Published in final edited form as:

Nature. 2016 October 13; 538(7624): 253–256. doi:10.1038/nature19789.

A cholinergic basal forebrain feeding circuit modulates appetite suppression

Alexander M. Herman¹, Joshua Ortiz-Guzman¹, Mikhail Kochukov², Isabella Herman^{1,3}, Kathleen B. Quast², Jay M. Patel^{3,4}, Burak Tepe¹, Jeffrey C. Carlson¹, Kevin Ung¹, Jennifer Selever^{2,5}, Qingchun Tong⁶, and benjamin R. Arenkiel^{1,2,4,5}

¹Program in Developmental Biology, Baylor College of Medicine, Houston, Texas 77030, USA

²Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, Texas 77030, USA

³Medical Scientist Training Program, Baylor College of Medicine, Houston, Texas 77030, USA

⁴Department of Neuroscience, Baylor College of Medicine, Houston, Texas 77030, USA

⁵Jan and Dan Duncan Neurological Research Institute at Texas Children's Hospital, Houston, Texas 77030, USA

⁶Institute of Molecular Medicine, University of Texas Health Science Center, Houston, Texas 77030, USA

Abstract

Atypical food intake is a primary cause of obesity and other eating and metabolic disorders. Insight into the neural control of feeding has previously focused mainly on signalling mechanisms associated with the hypothalamus¹⁻⁵, the major centre in the brain that regulates body weight homeostasis^{6,7}. However, roles of non-canonical central nervous system signalling mechanisms in regulating feeding behaviour have been largely uncharacterized. Acetylcholine has long been proposed to influence feeding⁸⁻¹⁰ owing in part to the functional similarity between acetylcholine and nicotine, a known appetite suppressant. Nicotine is an exogenous agonist for acetylcholine receptors, suggesting that endogenous cholinergic signalling may play a part in normal physiological regulation of feeding. However, it remains unclear how cholinergic neurons in the brain regulate food intake. Here we report that cholinergic neurons of the mouse basal forebrain potently influence food intake and body weight. Impairment of cholinergic signalling increases food intake and results in severe obesity, whereas enhanced cholinergic signalling decreases food consumption. We found that cholinergic circuits modulate appetite suppression on downstream

Reprints and permissions information is available at www.nature.com/reprints.

Correspondence and requests for materials should be addressed to B.R.A. (arenkiel@bcm.edu).

Author Contributions B.R.A., A.M.H., and Q.T. designed all experiments. J.C.C., J.O.-G., A.M.H., I.H., M.K., J.M.P., K.Q., B.T., and K.U. performed experiments. B.R.A., A.M.H., and K.Q. analysed the data. Genetic reagents and viral constructs were engineered by B.R.A., A.M.H., I.H., and J.S. A.M.H. and B.R.A. wrote the manuscript.

Reviewer Information Nature thanks L. de Lecea and the other anonymous reviewer(s) for their contribution to the peer review of this work.

The authors declare no competing financial interests.

targets in the hypothalamus. Together our data reveal the cholinergic basal forebrain as a major modulatory centre underlying feeding behaviour.

The diagonal band of Broca (DBB) (Extended Data Fig. 1a–g) is a major component of the cholinergic basal forebrain¹¹. Studies have shown that cell types associated with feeding are connected with the DBB^{12,13}. Given this correlation, we sought to determine if cholinergic neurons in the mouse DBB modulate feeding. With feeding after an overnight fast, we observed co-localization between c-Fos and the cholinergic-specific marker, choline acetyltransferase (ChAT) (Fig. 1a–f). Given their activation in response to food intake, we next assessed the necessity of cholinergic DBB neurons in feeding modulation. We stereotactically targeted cholinergic DBB neurons for ablation using a Cre-dependent¹⁴ adeno-associated virus (AAV) that expressed an enhanced yellow fluorescent protein (EYFP)-linked diphtheria toxin receptor in *Chat-cre^{+/-} ; R26^{LSL-tdTomato/+}* mice. DBB cholinergic neurons were mainly decimated after treatment with diphtheria toxin, whereas those in surrounding areas of the brain were unaffected (Fig. 1g–n). Within two weeks after ablation, we observed hyperphagia (Fig. 1o) and severe obesity (Fig. 1p–q). To assess the extent that food consumption contributed to this phenotype, we conducted paired feeding assays on obese DBB-ablated animals versus their non-ablated controls. Control mice exhibited normal food intake and minimal weight gain, whereas DBB-ablated animals lost weight when available food was limited to control levels (Fig. 1r–s), suggesting that hyperphagia was required to maintain obesity. This effect did not appear to be mediated by changes in energy expenditure, as overall levels of activity (Fig. 1t) and oxygen consumption (Extended Data Fig. 2a) were unaffected during early stages post-DBB ablation. Metabolic activity was only altered once obesity became a burden (Extended Data Fig. 2b–k).

