loss (supplementary materials). However, the prominent mountains at the western margin of SP, and the strange, multikilometer-high mound features to the south are both young geologically and presumably composed of relatively strong, water-ice–based geological materials. Their origin, and what drove their formation so late in solar system history, remain uncertain. What is more certain is that all three major Kuiper belt bodies (past or present) visited by spacecraft so far—Pluto, Charon, and Triton—are more different than similar and bear witness to the potential diversity (past or present) visited by spacecraft so far.

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0.25 ± 0.02 g; n = 330 OGT knockout mouse meals (7 mice), 0.43 ± 0.02 g (Fig. 1E and fig. S4, C to E). The point at which a meal is terminated depends on the total caloric content of the food but also noncaloric determinants of the food, mainly its volume and composition. When fed either regular carbohydrate-rich pellets or fat-based food paste with higher caloric density, the OGT knockout mice overate the same amount of calories (fig. S4F).

Immunohistochemistry for OGT showed that within the core feeding circuitry, the major loss of OGT in the knockout mice occurred in medial nuclei of the hypothalamus, most notably in the paraventricular nucleus (PVN) (Fig. 2A and fig. S5A). There was only minor loss of OGT from nuclei of the midbrain and the brainstem (Fig. 2A and fig. S5A). Using oCaMKII-CreER+ × TdTh mice and immunohistochemistry, we noticed some oCaMKII PVN cells expressed thyrotropin-releasing hormone (TRH) and some oxytocin (fig. S6A). Before any major weight gain, OGT deletion reduced the expression of TRH, as determined with in situ hybridization, in a subpopulation of cells in the PVN without affecting their viability, whereas several other known neuropeptides regulating feeding behavior were largely unaltered: proopiomelanocortin (POMC), cocaine- and amphetamine-regulated transcript (CART), orexin, agouti-related peptide (AgRP), neuropeptide Y (NPY), vasopressin (AVP), and oxytocin (TRH, n = 4 WT mice, 40 ± 4.1 cells/150 × 10^3 μm²; n = 4 OGT knockout mice, 23 ± 2.9 cells/150 × 10^3 μm²) (Fig. 2B and figs. S6, B to E, and S7A) (J2, J3). Next, we infected hypothalamic organotypic cultures with a virus that expresses green fluorescent protein (GFP) under the oCaMKII promoter and treated the explants with varying concentrations of glucose. The PVN retained its normal morphology and expression of neuropeptide during the extent of the experiment (~2.5 weeks) (fig. S8A). Immunohistochemistry for O-GlcNAc demonstrated that 1 hour of treatment with 5 mM glucose, simulating physiological and meal-derived fluctuations in glucose, increased O-GlcNAc in oCaMKII-positive cells [1 mM glucose, n = 300 cells, 134 ± 10 intensity/cell (arbitrary units, a.u.); 5 mM glucose n = 330 cells, 211 ± 12 intensity/cell (a.u.)] (Fig. 2C) (H4–H6). In contrast, there was no change in O-GlcNAc levels in neighboring oCaMKII-negative cells (fig. S8B). Incubation for 16 hours with 25 mM glucose raised the O-GlcNAc levels in oCaMKII cells to a larger extent [5 mM glucose, n = 390 cells, 156 ± 9 intensity/cell (a.u.); 25 mM glucose, n = 375 cells, 298 ± 14 intensity/cell (a.u.)] (fig. S8, C and D). Unlike cortex and the hippocampus, where O-GlcNAc levels mirror meal intake, it has recently been reported that fasting increased O-GlcNAc in AgRP neurons, which has been proposed to be due to stimulation of OGT expression by ghrelin in these cells (6, 7, 15). In the PVN, fasting decreased O-GlcNAc, but only in oCaMKII-positive cells [oCaMKII-positive cells, fed mice n = 455 cells, 158 ± 5 intensity/cell (a.u.); starved mice n = 259 cells, 81 ± 6 intensity/cell (a.u.)] (fig. S8E). In addition, we compared the expression of the immediate early gene c-Fos in oCaMKII PVN cells in vivo in mice fed ad libitum or refed upon starvation. This enabled us to characterize cellular responses to food intake as mice start eating after a period of starvation. Food intake appeared to activate oCaMKII cells in the PVN, and loss of OGT blocked this activation completely (fig. S8, F and G).

O-GlcNAc is highly expressed in neuronal synapses in the brain (17). We crossed oCaMKII-CreER+ × OGTfl/fl × TdTh mice to assess whether OGT regulates excitatory synaptic input onto oCaMKII PVN cells. If deleting OGT attenuates excitatory input, it would explain, at least in part, how OGT regulates the activity of these cells. The mean capacitance of labeled cells averaged ~15 pF and did not differ between WT and knockout mice (fig. S9A). The mean miniature excitatory post-synaptic current (mEPSC) frequency decreased by 72% in OGT knockout cells (n = 6 WT cells, 1.75 ± 0.11 Hz; n = 6 OGT knockout cells, 0.50 ± 0.10 Hz) (Fig. 3, A and B). The sharp decrease in mEPSC frequency suggests that OGT is essential for maintaining the number of functional excitatory synapses onto oCaMKII PVN neurons.

![Fig. 1. Acute deletion of OGT in oCaMKII-positive neurons causes hyperphagia-dependent obesity.](http://science.sciencemag.org/)

![Fig. 2. OGT-mediated hyperphagia is associated with feeding circuitry function in the PVN.](http://science.sciencemag.org/)
The mean mEPSC amplitude was also decreased, but to a much smaller degree (n = 6 WT cells 19.7 ± 1.2 pA; n = 6 knockout cells, 15.6 ± 0.9 pA) (Fig. 3, A and C). Neither the rise time nor the decay time of the EPSCs changed upon deletion of OGT (fig. S9, B and C). pharmacological inhibition of glutamatergic signaling in the PVN has previously been shown to elicit intense feeding (36).

