Materials and Methods

Generation of POMC-and NPY GFP transgenic mice: The POMC-Tau-Topaz GFP and NPY-Tau-Sapphire GFP transgenic mice were created using the BAC transgenic technology developed by Yang, et al. \( (S1) \). Topaz GFP and sapphire GFP are yellow- and blue-shifted GFP variants, respectively \( (S2) \) from Aurora Biosciences (La Jolla, CA). A 2 kb fragment of the POMC gene was subcloned into pGEMzf11- (Promega, Inc., Madison, WI) using HindIII sites, and a PacI restriction site was engineered after AGAGAGCTG in the first exon of the POMC gene, leaving 1.3kb of POMC sequence 5’ and 0.7 kb of POMC sequence 3’ to the PacI site. The Tau-Topaz GFP fusion protein followed by a poly A signal were then inserted into the PacI site, and the entire fragment was inserted to the pSV shuttle vector \( (S1) \). Kozak sequence was added before Tau, a microtubule-binding protein \( (S3) \), and the poly A signal was PCR amplified from nucleotides 697-1088 of SV40 poly A of pREP7 (Invitrogen, Carlsbad, CA). POMC BAC clones were obtained after screening BAC filters from Genome Systems (St. Louis, MO), and after homologous recombination occurred between the shuttle vector and the POMC BAC in DH10B \( E. coli \) host, the cointegrate POMC BAC was generated. Subsequently a second homologous recombination produced a modified BAC with Kozak-Tau-Topaz-poly A sequence inserted into the first exon of the POMC gene (Fig. 1B).

The NPY-Tau-Sapphire GFP transgenic mice were created similar to the POMC-Tau-Topaz GFP transgenic mice. A ~3.8kb fragment of the NPY gene was subcloned into pGEMzf11-. The ATG translation start site at the start of the second exon of the NPY gene was mutated to ATT, and a PacI restriction site was inserted immediately after the
mutated site. This left 1 kb of 5’ NPY sequence and 2.8 kb of 3’ NPY sequence relative to the PacI site. The Tau-Sapphire fusion protein and the poly A signal were then inserted into the PacI site, and the entire fragment was inserted to the pSV shuttle vector. NPY BAC clones were obtained after screening BAC filters from Genome Systems (St. Louis, MO). As with the POMC transgenic construct, homologous recombination steps then produced the modified NPY BAC with the Kozak-Tau-Sapphire-polyA sequence inserted near the mutated translation start site of the NPY gene (Fig. 1A).

The homologous recombination of the modified BACs was confirmed through Southern analysis. The modified BAC DNAs were then purified with Cesium Chloride gradient and Sepharose CL4b column (Sigma, St. Louis,MO), and injected into pronuclei of CBA/C57Bl6 F1 mice by the Rockefeller transgenic facility. The incorporation of the transgene in mouse genome was identified through Southern blot analysis and PCR.

**Immunofluorescence and GFP Colocalization**

Anesthetized animals were perfused transcardially with saline followed by fixative containing 4% paraformaldehyde (PFA) in PBS. Brains were post-fixed overnight. Serial coronal sections were cut using a vibrotome (Leica VT1000S). Immunofluorescence was carried out as follows: free-floating 50 μm brain sections were incubated in blocking solution (0.1% Triton X-100, 3% bovine serum albumin, 2% goat serum in PBS) for 1 hr at room temperature, followed by an overnight incubation with primary antibody at 4°C. Sections were then washed and incubated with the secondary antibody at room temperature for two hours. Primary antibodies and their final concentrations were as follows: anti-POMC antibody (1:200, Phoenix Pharmaceuticals, Belmont, CA), anti-NPY antibody (1:1000 Phoenix Pharmaceuticals, Belmont, CA).
Secondary antibody’s final concentration is as follows: rhodamine anti-rabbit immunoglobulin (Jackson ImmunoResearch Laboratories Inc., West Grove, PA). NPY-GFP animals were treated with colchicine to enhance cell body staining. Briefly, 43 hours before perfusion, colchicine (40 µg in 10 µl saline) was injected into the lateral ventricle using a Hamilton syringe over a period of 30 min. The needle was removed after 10 minutes. Sections were examined using a Zeiss Axioplane microscope or a Zeiss LSM 510 confocal microscope (for POMC colocalization). POMC-GFP/+; NPY-GFP/+ mice were perfused and sections were prepared as described above. Sections were visualized directly (without immunohistochemistry) using the appropriate filters (see below). Images were collected using the multichannel module of the AxioVision Zeiss software.