We sought to determine whether conditional removal of cholinergic neurotransmission selectively from the DBB, without cell death, led to increased food consumption and obesity. We stereotactically delivered an EGFP-Cre-expressing AAV into the DBB of *Chat^{loxP/loxP}* homozygous mice (Fig. 2a), and evaluated ChAT knockout by immunohistochemistry (Fig. 2b–k). Counts of ChAT-immunopositive neurons from control and experimental brains showed a 72% and 55% decrease in ChAT expression in the DBB of Cre-expressing female and male mice, respectively, compared to controls (Fig. 2l). Consistent with a role for cholinergic signalling in feeding behaviour, Cre-expressing animals displayed increased food intake (Fig. 2m) and subsequent weight gain (Fig. 2n). Because knockout is restricted to the *Chat* gene, these data imply that the observed effects on feeding and body weight were mediated by cholinergic signalling, although it does not rule out a role for GABAergic neurotransmission from cholinergic DBB neurons, which have recently been reported to co-release GABA¹⁵ (Extended Data Fig. 3a–c).

To test how acute inactivation of these neurons affected food intake, *Chat-cre^{+/-}* mice were DBB-targeted for conditional expression of hM4D-EGFP (Extended Data Fig. 4a). Following CNO treatment, hM4D-EGFP-expressing mice consumed more food compared to controls (Extended Data Fig. 4b), suggesting that both acute and chronic inactivation of cholinergic DBB neurons were sufficient to increase food intake.

We sought to determine if activating DBB cholinergic neurons was sufficient to suppress food intake *in vivo*. To test this, we expressed channelrhodopsin-2 (ChR2) in DBB cholinergic neurons, followed by monitoring feeding behaviour during photostimulation (Fig. 3a–g). We found that prolonged (48 h) activation of cholinergic DBB neurons significantly decreased daily food intake under conditions of normal food access (Fig. 3h, i), whereas mock-stimulated, non-ChR2-expressing controls showed no significant changes in feeding behaviour (average daily food intake was 4.26 g (± 0.22 g, s.e.m.); 112.48% of baseline ($\pm 5.91\%$, s.e.m.), $n = 3$). To test the acute effect of DBB stimulation on feeding, we fasted ChR2-expressing mice overnight and presented them with chow in the morning. We observed that acute cell body stimulation resulted in a similar overall decrease (approximately 25%) in food intake (Fig. 3j–k).

