fluor 594 (10–20 μM) was added to the internal solution to visualize cell morphology and to confirm dendritic structures under 2-photon laser scanning microscope. Bath solutions for whole-cell recordings did not contain drugs unless specified otherwise. For mEPSC recordings, ACSF contained 1 μM tetrodotoxin (TTX, Sigma) and 50 μM SR 95531/gabazine (Tocris). Recordings were made using an Axoclamp 700B amplifier (Axon Instruments) at room temperature. Data were filtered at 3 kHz and sampled at 10 kHz. Series resistance, measured with a 5 mV hyperpolarizing pulse in voltage clamp, was on average under 20 MΩ and less than 25 MΩ, uncompensated. All voltage-clamp recordings were made from cells held at −60 mV. For current-clamp recordings, membrane potentials were corrected for a ~8 mV liquid junction potential.

Two-photon imaging

Two-photon laser-scanning microscopy was performed using a custom microscope as previously described(Kozorovitskiy et al., 2012). Cell morphology was visualized using Alexa Fluor 594 (10–20 μM) excited with 810–840-nm light and introduced into the cell through whole-cell recording. Briefly, a pipette containing Alexa Fluor 594 in the internal

solution was guided to an AgRP neuron cell body with low positive pressure. Pressure was released and whole-cell patch was set up to fill the soma with dye, which rapidly diffused into dendrites and spines (~5min). A two-photon laser-scanning microscopy three-dimensional image stack through each neuron was collected, followed by stacks through dendritic segments at a tenfold higher magnification. For dendritic spine density analysis, cells were coded and analyzed with MATLAB and ImageJ software.

Food intake, body weight and body composition analysis

Food intake studies on chow were performed as previously described(Krashes et al., 2014; Liu et al., 2012). Following bilateral AAV viral injections, the animals (5- to 12-week-old male mice) were singly housed 1-week after surgery and allowed for 2-3 weeks to express AAV-mediated genes. Feeding studies were performed in home cages with *ad libitum* food access. Mice with 'missed' injections or incomplete 'hits' were excluded from analysis after post hoc examination of mCherry or GFP expression. In this way, all food intake measurements were randomized and blind to the experimenter. For fast-refed measurements, animals were fasted for 24 hours from 9am to 9 am. At 9am on the following day, weighed food pellets were placed back in the cages and food intake was monitored at 1, 2, 4, 6, 12 and 24 hours. Following fast-refed experiments, the animals were allowed for 1 week to recover from fasting. Body weight was measured weekly. Body composition was analyzed with a small rodent EcoMRI (Echo Medical Systems). For AAV-DIO-CA-AMPK injected animals (Figure 1), daily food intake was measured by the average of daily food eaten during day 13-17 after viral

injection, and body fat composition was assessed 6 weeks after surgery. In Figure 4, body weight and food intake were assessed 4 weeks after surgery.

Fixed-tissue preparation, imaging and immunohistochemistry

Immunohistochemistry was performed as described (Kong et al., 2012). In brief, Mice were transcardially perfused with 4% paraformaldehyde and the brains were post-fixed for 1–2 days. Brains were sectioned coronally at 40 µm using a Leica microtome. Brain sections were washed in PBS with 0.25% Triton X-100 (PBT, pH 7.4) and incubated in 3% normal donkey serum (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) in PBT-azide for 2 hr. Slides were then incubated overnight at room temperature in a primary antiserum. After washing in PBS, sections were incubated in fluorescein-conjugated donkey IgG. Sections were mounted onto SuperFrost slides and then visualized with Zeiss Confocal Microscope or Leica SPE Confocal Microscope. Primary antibodies used in the current study and their dilutions are: rabbit anti-DsRed (Invitrogen, 1:2500, RRID: AB_10013483), rabbit anti-hrGFP (Agilent Tech, 1:2500, RRID: AB_10598674), chicken anti-EGFP (AVES, 1:5000, RRID: AB_2571867), and rabbit anti-pLIMK2 (Abcam, 1:200, RRID: AB_776030). Secondary antibodies include Alex594-Donkey anti rabbit IgG (Invitrogen, 1:200, RRID: AB_2571868) and FITC-Donkey anti-chicken IgG (Jackson ImmunoResearch Laboratory, 1:200, RRID: AB_2571869).

For the studies in Supplemental Figure S1, 150 nl AAV-DIO-DN-AMPK virus was unilaterally injected into the arcuate nucleus of 5~6 wks old male *Agrp-ires-cre/Npy-*

hrGFP or Pomc-cre/lox-GFP mice. 3 wks later, animals were perfused and the brains were sectioned coronally at 40 µm following fixation. Totally 4 parallel series of brain slices containing the hypothalamus from each mouse were collected and 1 of them was subject to immunofluorescence staining using antibodies against mCherry, hrGFP (for AgRP –related animals), and EGFP (for POMC – related studies). GFP+ neurons from all brain slices containing the arcuate nuclei were then counted under epifluorescence microscope.