To assess whether the NPY innervations of POMC neurons are of local origin, we performed immunohistochemistry on sections from POMC-GFP brains using antibodies against AgRP or NPY. Sections were processed as above and incubated with rabbit anti-AgRP (1:5000; Calbiochem, San Diego CA) or anti-NPY (as above) antisera followed by secondary labeling with rhodamine anti-rabbit immunoglobulin (Jackson ImmunoResearch Laboratories Inc., West Grove, PA). Sections were analyzed using a fluorescence microscope and the contacts between AgRP-immunolabeled axon terminals and POMC-GFP neurons were counted.

**Mouse crosses**

NPY-GFP or POMC-GFP; ob/ob were generated by two successive crosses. First, NPY-GFP/+ or POMC-GFP/+ mice were crossed to ob/+ mice (C57BL/6 background) purchased from Jackson Laboratory (Bar Harbor, ME). Then, progeny were intercrossed
to generate NPY-GFP; *ob*/ob or POMC-GFP; *ob*/ob mice. Genotypes were determined by genomic PCR.

**Leptin treatment:** For the 6 hour leptin treatment, a single intraperitoneal injection of 25 µg/g leptin or a similar volume of PBS were given to 8 week old sex matched- obese transgenic mice at 10 AM. The mice were then perfused 6 hrs later. Serum samples were taken just prior to perfusion. For the 2 and 12-day leptin treatment, 8 weeks old sex matched- obese transgenic mice were anesthetized and implanted with subcutaneous 2002 Alzet mini-osmotic pumps (Palo Alto, CA) filled with either PBS (control) or 420 ng/µl leptin (Amgen, Thousand Oaks, CA). Pumps were incubated the night before at 37°C in sterile 0.9% NaCl solution. Animals and food were weighed daily at midday, and the animals were perfused 2 or 12 days after implantation.

**Ghrelin treatment**

8-10 wk old wild type mice carrying either the NPY-GFP or POMC-GFP transgene were given intraperitoneal injections of 100 µl of 0.1 µg/µl octanoylated ghrelin in PBS (generous gift of M. Tschöp) or with PBS alone at 9 am and 3 pm for four consecutive days. Food intake was measured at 15, 30, 60 and 120 minutes after each injection. Body weight was taken prior to each injection. Animals were perfused between 30 to 60 minutes following the last injection of the fourth day.

**Leptin measurement**

Serum leptin levels were measured using the Quantikine M mouse leptin immunoassay ELISA kit as described by the manufacturer (R&D systems Minneapolis MN).
Slice Preparation for Electrophysiology

Adult NPY-GFP and POMC-GFP mice (4-8 weeks old) were deeply anesthetized with halothane prior to decapitation and removal of the entire brain. The brain was immediately submerged in ice cold, carbogen-saturated (95%O₂/5%CO₂) artificial cerebrospinal fluid (aCSF), and a brain block containing the hypothalamus was made. The aCSF contained (in mM): 126 NaCl, 2.5 KCl, 2.4 CaCl₂, 1.2 NaH₂PO₄, 1.2 MgCl₂, 21.4 NaHCO₃, and 11.1 glucose. Coronal sections (180 µm) were cut with a Leica VT1000S vibratome, and the slices were incubated at 37°C for approximately 30 minutes followed by incubation at room temperature until used.