We selectively targeted cholinergic DBB neurons for conditional viral expression of a presynaptically localized, synaptophysin–EGFP (Extended Data Fig. 5a–e), allowing the identification of their presumptive downstream synaptic targets. Using this method, we observed DBB neuron terminals in previously reported areas of the brain¹¹ (Extended Data Fig. 5f–n), as well as in ventral-medial domains of the hypothalamus (Extended Data Fig. 5o–u), which have previously been shown to express markers for cholinergic terminals of unknown origin¹⁶. Within this region resides the arcuate nucleus of the hypothalamus, a site that contains well-characterized cell populations that conversely regulate hunger and satiety through NPY/AgRP-expressing and POMC-expressing neurons, respectively¹⁷. As cholinergic basal forebrain neurons co-express GABA, it is possible that loss of cholinergic neurons from the DBB could disinhibit AgRP neurons, thereby promoting food intake through elevated expression levels of *Agrp*. To test this, *Agrp* transcripts were assessed in DBB-ablated animals that were pair-fed to respective controls shortly after ablation, before hyperphagic-induced obesity. Under these conditions, *Agrp* transcript analysis from the arcuate nucleus of DBB-ablated animals showed no significant decrease in levels of *Agrp* compared to controls (Extended Data Fig. 6a). Interestingly, however, *Pomc* transcript levels were strikingly reduced in DBB-ablated mice (Extended Data Fig. 6a), suggesting that cholinergic DBB neurons may normally act to modulate downstream satiety pathways. Previous reports show that POMC neurons are activated by cholinergic nicotinic agonists, however, their effect on AgRP/NPY neurons appears much more variable. From our own recordings (Extended Data Fig. 6b), acetylcholine did not show fast excitation on NPY neurons, perhaps due to muscarinic acetylcholine receptor (mAChR) activity or through broader network effects.

Recent studies have implicated the arcuate nucleus in appetite suppression via AChR signalling onto POMC neurons¹⁸, and conditional rabies virus tracing experiments suggested that arcuate POMC neurons receive significant input from neurons in the DBB¹². Whole-cell recordings from labelled POMC neurons in *Pomc-EGFP^{+/−}* mice showed that neuronal firing increased in the presence of acetylcholine (Extended Data Fig. 6c–d), and this response was blocked by AChR blockers (Extended Data Fig. 6e). To assess potential connectivity between the DBB and arcuate nucleus, we implemented retrogradely transported canine adenovirus (CAV-2)¹⁹. Using conditional *R26^{L^{SL}-tdTomato/+}* mice, we delivered CAV-Cre into the arcuate nucleus, and found that cholinergic DBB neurons provide input (Fig. 4a–e). Non-cholinergic cell types in the DBB were also labelled,

suggesting that both cholinergic and non-cholinergic cell types from the DBB provide input into the hypothalamus to regulate its functions. To investigate this connectivity *in vivo*, *Chat-cre^{+/-}* mice were targeted for conditional ChR2::EYFP expression in DBB cholinergic neurons, and a fibre optic was implanted into the ventral space of the third ventricle to stimulate DBB terminals within the ventral hypothalamus (Fig. 4f). ChR2-expressing and non-ChR2-expressing (mock-stimulated) implanted animals were fasted overnight and presented with chow for two hours. Mock-stimulated animals showed no significant changes in feeding behaviour during photostimulation (average was 1.381 g (± 0.1429 g, s.e.m.); 117.1% of baseline ($\pm 12.12\%$, s.e.m.), $n = 6$ animals), whereas stimulated ChR2-expressing animals exhibited diminished food intake compared to non-stimulated conditions (Fig. 4g–h), consistent with decreases observed from DBB cell body stimulation, and supporting anatomical tracing data. Although these data support a role for cholinergic modulation of the arcuate nucleus, given sparse labelling of terminals throughout the hypothalamus, as well as dense innervation of the median eminence (Extended Data Fig. 7a–h), these results do not rule out an influence of other hypothalamic sites and cell types. Lastly, to test if this effect was AChR-mediated, we systemically injected photostimulated animals with the nicotinic cholinergic receptor (nAChR) antagonist mecamylamine, which has previously been shown to block nAChR-mediated decreases in food intake¹⁸. In the presence of mecamylamine, photostimulated animals showed a blunted response compared to stimulation alone (Extended Data Fig. 8a–b), suggesting that decreased food intake after stimulation was mediated in part by AChR signalling. Notably, however, we did not observe a complete phenotypic blockade, suggesting that mAChR signalling may play a synergistic role in modulating food intake, and may therefore explain why nAChR antagonism alone was not sufficient to fully suppress feeding.