Selectively deleting OGT in the oCaMKIIa/CaMKIIa neurons of the PVN by means of stereotactic virus injection caused concurrent obesity and hyperphagia (Fig. 4, A to C, and fig. S9D). Because deletion of OGT in oCaMKIIa/CaMKIIa neurons in the PVN prevents their feeding-induced activation and leads to hyperphagia, we predicted that stimulating those cells would decrease food intake. Activating oCaMKIIa/CaMKIIa PVN cells optogenetically decreased cumulative food consumption over 24 hours (baseline, n = 11 experiments (5 mice), 3.6 ± 0.14 g; 50 Hz, n = 11 experiments (5 mice), 2.6 ± 0.33 g) (Fig. 4, D and E) (19). The amount of food ingested per meal was smaller; whereas there was no significant effect on meal frequency (meal size, baseline, n = 125 meals, 0.25 ± 0.014 g; 50 Hz, n = 110 meals, 0.20 ± 0.014 g) (Fig. 4F and fig. S9E). In a second experiment, we started the stimulation at the onset of darkness after fasting the mice during the light phase. Fasting increased food intake, and 20 Hz optogenetic stimulation for 4 hours produced a significant decrease in food intake, whereas 50 Hz stimulation for 1 or 4 hours produced large, significant drops in feeding (fig. S9F). The decrease in meal size with 50 Hz stimulation was evident in the first meal, without any change in latency to initiation of feeding (fig. S9, G and H).

Together, our data favor a model in which the function of OGT is to couple energy intake with energy need, at least in part, by regulating the excitatory synaptic input in oCaMKIIa/CaMKIIa PVN cells (Fig. 4G). The select effect on meal termination suggests that OGT regulates satiation. oCaMKIIa/CaMKIIa neurons are often excitatory. Although vGlut2-positive excitatory neurons in the PVN decrease food intake, there appears to be only partial overlap between oCaMKIIa/CaMKIIa and vGlut2 expression in the PVN (20, 21). Because glucose remains elevated in the cerebrospinal fluid more than 1 hour upon eating, the feeding-related changes in O-GlcNAc integrates nutrient availability on a time scale longer than a single meal (14, 22, 23). The brain promotes satiation by coordinating adipokines, reflecting body energy deposits, and acute food-derived signals into circuits that turn off feeding. Rather than constituting a link in meal-to-meal satiety feedback loops, our observations suggest that OGT controls the threshold of such loops through its regulation of excitatory synaptic transmission onto oCaMKIIa/CaMKIIa PVN neurons. The observation that OGT knockout mice quickly reached a plateau in daily food intake supports this idea. Thresholding satiation between meals confers the behavioral advantage of stabilizing caloric intake over time so that the previous meal informs on the caloric need of the next. These data do not exclude additional, faster regulation of OGT activity and levels via metabolic hormones. These findings identify the regulation of excitatory synapses onto oCaMKIIa/CaMKIIa PVN neurons by OGT as an important mechanism underlying satiation, representing a potential medicinal target for human obesity.

**REFERENCES AND NOTES**

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The maternal microbiota drives early postnatal innate immune development

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Postnatal colonization of the body with microbes is assumed to be the main stimulus to postnatal immune development. By transiently colonizing pregnant female mice, we show that the maternal microbiota shapes the immune system of the offspring. Gestational colonization increases intestinal group 3 innate lymphoid cells and F4/80+CD11c+ mononuclear cells in the pups. Maternal colonization reprograms intestinal transcriptional profiles of the offspring, including increased expression of genes encoding epithelial antibacterial peptides and metabolism of microbial molecules. Some of these effects are dependent on maternal antibodies that potentially retain microbial molecules and transmit them to the offspring during pregnancy and in milk. Pups born to mothers transiently colonized in pregnancy are better able to avoid inflammatory responses to microbial molecules and penetration of intestinal microbes.

During pregnancy, the eutherian fetus inhabits a largely sterile environment in utero, protected from infections by maternal immunity. Rejection of the allogeneic fetus is avoided through maternal and fetal vascular separation, the immune privileged status of the placental trophoblast, and gestational maternal tolerance mechanisms (7). At birth, the situation changes dramatically as body surfaces become progressively colonized with microbes, directly exposing the immature neonatal immune system to potential pathogens (2, 3). Despite continued protection from the immunoglobulins and antibacterial peptides in milk, the consequence of this transition for human health is that most of the worldwide mortality in children up to 5 years old is due to infectious disease (4–6).

Immune system development is both programmed in neonatal tissues and driven later by exposure to pathogenic and nonpathogenic microbes (3). Germ-free mice have low immunoglobulin concentrations; lymphopenia of lymphoid structures; reduced bone marrow leukocyte pools; and aberrant innate and adaptive immune functions (7, 8). It has been widely assumed that most microbiota-driven immune alterations are postnatal effects induced by the neonate’s own microbiota (2, 9, 10). Here, we challenge this assumption by asking how the maternal microbiota in pregnancy alone affects the early postnatal immune system of the offspring.

To achieve gestation-only colonization under conditions where the mice deliver their pups spontaneously at term, we used a system in which pregnant dams are transiently colonized with genetically engineered *Escherichia coli* HA107 (11). Because this strain does not persist in the intestine, pregnant dams become germ-free again before term and naturally deliver germ-free pups (fig. S1A). Although *E. coli* is a minor component of the adult human microbiota, it is commoner in the neonatal intestine (12) and a frequent cause of human neonatal sepsis (13).

**Gestation-only colonization shapes the intestinal mucosal innate immune composition**

Gestation-only colonization with *E. coli* HA107 altered the numbers of early postnatal intestinal innate leukocytes in wild-type C57BL/6 mice. At postnatal day 14, there was an increase in small intestinal innate lymphoid cell (ILC) proportions and total numbers compared with germ-free controls, particularly the NKP46+RORγt+ ILC3 subset (Fig. 1, A and B, and fig. S1B). Small intestinal NKP46+RORγt+ ILC3 are described in germ-free mice (14), but persistently increased following transient gestational colonization, reaching a maximum in 14- to 21-day-old pups: This increase persisted even after weaning (Fig. 1C and fig. S1C), consistent with increased small intestinal ILC3 content of colonized compared with germ-free mice (15) and the microbiota-dependent modulation of RORγt expression in this subset (16). Increases in the expression of the cytokine interleukin-22 (IL-22) in this population have been observed following permanent colonization or the introduction of segmented filamentous bacteria to the microbiota (17, 18). Total numbers of IL-22–expressing cells increased in line with the increased NKP46+RORγt+ ILC3 numbers as a result of gestational colonization, although individual IL-22 expression levels did not change, likely because the pups were born and raised germ-free (fig. S1, D to F).