Electrophysiological Recording

Slices were transferred to the recording chamber and allowed to equilibrate for 10-20 min prior to use. The slices were bathed in oxygenated aCSF heated to approximately 29-30°C at a flow rate of 1.7-2 ml/min. GFP-positive NPY and POMC cells in the Arc were visualized using epifluorescence and IR-DIC imaging on an upright Zeiss Axioskop 2FS Plus microscope equipped with a fixed stage and a Sony XC-75 CCD camera. Filter sets were from Chroma Technology Corp (Rockingham, VT). The characteristics of the filter sets were as follows (values are exciter, emitter, beamsplitter): Sapphire GFP (NPY) D395/40, D510/40, 425dclp; Topaz GFP (POMC) HQ500/20, HQ535/30, Q515lp. GFP neurons were identified via epifluorescence and then patched under IR-DIC optics. Recordings were made using a HEKA EPC9/2 amplifier under the control of Pulse software. Cells were voltage clamped at -60mV, and sampled at a frequency of 20 kHz. Currents were recorded for 1 second every 3 seconds, and PSCs were detected using the MiniAnalysis Program. The frequency of PSCs was then
calculated at either 15-second intervals or for 3-minute periods before and after TTX addition. All membrane potentials reported were not corrected for liquid junction potentials.

For measurement of EPSCs, a cesium-methanesulfonate based internal solution (in mM: 125 CsMeSO₃, 10CsCl, 5 NaCl, 10 HEPES, 1 EGTA, 2 MgCl₂, 5 MgATP, 0.3 NaGTP, pH~7.35 with NaOH) was used, and picrotoxin (100µM) was added in the bath to block IPSCs. For measurement of IPSCs, a cesium-chloride based internal solution (in mM: 140 CsCl, 10 HEPES, 5 MgCl₂, 1 BAPTA, 5 MgATP, 0.3 NaGTP, pH~7.35 with NaOH) was used, and D-AP5 (50µM) and CNQX (10µM) were added in the bath to block EPSCs. Electrodes had resistances of ~2.7-3.3 mΩ when filled with the Cs-Methanesulfonate internal solution and ~1.8-2 MΩ when filled with the CsCl internal solution. Series resistance was monitored over the course of the recordings, and values were generally <10 MΩ and were not compensated. Cells were excluded if the series resistance increased significantly during the experiment. TTX (1µM) was added in the bath to isolate mPSCs. All values reported are means +/- SEM. Data analysis was performed using PulseFit, IgorPro, Microsoft Excel, and S-Plus. All figures were created using PulseFit and IgorPro. Wilcoxon rank sum tests and t-tests were used to compare mean frequencies.

**Electron Microscopy**

A tissue block containing the hypothalamus was dissected from each brain. Fifty µm thick vibratome sections were cut and thoroughly washed in 0.1 M phosphate buffer (PB). To eliminate unbound aldehydes, sections were incubated in 1% sodium-borohydride for 15 min, then rinsed in PB. Next, sections were incubated in rabbit anti-
Green Fluorescent Protein (GFP) (Molecular Probes Inc., Eugene, OR) (dilution 1:1000 in PB) for 24 h at room temperature. Subsequently, sections were incubated in biotinylated goat anti-rabbit immunoglobulin (dilution 1: 250; Vector Laboratories, Burlingame, CA, USA) at room temperature.

After a thorough wash in PB, sections were placed in avidin-biotin-complex (ABC Elite Kit, Vector Labs) for 2 hr at room temperature. The tissue-bound peroxidase was visualized by a diaminobenzidine reaction. After the immunostaining, the sections were osmicated (15 min in 1% osmic acid in PB), and dehydrated in increasing ethanol concentrations. During the dehydration, 1% uranyl-acetate was added to the 70% ethanol to enhance ultrastuctural membrane contrast. Dehydration was followed by flat-embedding in Araldite. Ultrathin sections were cut on a microtome, collected on Formvar-coated single-slot grids and analyzed with a Tecnai 12 Biotwin (FEI Company) electron microscope.