α2AMPK activity measurement

To measure a2AMPK activity in AgRP neurons (Figure 1D), DIO-Tag-α2AMPK AAV virus was injected bilaterally into the Arcuate nucleus of *Agrp-IRES-Cre* knockin mice as described above in the “Stereotaxic injections” paragraph. After 4 weeks, fed or 24 h fasted mice were sacrificed by decapitation and exogenous Flag-tag a2AMPK was immunoprecipitated from Arcuate lysates with an anti-Flag antibody (Sigma-Aldrich, RRID: AB_259529) bound to protein-G sepharose beads. Immunoprecipitated Flag-tag a2AMPK was analyzed by western-blot with anti-Flag antibody and by a kinase activity assay (as described below). For other studies, endogenous a2AMPK was immunoprecipitated from protein lysates of the arcuate nucleus with a specific a2AMPK antibody (Santa Cruz, RRID: AB_2169717) bound to protein-G sepharose beads. Kinase activities of both exogenous Flag-tag a2AMPK (immunoprecipitated from AgRP neurons) and endogenous a2AMPK (immunoprecipitated from Arcuates) were measured using ‘SAMS’ peptide and γ-32P as described previously (Dagon et al., 2012).

In-vivo expression of constitutively active AMPK in the hypothalamus

Adenovirus expressing constitutively active HA-tagged γ1AMPK (5 x 10^6 pfu/0.1 µl) or control vector without DNA insert (5 x 10^6 pfu/0.1 µl) was injected bilaterally into the medial hypothalamus of male wildtype mice (age 8–9 weeks) as described above in the "Stereotaxic injections" paragraph. After 8 days, mice were sacrificed by decapitation and arcuate nuclei were quickly dissected and frozen in liquid nitrogen.

In-vivo fasting-refeeding studies

For the studies in Figure 3B-D & K, male wildtype mice were fasted overnight and refed for 6h. In all studies mice were sacrificed by decapitation and arcuate nuclei were quickly dissected as described previously and frozen in liquid nitrogen (Minokoshi et al., 2004).

In-vitro AMPK signaling studies

GT1-7 cells were cultured at 37 °C with 5% CO_2 in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS). GT1-7 cells were plated subconfluently overnight and glucose starved for 0.5 and 1 hr or treated with 0.25 or 1 mM AICAR. For other studies GT1-7 cells were: A) Transfected with CA-AMPK or empty vector for 24 h and collected for analysis, or B) Transfected with WT-PAK2 or phospho-defective S20A-PAK2 for 24h and then treated with 1 mM AICAR for 1 h. All DNA vectors were transfected with lipofectamine according to the manufacturer's protocol.

Immunoprecipitation and western blot analysis

Arcuate lysates were subjected to immunoprecipitation with a2AMPK antibody (Santa Cruz, RRID: AB_2169717) and immunoblotted with antibodies against PAK2 antibody (Pierce, RRID: AB_257183). Protein levels were analyzed with a2AMPK (RRID: AB_2169717), GAPDH (Santa Cruz, RRID: AB_627679), LIMK (Cell Signaling, RRID: AB_2297044), p-Thr^{505} LIMK (Cell Signaling, RRID: AB_2136943), p-Ser^{79} ACC (Cell Signaling, RRID: AB_2288840), ACC (Cell Signaling, RRID: AB_2219400), p-Ser^{20} PAK2 (Thermo Fisher Scientific, RRID: AB_10984997) and PAK2 (Pierce, RRID: AB_2571863).

Single-Cell RT-PCR

Transgenic *Npy-hrGFP* male mice (n=2; 5 weeks old) were deeply anesthetized by isoflurane and decapitated. Brains were rapidly harvested, immersed in ice-cold Earle's Balanced Salt Solution (EBSS, Sigma-Aldrich, E2888) for 5 minutes, and then blocked in a chilled brain matrix to obtain 1mm-thick coronal sections of arcuate nucleus. Arcuate sections were imaged through a fluorescence stereoscope (Zeiss SteREO Discovery.V8) with a 1.25x lens (Zeiss Objective Achromat S, FWD 50mm), and portions of arcuate nucleus containing GFP-labeled cells were microdissected. Tissue samples were enzymatically digested according to a published protocol (Saxena et al., 2012) and then gently triturated with fire-polished Pasteur pipets of incrementally smaller diameters (Hempel et al., 2007) (approximately 600µm, 300µm, and 150µm at tip). The resulting cell suspension was washed and re-suspended in a media solution (Saxena et al., 2012) then plated in a 35mm cell culture dish (Nunc, Thermo Scientific, 150318). Dissociated cells were visualized by fluorescence stereoscope and manually