There was also an increase in the small and large intestinal F4/80+CD11c+ mononuclear cells (iMNCs) in day 14 (d14) pups born to gestation-only colonized dams (Fig. 1, D and E, and fig. S2, A to C), whereas the F4/80+CD11c+ macrophages, F4/80+CD11c+ dendritic cells (DCs), and the CD103+ or CD11b+ DC subpopulations were not significantly affected (Fig. 1, D and E, and fig. S2, B to E). The gestational effects on increased F4/80+CD11c+ iMNCs were also maximal between postnatal days 14 to 21, and they persisted until at least 8 weeks of age in the colon (Fig. 1F). Gestational colonization caused no significant changes in small intestinal ILC2 numbers (fig. S3, A and B) or in other early postnatal innate leukocyte populations in either systemic or intestinal tissues (table S1). These results showed that temporary colonization of a pregnant dam has long-term consequences for certain populations of innate lymphoid and monocytic cells in the intestines of her offspring.

We next sought to verify that the effects of gestational *E. coli* on early postnatal innate leukocytes would also be seen with animals stably colonized by a different microbiota both in the mother and after birth. We compared C57BL/6 animals carrying the defined altered Schaedler flora (ASF) of eight microbes with germ-free controls. Both small intestinal NKP46+ ILC3 and intestinal F4/80+CD11c+ MNC populations were increased in pups born to stably colonized ASF
Editor's Summary

When enough isn't enough

Overeating and obesity are rapidly becoming worldwide problems. Normally, mice do not overeat—they balance their caloric intake with their caloric needs. Lagerlöf et al. deleted an enzyme called O-GlcNAc transferase (OGT) from a subset of neurons in the mouse hypothalamus (see the Perspective by Schwartz). After the loss of OGT, the animals began to overeat and rapidly gained weight. The animals ate more at meal times, rather than eating more often. Thus, OGT seems to regulate satiety and helps to couple caloric intake to caloric need.

Science, this issue p. 1293; see also p. 1268
Supplementary Material for

The nutrient sensor OGT in PVN neurons regulates feeding

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Materials and Methods

Animals

OGT\textsuperscript{Fl} and αCaMKII-CreER\textsuperscript{T2} mice have been derived previously (8, 24). They were fully backcrossed (>N11) to the C57BL6 background. Only males were used and they were about 6 weeks old at the start of experiments. Tamoxifen was injected accordingly: 2mg twice per day for 5 days (20mg total dose). Control mice were either vehicle (sunflower seed oil injected into OGT\textsuperscript{Fl} x αCaMKII-CreER\textsuperscript{T2} mice) or genetic (tamoxifen injected into OGT\textsuperscript{Fl} x αCaMKII-CreER\textsuperscript{T2}) controls. No difference between controls was seen. Therefore, all control animals were combined. Mice were fed standard chow (24% protein, 18% fat, 58% carbohydrate) and had free access to food unless otherwise specified. Only littermates were compared. Apart from noted exceptions, the animals were kept on a 14 h/10 h light/dark cycle. For all animal studies, animals where randomly allocated to experimental groups. The experimenter was aware of the animal genotype when conducting experiments. The TdT\textsuperscript{Fl} mice express the fluorescent reporter TdTTomato upon exposure to Cre recombinase and were originally developed by the Allen Institute (Ai9). All animals were housed according with the Johns Hopkins University Animal Care and Use Committee guidelines. All details pertaining to optogenetics experiments, including animal work, are described below under Optogenetics.

Biochemistry

Primary neuronal cell culture. Cortical neurons were prepared from timed E18 embryos, as previously described (25). Lentivirus was added at DIV (days in vitro) 2. Cells were harvested for analysis at DIV 11-13.

Western blot. Cultured cells were harvested in RIPA buffer (50mM tris, 150mM sodium chloride, 1% NP-40, 1% deoxycholate, 0.1% SDS, 1mM EDTA, including inhibitors for proteases, phosphatases and O-GlcNAcases), solubilized for 20 min at 4\textdegree{}C and then spun at 13000 rpm. The supernatant was used for further analysis. When tissue was analyzed, unless otherwise noted, after removal from the animal, it was homogenized in homogenization buffer (HB; 0.32M sucrose, 10mM HEPES, including inhibitors for proteases, phosphatases and O-GlcNAcases). 2X RIPA was then added to a final concentration of 1X RIPA for solubilization at 4\textdegree{}C for 20 min, before spun at 13000 rpm. Clear lysate, with top lipid layer discarded, was used for further analysis. Note that the muscle indicated in Fig. S1A is musculus femoris. Western blotting was performed according to standard procedures. Briefly, after separation by SDS-PAGE and transfer to polyvinylidene fluoride (PVDF) membranes, membranes were blocked in 3% bovine serum albumin (BSA), or 5% bovine milk when blotting for O-GlcNAc, and then probed with the following antibodies: OGT (AL25, produced in-house, 1:5000), O-GlcNAc (CTD110.6, produced in-house, 1:10000), HSC70 (Santa Cruz Biotechnology, sc7298, 1:5000).

Virus

For cell culture experiments, lentivirus was used. VSVG pseudotyped lentiviral particles were produced by standard procedures. In short, a FUGW vector expressing enhanced (eGFP) alone (Wt) or eGFP and Cre recombinase (KO) together with cDNA plasmids VSVG and delta 8.9 were transfected into HEK 293T cells using Lipofectamine
The culture supernatant was collected 24 h and 48 h after transfection, then subjected to ultracentrifugation where the resulting pellet was resuspended in Neurobasal medium. For viral infections in vivo, adeno-associated virus (AAV) was used. All AAV were of serotype 2.1 and purchased from University of Pennsylvania Vector Core: For Wt; #AV-1-PV1917 (AAV1.CaMKII0.4.eGFP.WPRE.rBG) and for KO; #AV-1-PV2521 (AAV1.CaMKII.HI.eGFP-Cre.WPRE.SV40). All details pertaining to optogenetics experiments are described below under Optogenetics.