Together, our data demonstrate a powerful role for cholinergic basal forebrain neurons in modulating food intake. Cholinergic systems are highly druggable, which may offer a new avenue for targeting cholinergic mechanisms to help treat eating disorders. AChR transcript analysis from the arcuate nucleus showed expression of common AChR subunits (Extended Data Fig. 9a), serving as possible targets for pharmacological interventions. Understanding the AChR profiles of prominent feeding-associated cell types will surely be useful in this respect²⁰. Additionally, revealing the contribution of other signalling mechanisms from cholinergic neurons will be essential in understanding their composite roles in feeding. Cholinergic and other neuromodulatory systems are known to co-express multiple neurotransmitters and/or neuropeptides^{15,21,22}. Although cholinergic signalling is sure to play an important role, it remains unclear how dual transmitter systems function in concert to modulate their targets. Although our studies indicate a modulatory effect of acetylcholine on POMC neurons to influence food intake, it is possible that other cell types of the hypothalamus and/or other brain regions may be affected in a manner yet to be determined by cholinergic innervation. As such, it is possible that our observed phenotypes are not due solely to downstream effects in the arcuate nucleus alone, but may be a combinatorial effect of altered cholinergic signalling at other projection sites or cell types of the hypothalamus or other innervated brain regions. It will be especially interesting to determine the role of alternative cholinergic centres in modulating feeding behaviour such as those associated with the reward and addiction pathways of the mesolimbic system. The boundary between

homeostatic and non-homeostatic (hedonic/aversive) control of feeding is not always distinct or clear-cut, and often converges^{23,24}. Given its role as a powerful modulator of reward^{25–28} and aversion^{26,27,29}, and its involvement in addiction^{26,27}, acetylcholine is a prime candidate that may link known canonical homeostatic mechanisms that govern feeding behaviour, with the reward and/or aversive aspects of food intake. Nonetheless, fully understanding the relationship between cholinergic systems and their role in modulating feeding behaviour is an important step towards uncovering a comprehensive profile of feeding regulation.

METHODS

Data reporting

No statistical methods were used to predetermine sample size. When relevant (such as experiments requiring multiple trials), randomization was carried out. Behavioural trial experiments were randomized. There was blinding of initial allocation of animals into groups, but not thereafter. Cell counts were blinded.

Experimental mouse lines

All animals used in this study were treated in compliance with US Department of Health and Human Services and Baylor College of Medicine IACUC guidelines. For the studies reported here, both male and female mice were considered for analyses. Standard pellet mouse chow (Harlan, 2920X) was used for all experiments, and all animals were maintained on a normal 12-h light–dark cycle. *Chat-cre* (B6;129S6-*Chat^{tm2(cre)Lowl/J}*), *Pomc-EGFP* (C57BL/6J-Tg(Pomc-EGFP)1Low/J), *Npy-hrGFP* (B6.FVB-Tg(Npy-hrGFP)1Lowl/J), *Chat^{loxP/loxP}* (B6.129-*Chat^{tm1Jrs/J}*), *R26^{LSL-tdTomato}* (B6.Cg-Gt(ROSA)26Sor^{tm14(CAG-tdTomato)Hze/J}), and *Vgat-cre* (Slc32a1^{tm2(cre)Lowl/J}) mice were originally purchased and are available from Jackson Laboratories. *Chat-cre^{+/-}*; *R26^{LSL-tdTomato/+}* mice were generated by crossing heterozygous male *Chat-cre^{+/-}* mice with female homozygous *R26^{LSL-tdTomato}* mice. *Chat^{loxP/loxP}* animals were bred and maintained as homozygotes. A *Vgat-cre* homozygous male was crossed to female C57BL/6J mice to generate heterozygous *Vgat-cre^{+/-}* animals. *Chat-cre^{+/-}*, *Pomc-EGFP^{+/-}* and *Npy-hrGFP^{+/-}* mice used in this study were maintained as heterozygotes and bred to wild-type C57BL/6J female mice. Genotyping for *Chat^{loxP/loxP}*, *Pomc-EGFP*, and *Npy-hrGFP* animals was done according to available Jackson Laboratory protocols for these strains. Genotyping for Cre was done using primers for Cre recombinase detection (forward primer: 5'-GCATTTCTGGGGATTGCTTA-3', reverse primer: 5'-GTCATCCTTAGCGCCGTAA-3').