sorted according to a published protocol(Hempel et al., 2007). Briefly, a glass micropipette with a tip diameter of 30-50μm was used to pick up individual GFP-labeled cells and transfer them through a series of washes to remove potential contaminants - *e.g*., extracellular mRNA from lysed cells. A clean micropipette was then used to deposit one cell *per* RNAse-free PCR tube (USA Scientific, 1402-2900) containing 4.5μL of lysis buffer(Tang et al., 2010). To control for mRNA contamination of the cell sorting media (*e.g*., from lysed cells), an equivalent volume of sorting media was also collected in a PCR tube and processed along with single-cell samples ("bath control"). Single-cell lysates and bath controls were immediately processed for cDNA synthesis and amplification. mRNA was reverse-transcribed into cDNA with poly-dT primers and then amplified with 20 PCR cycles(Tang et al., 2010). To control for inadvertent amplification of genomic DNA, additional single-cell lysates were processed concurrently without reverse transcriptase ("no RT control"). Additionally, as a positive control, cDNA was simultaneously synthesized and amplified from a single-cell amount (10pg) of arcuate RNA purified with a commercial kit (Purelink RNA Micro Scale Kit, Life Technologies,12183016). PCR was then performed by using the following primers: *Agrp: F:* TGGCTCCACTGAAGGGCATCA and *R:* TGCAGCCTTACACAGCGACG; *Pak1: F:* TGCGGGTGTTTGCTACCTCC and *R:* GCAATGGGGCTGGCATGACA; *Pak2: F:* TCCTCAATCCTCGAGAGCACCG and *R:* TCCGTCGCCAGCAATGGACA; *Pak3: F:* GCTGCCAGATTACATGGATGCCG and *R:* TGCACAGCCAAATGAGATACAACT.

Data analysis

Offline data analysis for electrophysiology was performed using custom software written in Igor Pro (Wavemetrics) and MATLAB. Statistical analyses were done using GraphPad PRIZM 6 software (GraphPad). All values are reported as means ± s.e.m.. Statistical significance was determined by unpaired two-tailed Student's *t*-tests or one-way ANOVA test. $P < 0.05$ was considered to be statistically significant.

REFERENCE

Balthasar, N., Coppari, R., McMinn, J., Liu, S.M., Lee, C.E., Tang, V., Kenny, C.D., McGovern, R.A., Chua, S.C., Jr., Elmquist, J.K.*, et al.* (2004). Leptin receptor signaling in POMC neurons is required for normal body weight homeostasis. Neuron *42*, 983-991.

Dagon, Y., Hur, E., Zheng, B., Wellenstein, K., Cantley, L.C., and Kahn, B.B. (2012). p70S6 kinase phosphorylates AMPK on serine 491 to mediate leptin's effect on food intake. Cell Metab *16*, 104-112.

Hempel, C.M., Sugino, K., and Nelson, S.B. (2007). A manual method for the purification of fluorescently labeled neurons from the mammalian brain. Nat Protoc *2*, 2924-2929.

Kong, D., Tong, Q., Ye, C., Koda, S., Fuller, P.M., Krashes, M.J., Vong, L., Ray, R.S., Olson, D.P., and Lowell, B.B. (2012). GABAergic RIP-Cre neurons in the arcuate nucleus selectively regulate energy expenditure. Cell *151*, 645-657.

Kozorovitskiy, Y., Saunders, A., Johnson, C.A., Lowell, B.B., and Sabatini, B.L. (2012). Recurrent network activity drives striatal synaptogenesis. Nature *485*, 646-650.

Krashes, M.J., Shah, B.P., Madara, J.C., Olson, D.P., Strochlic, D.E., Garfield, A.S., Vong, L., Pei, H., Watabe-Uchida, M., Uchida, N.*, et al.* (2014). An excitatory paraventricular nucleus to AgRP neuron circuit that drives hunger. Nature *507*, 238-242.

Liu, T., Kong, D., Shah, B.P., Ye, C., Koda, S., Saunders, A., Ding, J.B., Yang, Z., Sabatini, B.L., and Lowell, B.B. (2012). Fasting activation of AgRP neurons requires NMDA receptors and involves spinogenesis and increased excitatory tone. Neuron *73*, 511-522.

Minokoshi, Y., Alquier, T., Furukawa, N., Kim, Y.B., Lee, A., Xue, B., Mu, J., Foufelle, F., Ferre, P., Birnbaum, M.J.*, et al.* (2004). AMP-kinase regulates food intake by responding to hormonal and nutrient signals in the hypothalamus. Nature *428*, 569-574.

Saxena, A., Wagatsuma, A., Noro, Y., Kuji, T., Asaka-Oba, A., Watahiki, A., Gurnot, C., Fagiolini, M., Hensch, T.K., and Carninci, P. (2012). Trehalose-enhanced isolation of neuronal sub-types from adult mouse brain. Biotechniques *52*, 381-385.

Tang, F., Barbacioru, C., Nordman, E., Li, B., Xu, N., Bashkirov, V.I., Lao, K., and Surani, M.A. (2010). RNA-Seq analysis to capture the transcriptome landscape of a single cell. Nat Protoc *5*, 516-535.

Tong, Q., Ye, C.P., Jones, J.E., Elmquist, J.K., and Lowell, B.B. (2008). Synaptic release of GABA by AgRP neurons is required for normal regulation of energy balance. Nat Neurosci *11*, 998-1000.

van den Pol, A.N., Yao, Y., Fu, L.Y., Foo, K., Huang, H., Coppari, R., Lowell, B.B., and Broberger, C. (2009). Neuromedin B and gastrin-releasing peptide excite arcuate nucleus neuropeptide Y neurons in a novel transgenic mouse expressing strong Renilla green fluorescent protein in NPY neurons. J Neurosci *29*, 4622-4639.