**Histology**

**Immunohistochemistry.** Animals were anesthetized with avertin (0.03ml/g) and then perfused with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS). The brain was removed and post-fixed in 4% PFA/PBS overnight. After the brain had been equilibrated in 30% sucrose/PBS, it was frozen and serial sections (25 µm) were cut on a microtome. Free-floating sections were blocked in IHC buffer (5-10% normal goat serum diluted in 0.25% Triton X-100/PBS) overnight at 4°C, incubated with the primary antibody overnight at 4°C and then with the secondary antibody either for 2 hours in room temp or overnight at 4°C. DAPI was included with the secondary antibody. Washes after the primary and secondary antibody were done in 0.25% Triton X-100/PBS and antibodies were diluted in IHC buffer. Primary antibodies used were OGT (AL25 produced in-house), 1:5000), TRH (Santa Cruz Biotechnology, 1:100), c-Fos (ABE457, Millipore, 1:500), Oxytocin (MAB5296, Millipore, 1:1000), GFP (Abcam, 1:1000), in Figs. 4 and 9 the signal from virally expressed GFP alone was imaged without using an antibody against GFP. For some experiments when staining for OGT alone, the animal was not perfused but fixed in 4% PFA/PBS overnight.

**In situ hybridization.** The brain was removed and fresh frozen in OCT and stored at -80°C. Serial sections were cut (25 µm) on a cryostat and stored at -80°C. Probes and hybridization protocol have been described at length previously (26-27). Briefly, slides were fixed in 4% PFA, treated with acetic anhydride and then blocked in pre-hybridization solution (hybridization buffer without probes added: 50% formamide, 5X saline sodium citrate (SSC), 5X Denhardt’s solution, 250ug/ml yeast tRNA, 500ug/ml sperm DNA) before application of respective probe diluted in hybridization buffer. Hybridization was done at 70°C overnight. The next day the slides were washed, re-blocked (0.1M Tris, pH 7.5, 0.15M sodium chloride, 5% sheep serum) and then incubated overnight at 4°C with an alkaline-phosphatase linked anti-digoxigenin antibody (Roche, 1:5000) diluted in blocking buffer. On the third day probes were developed after washing. Probe development was done by applying a mix of NBT (nitro-blue tetrazolium chloride), BCIP (5-bromo-4-chloro-3'-indoylphosphate p-toluidine salt) and levamisole until sufficient stain had precipitated (within the same probe, all slides were treated for the same length of time). Afterwards, slides were DAPI stained.

**Brain region coordinates.** Different brain regions were identified by taking advantage of the Paxinos and Franklin atlas (The Mouse Brain, in stereotaxic coordinates, second edition). Counted as distance from Bregma, the following coordinates were used: SCN (-0.34 to -0.82), PVN (-0.60 to -1.20), Arc (-1.46 to -2.20), VMH (-1.30 to -2.06), LH (-1.34 to -1.75), DMH (-1.46 to -2.06). The PBN and the NTS were identified on sagittal slices, PBN (Bregma: -5.00 to -5.45, lateral: 0.85 to 1.35), NTS (Bregma: -6.30 to -7.80, lateral: 0.36 to 0.96). Abbreviations used: Paraventricular nucleus (PVN),
Dorsomedial hypothalamic nucleus (DMH), Arcuate nucleus (Arc), Ventromedial hypothalamus (VMH), Lateral hypothalamus (LH), Nucleus of the solitary tract (NTS), Parabrachial nucleus (PBN).

Organotypic cultures

Acute coronal slices were cut from male mice around postnatal day 10 on a vibratome. The slices were cut in oxygen-bubbled dissection medium (1 mM CaCl$_2$, 5 mM MgCl$_2$, 10 mM glucose, 4 mM KCl, 26 mM NaHCO$_3$, 218 mM sucrose, 1.2 mM Na$_2$HPO$_4$(H$_2$O), 30 mM HEPES). After cutting, the dorsal half of each slice was removed and then mounted on membrane inserts in a 6 well plate (one slice per insert). Each well was filled with 1 ml slice culture media (SCM: 4.16 g/500ml MEM, 20% horse serum, 1 mM L-glutamine, 0.5 mg/500ml insulin, 2 mM MgCl$_2$, 1 mM CaCl$_2$, 3 mM glucose, 1.3 mM NaHCO$_3$, 3.58 g/500ml HEPES) and changed every 2-3 days. Explants were incubated at 35 °C. On DIV 10, virus was added by direct application on the top of each slice to label αCaMKII-positive cells (UPenn, #AV-1-PV1917 (AAV1.CaMKII0.4.eGFP.WPRE.rBG)). After about 1 week, cultures were submitted to 1 mM glucose SCM for 24 h (media changed once during those 24 h) and then changed into either fresh 1 mM or 5 mM glucose SCM for 1 h. In a second round of experiments, slices were put in 5 mM glucose SCM for 24 h and then transferred to either fresh 5 mM or 25 mM glucose SCM for 16 h. Slices were then fixed in 4% PFA in PBS for 2-7 h. The area around the fixed slices were cut out using a scalpel and then subjected to immunohistochemistry (O-GlcNAc and TRH) as described for free-floating slices.

Stereotactic virus injection

Anesthesia was induced using avertin (0.03ml/g) and thereafter kept by 1-1.5% isoflurane gas inhalation. The coordinates for the PVN were: distance from Bregma, -0.80 mm; distance from midline, 0.20 mm; depth from surface, -4.75mm. The stereotaxic instrument used was the Leica Angle Two stereotaxic instrument. The scalp was exposed by a small incision through the skin. Bregma and Lambda were identified and two small holes drilled over the injection site. Stored virus was thawed, diluted with sterile saline to a final concentration of 1x$10^9$ particles/ul and then spun for 5 min at 3000 rpm in room temperature. The virus was then loaded into a pulled glass pipette (20-40 µm) and connected to a syringe pump. In total, 2ul virus was injected at 0.300 ul/min. After the surgery, the scalp was sutured and the animal rehydrated with saline (0.02 ml/g, intraperitoneal injection) and given buprenorphine to relieve pain (0.01 ml/g, subcutaneous injection). Upon animal harvest, the injection site and spread of the virus was controlled by immunohistochemistry. In total, 5 Wt animals and 8 KO animals were injected. In all but one case, the PVN was successfully targeted. Although there was minor spread outside the PVN, the PVN was the common denominator of areas hit across animals. In the one case when the PVN was hit only partially, the daily food intake and body weight increased but to a lesser degree, as expected (data not shown). This animal was removed from analysis. Excluding animals where the PVN had not been successfully targeted was a pre-established criterion. All details pertaining to optogenetics experiments are described below under Optogenetics.