The quantitative analysis of synapse numbers was performed in a double-blind fashion on electron micrographs from mice of different experimental groups. To obtain a complementary measure of axo-somatic synaptic number, unbiased for possible changes in synaptic size, the disector technique was used (S4). On consecutive 90-nm-thick sections, we determined the average projected height of the synapses and used about 30% of this value as the distance between the dissectors. On the basis of this calculation, the number of axo-somatic synapses was counted from 7 perikaryal profiles in two consecutive serial sections roughly 270 nm apart (the "reference" and "look-up" sections) from each tissue block. In order to increase the sampling size, the procedure was repeated in such a way that the reference and look-up sections were reversed. The number of
symmetrical, asymmetrical and total contacts were collected independently from serial
sections. Synapse characterization was performed at 20,000 magnification, while all
quantitative measurements were performed on electron micrographs at a magnification of
11,000. Symmetric and asymmetric synapses were counted on all selected neurons only
if the pre- and/or postsynaptic membrane specializations were clearly seen and synaptic
vesicles were present in the presynaptic bouton. Synapses with neither clearly symmetric
nor asymmetric membrane specializations were excluded from the assessment. The
plasma membranes of selected cells were outlined on photomicrographs and their length
was measured with the help of a chartographic wheel. Plasma membrane length values
measured in the individual animals were added and the total length was corrected to the
magnification applied. Synaptic densities were evaluated according to the formula
\[ NV = \frac{Q - V_{dis}}{V_{dis}} \]
where \( Q \) represents the number of synapses present in the "reference"
section that disappeared in the "look-up" section. \( V_{dis} \) is the disector volume (volume of
reference) which is the area of the perikaryal profile multiplied by the distance between
the upper faces of the reference and look-up sections, i.e. the data are expressed as
numbers of synaptic contacts per unit volume of perikaryon. Section thickness was
determined by using the Small's minimal fold method. The synaptic counts were
expressed as numbers of synapses on a membrane length unit of 100 μm. Since an F-test
analysis of our synaptic counts in the arcuate nucleus of the mice has revealed a
significant nonhomogeneity of variances between groups, the Kruskal-Wallis one-way
non-parametric analysis of variance test was selected for multiple statistical comparisons.
The Mann-Whitney U-test was used to determine significance of differences between the
groups. A level of confidence of P < 0.05 was employed for statistical significance. The morphometric analysis was carried out in a blinded fashion.

References


Figure legends:

**Fig. S1. AgRP input onto the POMC neurons.**

Immunolabeling for NPY (red, top) or AgRP (red, bottom) revealed multiple contacts between AgRP and NPY boutons with GFP-labeled POMC perikarya (green) in the arcuate nucleus. The larger image demonstrates multiple contacts between AgRP boutons (red) and POMC-GFP perikarya (green). Bar scale represents 100 µm. Contacts between AgRP-immunolabeled axon terminals and GFP-labeled POMC neurons were counted in 3 different brains. 273 of the 300 GFP-POMC perikarya evaluated were contacted by numerous AgRP boutons. Bar scale represents 100 µm.

**Fig. S2. Food intake and body weight 6 hrs, 2 days and 12 days post leptin treatment.** No significant changes in food intake or body weight were observed 6 hrs after intraperitoneal leptin injection. Leptin levels were measured just prior to perfusion and found to be 12.7±2.7 ng/ml. Food intake and body weight were taken during 2 or 12 days of continuous treatment with leptin or saline using a subcutaneous osmotic pump. A
significant reduction in food intake was observed at the end of the second day of the 2
days treatment relative to saline treated mice ($P < 0.05$, saline versus leptin on day 2) with
no change in body weight in comparison to the saline treated mice. At 12 days post
leptin treatment, mice showed a significant reduction in body weight and food intake
($p<0.0005$ saline versus leptin at day 12). Food consumption of NPY-GFP and POMC-
GFP was indistinguishable and has been pooled. Error bars indicate SEM. n$\geq$6 for each
group.

**Fig. S3. Peripheral injection of ghrelin effect on food intake and body weight**

A significant increase in food intake was evident 30 min following intraperitoneal
injection of 10µg of octanoylated ghrelin. However, no significant changes were
observed in body weight after 4 days of twice-daily injection. The food consumption
data was pooled, as there were no differences observed between different sexes and
genotypes. Error bars indicate SEM, n=6-8 for each group.