Microscopy and immunohistochemistry

Animals were deeply anaesthetized using isoflurane and were transcardially perfused with PBS followed by 10% neutral buffered formalin (NBF, Azer Scientific). Brains were dissected and post-fixed in 10% NBF overnight at 4 °C. Brains were cryoprotected in a 20% sucrose/PBS solution at 4 °C for one day, followed by a 30% sucrose/PBS solution at 4 °C for one more day. Brains were then embedded and frozen in OCT and stored at –80°C. Brains visualized using endogenous or virally-expressing fluorescent reporters were cut using a cryostat (Leica CM1860) in coronal sections at 25–30 µm. For ChAT and β-endorphin immunohistochemistry, 40 µm free-floating sections were blocked for 1 h at room

temperature in 10% horse serum blocking solution, made in PBS-TC (1× PBS, 0.5% Triton-X 100, 0.1 mM CaCl₂, pH 7.35). Sections were then incubated overnight at 4 °C at a 1:200 dilution of block solution containing goat anti-ChAT primary antibody (Millipore, AB144P) or rabbit anti-β-endorphin primary antibody (Phoenix Pharmaceuticals, H-022-33). Sections were then washed 4 times, 30 min each in plain PBS-TC. Sections were then incubated in secondary antibody (donkey anti-goat Alexafluor-488 or Alexafluor-555, Life Technologies) at a 1:200 dilution for 3 h at room temperature. Sections were then washed 4 times for 30 min each in PBS-TC. All sections were mounted using DAPI Fluoromount-G (Southern Biotech, 0100-20). Detection of fluorescent expression was performed using a Leica TCS SPE confocal microscope under a 10× or 20× objective.

Stereotaxic injections and viral constructs

For all stereotaxic injections, mice were anesthetized using a ketamine/dormitor mixture and were maintained under anaesthesia using vaporized isoflurane with O₂. All injections were performed using a stereotaxic apparatus synced to Angle Two software for coordinate guidance. For DTR-mediated cell death of cholinergic neurons, female *Chat-cre^{+/-}* or *Chat-cre^{+/-}; R26^{LSLtdTomato/+}* mice (8–10 weeks old) were bilaterally injected into the horizontal limb of the diagonal band of Broca (HDB, right hemisphere, from bregma: AP = +0.14, DV = -5.80, ML = -1.29; left hemisphere, from bregma: AP = +0.14, DV = -5.74, ML = +1.17) with 500 nl per hemisphere of a Cre-dependent *AAV-EF1α-FLEX-DTR-P2A-EYFP-WPRE-hGHpA*, serotype DJ/8. For conditional *Chat* knockout experiments, *Chat^{loxP/loxP}* animals (8–10 weeks old) were bilaterally injected into the HDB with 300 nl per hemisphere of *AAV-EF1α-EGFP-P2A-CRE-WPRE-hGHpA* for experimental animals, or *AAV-EF1α-EGFP-WPRE-hGHpA* for control animals (serotype DJ/8 for both AAVs). For synaptophysin tracing experiments using the EGFP variant, *Chat-cre^{+/-}* mice (8–16 weeks old) were injected bilaterally into the HDB with 500 nL per hemisphere of a Cre-dependent *AAV-EF1α-FLEX-Syn::EGFP-WPRE-hGHpA*, serotype DJ/8. For synaptophysin tracing experiments using the mRuby2 variant, *Chat-cre^{+/-}; Pomc-EGFP^{+/-}* mice (12 weeks old) were injected bilaterally into the HDB with 500 nl per hemisphere of a Cre-dependent *AAV-EF1α-FLEX-Syn::mRuby2-WPRE-hGHpA*, serotype DJ/8. Lastly, for *in vivo* ChR2 behaviour experiments, male *Chat-cre^{+/-}* mice (12–14 weeks old) were bilaterally injected into the HDB with 500 nl per hemisphere of a Cre-dependent *AAV-EF1α-DIO-hChR2(H134R)-EYFP-WPRE-hGHpA* (Addgene, plasmid number 20298) serotype 2/9.