Behavior
General feeding behavior. Animals were kept in individual cages after tamoxifen treatment or virus injection. Food intake was measured by weighing leftover food pellets. The bottom of the cage was searched to include all leftover food. For some Wt and KO tamoxifen treated animals, the animals were not separated to individual cages until two weeks after the treatment had finished. Data from these animals were compiled with the rest as the effect on feeding behavior and body weight for all animals followed the same time course. Whereas tamoxifen treated animals were kept on a 14 h/10 h light/dark cycle, the virus injected animals were kept on a 12 h/12 h light/dark cycle.

Restricted feeding. 10 days after tamoxifen treatment, the KO animals were split into two groups. One group had continuous free access to food whereas the other group was fed 1 gram at the start of light hours and 2.5 grams at the start of dark hours, totaling 3.5 grams/day. At 21 days, free access to food was reintroduced to the latter group. Animals were kept on a 12 h/12 h light/dark cycle.

Comprehensive laboratory animal monitoring system (CLAMS). CLAMS is a closed chamber system that measures ventilation gases and food intake automatically. Our system was also equipped with laser beams that register physical movement. We submitted 7 Wt and 7 KO mice to CLAMS analysis two weeks after tamoxifen treatment. After several days of acclimatization, data were collected for 3 days. Data were binned in 15 min increments. Values that were the obvious result of aberrant mouse behavior or technical failure, such as spillage off the scale, were removed from analysis. There were a few small negative values recorded. These were included in the analysis, except for Fig. 1E for which negative values were removed. The total number of beam breaks is shown as ‘physical activity’ in all figures. A ‘meal’ was defined as any multiplicative of 15 min where the amount of intake the first 15 min was 50 mg or larger and the previous 15 min had no intake registered. A meal was considered finished when no intake had been registered for another 15 min. The formulas used to calculate different endpoints can be found at www.colinst.com. Animals were kept on a 12 h/12 h light/dark cycle.

Refeeding. For refeeding experiments in Fig. S8, the food was removed for 24 h and then reintroduced for 2.5 h before animal harvest. Throughout the experiment, the animals had free access to drinking water. For the refeeding paradigm coupled with optogenetics, see Optogenetics.

Fat-based diet. Mice were fed either regular chow (3.1 kcal/g, 24% protein, 18% fat, 58% carbohydrate) or energy-dense fat-based food (6.7 kcal/g, 9.1% protein, 90.5% fat, 0.4% carbohydrate). The mice were raised on regular chow and then switched to fat-based diet either about 1 week before injected with tamoxifen or about 14 days after injections had finished. The same behavior was noticed in both regiments and the data were combined. As the data for regular chow mimicked the data in general feeding behavior experiments, all those data were combined also. The data shown are the averages during the period when daily food intake had stabilized (days 20-26 after tamoxifen injection).

Optogenetics

Animals. All experimental protocols were conducted according to U.S. National Institutes of Health guidelines for animal research and were approved by the Institutional Animal Care and Use Committee at NIDA. Male C57BL/6J mice (2–5 months old, Jackson Laboratories) were used for optogenetic experiments.
Stereotactic viral delivery and fiber guide system implantation. Mice were anesthetized with isoflurane and were placed into a stereotaxic apparatus, as previously described (28). A pulled glass pipette with 30-40µm tip diameter was inserted into the brain, and one unilateral injection (30nL) of AAV2/1-CamKIIα-hChR2(H134R)-EYFP virus (titer: 3.3x10¹² viral genomes/mL, Optogenetics and Transgenic Technology Core, NIDA) was made at coordinates in the paraventricular nucleus of the hypothalamus (PVN, bregma: -0.70mm, midline: +0.15mm, dorsal surface: -4.60mm). A micromanipulator was used to control the injection speed (30nL/min), and the pipette was withdrawn 15 min after the injection. This was followed by the insertion of a ferrule-capped optical fiber (200 µm diameter core; NA 0.48) through the craniotomy. Metabond cement was used to anchor the ferrule to the skull. The fiber tip was implanted 0.7mm above the PVN. Mice were returned to their home cages for 10 – 14 days to recover and for expression of ChR2:EYFP.

Components for food consumption and photostimulation. In home cages, mice had ad libitum access to rodent chow. For behavioral testing, mice were transferred into feeding cages with automatic pellet dispensers and supplied with food pellets (20 mg each) of identical composition to the food in the home cage. Pellet removal was sensed by the offset of a beam break and an additional pellet was administrated after a delay (10 s). Food consumption was monitored continuously using Graphic State software (Coulbourn Instruments). Water was also available ad libitum during behavioral experiments, and in some cases, was monitored by optical detection (Coulbourn Instruments) of licks emitted towards the drinking spout. In addition, water bottles were manually weighed to estimate water intake every 24 hours. Mice were allowed to acclimate for 3 days before initiating the photostimulation protocols. Light was delivered to the brain through an optical fiber. The relationship of light scattering and absorption in the brain as a function of distance has been described previously (29). Using this relationship, the light power exiting the fiber tip (10 mW) was estimated to correspond to 2.0 mW/mm² at the PVN, which was sufficient to drive a behavioral response. For optical delivery of light pulses with millisecond precision to multiple mice, the output beam from a 300 mW diode laser (473 nm) was split into four beams using a combination of 50/50 beam splitters and turning mirrors. Each beam was controlled using an acousto-optic modulator (AOM) to generate light pulses that were launched into separate fiber ports and their corresponding optical fibers. Using these components, four mice could be simultaneously and independently photostimulated. LabVIEW software was used control the AOMs.

Behavioral experiments. General feeding behavior. Food intake was recorded during a pre-stimulus (baseline) for 3 days followed by a 24 h photostimulation period. In two experiments, stimulation started during light hours and in one experiment stimulation started at the onset of darkness. In all experiments, the same behavioral response was recorded and the data were averaged over all experiments. The primary photostimulation protocol consisted of 50 light pulses (10 ms) for 1 s at 50 Hz, with the sequence repeated every 4 s for 24 h. Meals were quantified as described for CLAMS.