**Table S1. Summary of the number of synapses after leptin replacement in ob/ob.**

(A) **Number of synapses per 100µm perikaryal membrane of NPY neurons**

<table>
<thead>
<tr>
<th></th>
<th>ob/ob+PBS- 6 hours</th>
<th>ob/ob+Leptin- 6 hours</th>
<th>ob/ob+PBS- 2 days</th>
<th>ob/ob+Leptin- 2 days</th>
<th>ob/ob+PBS- 12 days</th>
<th>ob/ob+Leptin- 12 days</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total</strong></td>
<td>$34.76\pm2.09$</td>
<td>$22.78\pm3.47^*$</td>
<td>$42.65\pm2.83$</td>
<td>$25.44\pm1.58#$</td>
<td>$38.86\pm1.67$</td>
<td>$20.34\pm0.76^*$</td>
</tr>
<tr>
<td><strong>Stimulatory</strong></td>
<td>$26.73\pm0.88$</td>
<td>$5.45\pm0.65^{**}$</td>
<td>$25.44\pm1.58$</td>
<td>$4.76\pm0.80^{#}$</td>
<td>$33.72\pm2.0$</td>
<td>$4.34\pm0.68^{^^}$</td>
</tr>
<tr>
<td><strong>Inhibitory</strong></td>
<td>$9.27\pm0.51$</td>
<td>$14.28\pm1.75^{***}$</td>
<td>$13.04\pm0.65$</td>
<td>$22.27\pm1.21^{##}$</td>
<td>$6.65\pm0.77$</td>
<td>$17.39\pm1.12^{^^^}$</td>
</tr>
</tbody>
</table>

* $P < 0.05$  ** $P < 0.0005$  *** $P < 0.05$  PBS, n=3, 21 cells; leptin, n=4, 28 cells

# $P < 0.01$, ##$P < 0.0005$, ###$P < 0.005$  PBS:n=3; Leptin:n=3, 21 cells; Leptin n=3, 21 cells

^$P < 0.0005$, ^^$P < 0.00005$, ^^^$P < 0.001PBS: n=3, 21 cells: Leptin:n=4, 28 cells

(n refers to the number of animals)

(B) **Number of synapses per 100µm perikaryal membrane of POMC neurons**
<table>
<thead>
<tr>
<th></th>
<th>ob/ob+PBS- 6 hours</th>
<th>ob/ob+Leptin- 6 hours</th>
<th>ob/ob+PBS- 2 days</th>
<th>ob/ob+Leptin- 2 days</th>
<th>ob/ob+PBS- 12 days</th>
<th>ob/ob+Leptin- 12 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>24.55±2.05</td>
<td>32.62±4.0</td>
<td>20.45±2.30</td>
<td>46.14±3.06 #</td>
<td>25.33±2.10</td>
<td>47.14±5.02 ^</td>
</tr>
<tr>
<td>Stimulatory</td>
<td>8.37±1.37</td>
<td>22.95±5.2 *</td>
<td>7.98±1.34</td>
<td>31.42±1.43 #</td>
<td>8.19±0.75</td>
<td>30.24±3.24 ^</td>
</tr>
<tr>
<td>Inhibitory</td>
<td>17.46±2.89</td>
<td>15.24±1.36</td>
<td>13.51±1.43</td>
<td>15.71±2.19</td>
<td>16.09±2.62</td>
<td>14.89±0.93</td>
</tr>
</tbody>
</table>

*P < 0.05   PBS: n=5, 35 cells; Leptin n=5, 35 cells

#P < 0.005, ##P < 0.0005 PBS:n=3, 21 cells; Leptin: n=5; 35 cells

^P < 0.05 , ^^P < 0.005 PBS: n=3, 21 cells; Leptin:n=6, 42 cells

(n refers to the number of animals)
Fig. S1
Fig. S2

Food intake

6 hrs

Food intake/6 hours (g)

saline

Leptin

Body weight

10 am 4 pm

Body weight (grams)

saline

leptin

48 hrs

grams

24 hrs 48 hrs

saline

leptin

12 days

grams

1 2 3 4 5 6 7 8 9 10 11 12

Day

saline

leptin

Body weight (grams)

0 24 hrs 48 hrs

0 24 hrs 48 hrs

saline

leptin

Body weight

0 5 10 15 20 25 30 35 40 45

0 5 10 15 20 25 30 35 40 45

** * * * * * * **

0 2 4 6 8

0 2 4 6 8

1 2 3 4 5 6 7 8 9

1 0 1 1 2

1 2 3 4 5 6 7 8 9

1 0 1 1 2

0 1 2 3 4 5 6 7 8 9 10 11 12

Day

0 1 2 3 4 5 6 7 8 9 10 11 12

Day

* * * * * *
Fig. S3

A

Accumulative food intake (gram)

Time after injection (min)

0-15 0-30 0-60 60-120

B

Body Weight (gram)

pre ghrelin post ghrelin

pre saline post saline