Neuronal activation after feeding and c-Fos IHC

Male wild-type animals were fasted overnight before being presented with standard pellet mouse chow (Harlan, 2920X) for 3 h the following morning (fed group), while a second group of mice was fasted overnight but not presented with chow (fasted group). Mice were then immediately euthanized and perfused with PBS and 10% NBF. Brains were fixed overnight in 10% NBF before two overnight fixations in 20% and 30% sucrose/PBS solutions. Brains were frozen in OCT cutting compound and cryosectioned at 35 μm. Sections were then blocked for 1 h at room temperature in 10% horse serum blocking solution, made in PBS-TC (1× PBS, 0.5% Triton-X 100, 0.1 mM CaCl₂, pH 7.35). Sections were then incubated overnight at 4 °C at a 1:200 dilution of block solution containing goat anti-ChAT primary antibody (Millipore, AB144P) and 1:500 dilution of rabbit anti-c-Fos

antibody (Calbiochem, PC38). Sections were then washed 4 times for 30 min each in plain PBS-TC. Sections were then incubated in secondary antibodies (donkey anti-goat Alexafluor-488, and donkey anti-rabbit Alexafluor-546) at a 1:200 dilution each for 3 h at room temperature. Sections were then washed 4 times for 30 min each in PBS-TC. All sections were mounted using DAPI Fluoromount-G (Southern Biotech, 0100-20). Detection of fluorescent expression was performed using a Leica TCS SPE confocal microscope under a 20× objective.

DTR-mediated cell death and Chat conditional knockout assays

After allowing 10–14 days for conditional viral expression (injections and viral construct described previously), mice were intraperitoneally (i.p.) injected 3 times daily for 5 days with 800 ng (4 ng μl^{-1} working solution) of diphtheria toxin (Sigma, D0564) for optimal cell death of targeted cholinergic neurons. Female *Chat-cre^{+/-}*; *R26^{LSL-tdTomato/+}* mice were used initially to validate DTR-mediated cell death by visualizing DBB cholinergic cell loss. For remaining experiments, female *Chat-cre^{+/-}* animals were used. For controls, age- and gender-matched *Chat-cre^{+/-}* mice (stereotactically injected identically into the DBB with *AAV-FLEX-DTR-P2A-EYFP*) were injected with equal volume (200 μl per injection) of sterile saline. Body weights and daily food intake were measured and averaged per group for each time point presented. For *Chat* conditional knockout assays, *Chat^{loxP/loxP}* mice were injected bilaterally into the HDB as previously described with either *AAV-EF1 α -EGFP-P2A-CRE-WPRE-hGHpA* for conditional knockout animals or *AAV-EF1 α -EGFP-WPRE-hGHpA* for controls. Body weights and daily food intake were measured and averaged per group for each time point presented. For cell counts after *Chat* conditional knockout, 3 mice from each group were euthanized and brains were sectioned at 40 μm for ChAT immunohistochemistry. 10 sections representing the anterior, central, and posterior areas of the DBB were chosen, and blinded, total cell counts based on ChAT immunoreactivity were tallied. A count from all 10 sections from a single mouse brain were totalled and averaged for each group of 3 mice. Data were normalized to control levels of expression and represented as a mean percentage $\pm$ s.e.m.

Activity monitoring and metabolic assays

Activity (reported as time active per day), O_2 consumption, and metabolic blood assays were performed by the Baylor College of Medicine Mouse Metabolism Core before obesity phenotypes (3 days after diphtheria toxin (DT) treatment), during early stages of obesity and hyperphagia (3 weeks post-DT treatment), and at late stages of obesity and hyperphagia (3 months post-DT treatment). Activity and O_2 consumption assays were performed using the OxyMax Comprehensive Laboratory Animal Monitoring System (CLAMS, Columbus instruments). Lean mass and body fat content was assessed using quantitative MRI. Blood panel assays for cholesterol, leptin, insulin, and glucose were also performed by the Baylor College of Medicine Mouse metabolism core. Blood was collected via the tail vein. Mice were fasted for 4 h before measuring blood glucose.