Hunger-induced feeding behavior. Mice were fasted for 9 hours during the light phase (with ad libitum access to water) before testing for evoked feeding behavior at the onset of feeding at the start of the dark phase. The primary photostimulation protocol consisted of 50 or 20 light pulses (10 ms each) which were delivered for 1 s (50 Hz or 20
Hz, respectively), and the sequence repeated every 4 seconds for 12 hours. For the no starvation control, we used the average intake during the equivalent hours over 2 days. For meals analysis, food intake was binned over 5 min. A ‘meal’ was defined as any multiplicative of 5 min where the amount of intake the first 5 min was 10 mg or larger and no intake had been registered prior to that period. A meal was considered finished when no intake had been registered for another 10 min.

Whole body composition analysis
Quantitative NMR to measure total fat and lean mass was done using EchoMRI. Animals were analyzed about three weeks after tamoxifen treatment.

Electrophysiology
Whole-cell patch-clamp recordings were performed to assess the excitatory synaptic function in OGT KO PVN neurons. For this, we took advantage of αCaMKII-CreER\(^T\) x OGT\(^{FF/W}\) x TdT\(^F\) mice. Acute PVN slices were prepared 17-19 days after tamoxifen injection at a similar time of day. Mice were anesthetized with isoflurane and decapitated. Brains were removed rapidly and placed in ice-cold cutting solution containing 210.3 mM sucrose, 2.5 mM KCl, 1 mM NaH\(_2\)PO\(_4\), 26.2 mM NaHCO\(_3\), 4 mM MgCl\(_2\), 0.5 mM CaCl\(_2\) and 11 mM D-\((+)-\)glucose and oxygenated with 95% O\(_2\)/5% CO\(_2\). Coronal slices (300 \(\mu\)m thick) were cut with a vibratome and were kept in ACSF (125 mM NaCl, 2.5 mM KCl, 2 mM MgCl\(_2\), 2 mM CaCl\(_2\), 1.0 mM NaH\(_2\)PO\(_4\), 26.2 mM NaHCO\(_3\) and 11 mM glucose, oxygenated with 95% O\(_2\)/5% CO\(_2\)) at 23–25 °C until recordings. Slices were placed in a submerged chamber and perfused with ACSF supplemented with 50 \(\mu\)M picrotoxin and 1 \(\mu\)M TTX. Targeted whole cell recordings of PVN tdTomato-positive neurons were made using pipettes of 2-5 \(\Omega\) resistance. The intracellular solution contained 115 mM CsMeSO\(_4\), 0.4 mM EGTA, 5.0 mM TEA-Cl, 1 mM QX314, 2.8 mM NaCl, 20 mM HEPES, 3.0 mM MgATP, 0.5 mM GTP and 10 mM sodium phosphocreatine (pH = 7.2 and osmolality of 285–290 mOsm). Cells were held at -70mV holding potential and recording was performed at room temperature. The junction potential was left uncorrected. Signals were measured with an amplifier and digitized using an analog-to-digital board. Data were acquired with pClamp 10 software and digitized at 20 kHz. Cell capacitance was measured. Miniature EPSCs (mEPSCs) were analyzed with MiniAnalysis using a detection threshold of 7 pA (>2 times root mean square noise). Decay times were obtained by single-exponential fitting of the average mEPSC traces.

Image analysis
Neuronal cell number. In vitro: Cell number was quantified by counting the number of DAPI stained primary cultured neurons. On each of three Wt and KO coverslips, 10 random images were taken on a confocal microscope. In ImageJ, after setting a pre-defined threshold, the number of cells was counted automatically by applying the ‘Analyze particles’ command. In vivo: Cell number was quantified by counting the number of DAPI stained cells within the CA1 region of the hippocampus and the PVN in several slices from 3 Wt and 3 KO animals (confocal microscope). All slices were matched in distance from Bregma between Wt and KO. The number of cells was counted.
manually in the CA1 region. For the PVN, cells were counted automatically in ImageJ as described above.

*Neuropeptide transcript expression*. Within the PVN, the expression of the following probes was quantified: TRH, AVP, Oxytocin. Within the arcuate nucleus, the expression of the following probes was quantified: POMC, CART, AgRP, NPY. In the lateral hypothalamus the expression of orexin was quantified. Images for quantification were taken on a Nikon Eclipse Ti, except for orexin where an Axiophot was used. Images shown in Fig. 2 and S7 were all taken on the Axiophot. Animals had been harvested in the early phase when KOs had just started to develop hyperphagia without developing any major obesity (about two weeks after tamoxifen treatment). Slices from the same 4 Wt and 4 KO animals were used for all probes, except for Wt CART where slices from 3 of the animals were used. Quantification was done in ImageJ. A pre-defined region of interest (ROI) was placed over each cell. The number of cells per image and the average intensity of each cell were collected. The right and left side of each brain region were imaged separately. Data were averaged per animal. Within the same probe, all sections were matched in distance from Bregma. The investigator was blinded to whether images came from Wt or KO animals during counting.

*c-Fos and OGT expression in virus-injected PVN*. Prior to harvest, αCaMKII-positive cells in the PVN had been labeled with virus expressing GFP under the αCaMKII promoter by stereotaxic injection. Within the PVN, in ImageJ, all c-Fos positive cells per brain slice of the PVN were counted manually and marked as GFP-positive or GFP-negative. All sections were matched in distance from Bregma. Note that the Wt virus GFP expression was higher than the KO virus GFP expression, a result we attributed to the combined GFP and Cre recombinase expression by the KO virus while the Wt virus expressed GFP alone. Therefore, for the GFP channel in KO slices, the microscope gain was set higher to include all GFP positive cells (confocal microscope).

*O-GlcNAc expression in organotypic slices and starved mice*. All image analysis was done on raw, unmanipulated images in ImageJ. Cells and background were marked with a pre-defined ROI. For TdT/GFP positive cells, the ROI was placed over positive cells in the TdT/GFP channel. For TdT/GFP negative cells, cells were picked randomly in the DAPI channel and then verified as TdT/GFP negative in the TdT/GFP channel. If the ROI marked a TdT/GFP positive cell, the ROI was moved to a cell nearby. The background in each image was quantified by measuring the average intensity of three ROIs placed over cell-empty areas as judged by no DAPI signal. After placing all ROIs, the mean intensity was measured in the O-GlcNAc channel. The background value was subtracted from each cell value. The experimenter was blinded to the O-GlcNAc channel when placing the ROIs.

*Image presentation*. All image analysis was done on raw, unmanipulated images. For presentation purposes, most shown immunohistochemistry images and DAPI stained images were improved *post hoc*. All manipulations were applied equally within the same experiment and to Wt and KO images. The manipulations were the following: change of Levels and Brightness/Contrast and Filtering (‘Despeckle’ and ‘Gaussian blur’ (2.0pixels)).

**Statistical analysis**
Statistical analyses were done using the program Prism 5. Repeated-measures two-way analysis of variance (ANOVA) analyses detected significant interaction of ‘time’ and ‘genotype’, and post hoc Bonferroni analyses detected statistical significance between individual time points. In Fig. S3A, one-way ANOVA was applied to calculate significant interaction between all groups and then the difference between all separate group combinations was tested post hoc with Tukey’s Multiple Comparison Test. For in situ hybridization, statistical analysis compared between animals mean data averaged over all imaged slices. All Student’s t-tests were two-tailed and unpaired. Star, ‘*’, signifies $P<0.05$. All error bars represent mean +/- standard error of the mean (s.e.m.). A few animals where either some food intake or body weight value had been missed were omitted in repeated-measures two-way ANOVA analyses and post-hoc Bonferroni analyses. However, these values did not change the overall result and were included in shown graphs. The normality distribution of data used for the F-test in Fig. S6C was verified using the D'Agostino-Pearson omnibus normality test.
Fig. S1. αCaMKII-CreER x OGTFl deletes OGT in αCaMKII-positive neurons of adult mice but does not affect OGT expression in peripheral organs. (A) Schematic of the Ogt locus, including loxP sites (triangles) in OGTFl mice and translational start site (empty circle). (B) PCR analysis showing the genotype of Wt (αCaMKII-CreER’ x OGTFl) and KO (αCaMKII-CreER’ x OGTFl) mice. (C) Immunohistochemistry for OGT. Top: neocortex. Bottom: the hippocampus. (D) Primary cultured OGTFl neurons were infected with either a wildtype (Wt) virus or a virus expressing Cre recombinase (KO). Western blot characterizing the O-GlcNAc and OGT expression in Wt and KO neurons. (E) Western blot characterizing O-GlcNAc and OGT expression in peripheral tissues. (F) Quantification of OGT expression in peripheral tissues from (E) (Wt n=7, KO n=8; two-tailed t-test: P<0.05). (G) PCR analysis detecting Cre-dependent Ogt recombination in the brain but not in the liver. Quantifications represent mean +/- s.e.m.
**Fig. S2. Deletion of OGT in postmitotic neurons does not affect cell number.** (A) Left: DAPI stain of primary cultured neurons that were infected with either a wildtype virus (Wt) or a virus expressing Cre recombinase (KO). Right: quantification of DAPI stained cells (Wt n=3, KO n=3; two-tailed $t$-test: $P<0.05$). (B) Left: DAPI stain of the CA1 region of the hippocampus, where almost all cells express αCaMKII. Right: quantification of DAPI stained cells (Wt n=3, KO n=3; two-tailed $t$-test: $P<0.05$). Quantifications represent mean +/- s.e.m.
Fig. S3. Loss of OGT leads to adiposity, hyperphagia and hyperactivity. (A-B) Induction of Cre recombinase by tamoxifen treatment leads to weight gain only in the presence of floxed Ogt. (A) Quantification of body weight; tamoxifen treatment alone does not affect body weight (n=3 for all groups; one-way ANOVA with post hoc Tukey’s Multiple Comparison Test: P<0.05). (B) Quantification of body weight; αCaMKII-CreER crossed with an unrelated floxed mouse and treated with tamoxifen does not affect body weight (Wt n=5, KO n=5; two-tailed t-test: P<0.05). (C-D) Quantitative NMR of total body fat (C) and total lean body mass (D) (Wt n=7, KO n=6; two-tailed t-test: P<0.05). (E-G) Quantification of amount fat in different regions the body. (E) Intraperitoneal fat. (F) Subcutaneous fat. (G) Liver. (H) Daily food intake. (I) Total energy expenditure as measured by exchange of respiratory gases, see Methods. (J) Physical activity. (K) Volume O₂ inhaled. (L) Volume CO₂ exhaled. (M) The respiratory exchange ratio (RER). (H-M: Wt n=17, KO n=20; two-tailed t-test: P<0.05). Quantifications represent mean +/- s.e.m.
Fig. S4. Diurnal rhythm and meal frequency are not perturbed in OGT KO mice while meal size and duration are increased. The OGT-dependent hyperphagia correlates with total caloric intake. (A) Quantification of total activity during CLAMS as dispersed over dark and light hours (Wt n=21, KO n=21; two-tailed t-test: P<0.05). (B) Quantification of food intake during CLAMS as dispersed over dark and light hours (Wt n=17, KO n=20; two-tailed t-test: P<0.05). (C) Quantification of the number of initiated meals during CLAMS (Wt n=17, KO n=20; two-tailed t-test: P<0.05). (D-E) Quantification of meal size (D) and meal length (E) during CLAMS (Wt n=268, KO n=330; two-tailed t-test: P<0.05). (F) Quantification of food intake as it differs between carbohydrate-based pellets and fat-based paste (KD). (Chow (3.1 kcal/g): Wt n=10, KO n=10; KD (6.7 kcal/g): Wt n=7, KO n=5). Quantifications represent mean +/- s.e.m.
Fig. S5. αCaMKII-CreER x OGT<sup>Fl</sup> deletes OGT from several core feeding nuclei.

(A) For each panel: left; immunohistochemistry for OGT in αCaMKII-CreER x OGT<sup>Fl</sup> mice. Arrowheads indicate cells that lack OGT. Right; schematics showing general anatomical landmarks for slices that were used for imaging. The areas within each slice containing purple dots were the areas that were imaged and used for analysis. Purple dots within the schematics indicate cells that lost OGT in KO mice. Abbreviations used: Paraventricular nucleus (PVN), Dorsomedial hypothalamic nucleus (DMH), Arcuate nucleus (Arc), Ventromedial hypothalamus (VMH), Lateral hypothalamus (LH), Nucleus of the solitary tract (NTS), Parabrachial nucleus (PBN).
Fig. S6 αCaMKII-CreER x OGT<sup>Fl</sup> deletes OGT from a subpopulation of cells in the PVN with no effect on total cell number in the PVN. (A) αCaMKII-positive cells (purple) were labeled using αCaMKII-CreER<sup>+</sup> x TdT<sup>Fl</sup> mice. Left: DAPI (green). Middle: immunohistochemistry for TRH (green). Right: immunohistochemistry for Oxytocin (green). Arrow; cell positive for both the cell-specific marker and TdT. Arrowhead; cell positive for the cell-specific marker but not TdT. (B) Double-labeling of TRH cells and αCaMKII cells. The PVN was injected stereotactically with Wt or KO virus. TRH cells were labeled with immunohistochemistry for TRH (purple) and αCaMKII cells with virus expressing GFP under the αCaMKII promoter (green). Arrow; cell positive for both TRH and GFP. Arrowhead; cell positive for TRH but not GFP. (C) Serial sections of the PVN were cut and subjected to in situ hybridization for TRH as shown in Fig. 2B. Shown is the quantification of the number of TRH positive cells per PVN slice. The distribution of TRH cells in Wts and KOs showed statistical un-equal variance, indicating that a particular subpopulation of TRH cells is affected by OGT deletion (Wt n=45, KO n=48; F-test: \( P < 0.05 \)). (D) The same data as in (C) but plotted is the number of TRH positive cells per PVN slice in relation to distance from Bregma. (E) Left: DAPI stain of the PVN. Right: quantification of DAPI stained cells (Wt n=3, KO n=3; two-tailed t-test: \( P < 0.05 \)). Quantifications represent mean +/- s.e.m.
**Fig. S7. Deletion of OGT in αCaMKII-positive neurons leaves several major feeding circuits intact.** (A) *In situ* hybridization for neuropeptide modulators in core feeding circuits two weeks after OGT KO prior to significant weight increase. For each peptide: left; image of probe staining, right; quantification of number of stained cells within the respective area (cells / 150 x 10^3 μm^2) and quantification of intensity of staining per cell (a.u.) (Wt n=4 (except for CART, and number of oxytocin positive cells, where n=3), KO n=4; two-tailed t-test: P<0.05). Quantifications represent mean +/- s.e.m.
Fig. S8. αCaMKII-positive PVN neurons are uniquely sensitive to physiological variations in glucose in terms of O-GlcNAc and deletion of OGT blunts feeding-induced activation of the same cells. (A-D) Hypothalamic explant with GFP expression (green) and immunohistochemistry for TRH (A) or O-GlcNAc (C) (purple). Boxed magnification in (A) shows the PVN. (B) Quantification of O-GlcNAc intensity in αCaMKII-negative cells in the PVN from Fig. 2C (1 mM n=360, 5 mM n=330; two-tailed t-test: P<0.05). (D) Quantification of O-GlcNAc intensity in αCaMKII-positive cells in the PVN from (C) (5 mM n=390, 25 mM n=375; two-tailed t-test: P<0.05). (E) O-GlcNAc expression in the PVN in fed and starved animals (αCaMKII-CreER+ x TdTFl mice). Left; double-labeling of TdT expression (purple) and immunohistochemistry for O-GlcNAc (green). Arrow; cell positive for TdT. Arrowhead; cell negative for TdT. Right; quantification of O-GlcNAc expression in TdT-positive and TdT-negative cells (TdT-positive: fed n=455, starved n=259. TdT-negative: fed n=357, starved n=375; two-tailed t-test: P<0.05). (F) The PVN was injected stereotactically with Wt or KO virus. Double-labeling of GFP expression (green) and c-Fos (orange) in the PVN in fed and refed animals. Arrow; cell positive for both c-Fos and GFP. Arrowhead; cell positive for c-Fos but not GFP. (G) Quantification of number of cells expressing both GFP and c-Fos from (F) (Fed: Wt n=8, KO n=7. Refed: Wt n=12, KO n=10; two-tailed t-test: P<0.05). Quantifications represent mean +/- s.e.m.
Fig. S9. OGT KO does not affect membrane capacitance or EPSC shape in αCaMKII-positive cells in the PVN and optogenetic stimulation of the same cells decreases food intake. (A-C) Patch-clamp analysis of αCaMKII-positive cells in the PVN (Wt n=6, KO n=6; two-tailed t-test: \( P<0.05 \)). (A) Membrane capacitance. (B) mEPSC peak rise time. (C) mEPSC peak decay tau. (D) GFP expression (green) and immunohistochemistry for OGT (purple) in the PVN after stereotactic injection of Wt or KO virus. Arrow; cell positive for OGT and GFP. Arrowhead; cell positive for OGT only. (E-H) Optogenetic stimulation of αCaMKII-positive cells in the PVN. (E) Quantification of meal frequency (baseline n=11, stimulation n=11; two-tailed t-test: \( P<0.05 \)). (F) Quantification of food intake after a light fast. Bars represent average intake over all mice and lines represent average intake per individual mouse (All conditions n=5; two-tailed t-test: \(*\) refers to \( P<0.05 \)). (G) Quantification of the size of the first meal after a light fast, 50 Hz stimulation (baseline n=5, stimulation n=5; two-tailed t-test: \( P<0.05 \)). (H) Quantification of the latency to the initiation of the first meal after a light fast, 50 Hz stimulation (baseline n=5, stimulation n=5; two-tailed t-test: \( P<0.05 \)). Quantifications represent mean +/- s.e.m.
References and Notes


20. Y. Xu, Z. Wu, H. Sun, Y. Zhu, E. R. Kim, B. B. Lowell, B. R. Arenkiel, Y. Xu, Q. Tong, Glutamate mediates the function of melanocortin receptor 4 on Sim1 neurons in body