Paired feeding assays

Paired feeding assays were performed with individually-housed, male DT-treated (DBB-ablated) and saline-treated (non-ablated) animals. Assays for determining the contribution of

food intake on maintaining obesity were conducted on animals 12-weeks post-ablation. First, daily body weight and *ad libitum* food intake for all animals was recorded for 7 days to establish baselines for all animals. Then 1 control mouse and 1 experimental mouse were then randomly paired. All food from experimental cages was removed and only an equivalent amount of food consumed the previous day by a mouse's respective control partner was introduced to the cage. This restrictive period was done for 21 days. Afterwards, all experimental mice were allowed to resume to feed *ad libitum* once again and food intake and body weight were measured daily for 2 weeks. Change in body weight over time was normalized as a percentage of day 1 initial starting weight for each individual animal. For paired feeding conducted on animals used for *Agrp* and *Pomc* transcript analysis, assays were performed 3 days post-ablation to prevent significant weight gain from hyperphagia. A restrictive feeding period was conducted for 21 days, after which mice were euthanized on the morning after the final day, and hypothalamic tissue was harvested for RNA purification and subsequent qRT-PCR (see below).

***In vivo* optogenetic behaviour assays**

Concurrent with *AAV-EF1 α -DIO-hChR2(H134R)-EYFP-WPRE-hGHpA* injections (as described previously), male *Chat-cre^{+/-}* mice were bilaterally implanted with 200 μ m silica fibre optic implants made in-house (Thor Labs, TS1249968); 230 μ m ferrules (Precision Fibre Products, MM-FER2007C-2300) and situated 0.1 mm above the viral injection site. Fibre optic implants were held in place by a cap made from adhesive cement (C&B Metabond Quick! Cement System (Parkell)) for initial base, and crosslinked flash acrylic (Yates-Motloid, 44115 and 44119) for headcap. Mice were allowed at least two weeks for recovery and expression of the virus before assays were performed. For prolonged 2-day stimulation, each mouse was allowed 48 h to acclimate in a behaviour box with free access to food and water (days 1 and 2: acclimation). In addition, acclimation occurred while tethered to a dual fibre optic cord (Doric Lenses) attached to a 473 nm laser source (CrystaLaser CL-2005). After the 48-h acclimation period, a pre-measured amount of food was placed into the chamber and weighed once every 24 h for two days without stimulation (days 3 and 4: pre-stimulation). Over the next 48 h, food was weighed once each day while mice were chronically stimulated with trains of blue light (5 mW, 10 ms pulses, 20 Hz, 5 s trains, 30 s intervals) (days 5 and 6: stimulation). Finally, food was weighed once every 24 h for two final days with no blue light stimulation (days 7 and 8: post-stimulation). As a control group, non-ChR2-expressing mice were injected and implanted in the identical way used for experimental mice. Control mice were acclimated identically and were subsequently subjected to a mock stimulation for 48 h, and food intake was measured each day. For comparisons between pre-stimulation, stimulation, and post-stimulation conditions, paired Student's *t*-tests were used. For comparisons between experimental conditions and the control (mock-stimulation) condition, unpaired Student's *t*-tests were used. For short-term 2-h stimulation experiments, mice were given 48 h to acclimate in their behaviour chamber. After acclimation, mice were fasted overnight and subsequently presented with a pre-measured amount of food in the morning. For control conditions, mice were not stimulated and food intake was recorded every 30 min for 2 h total. For experimental conditions, mice were stimulated with trains of blue light (5 mW, 10 ms pulses, 20 Hz, 5 s trains, 30 s intervals) for 15 min before presentation of pre-measured chow, and food intake

was recorded every 30 min for 2 h total in presence of continued blue light illumination. Trials were randomized and conducted one week apart on the same animals. For experiments targeted at terminal stimulation in the arcuate nucleus of the hypothalamus, male animals were bilaterally injected into the HDB with *AAV-EF1 α -DIO-hChR2(H134R)-EYFP-WPRE-hGHpA*, and a single fibre optic was implanted into the third ventricle at the level of the arcuate (from bregma: AP = -1.70, DV = -5.75, ML = 0.00). For behavioural assays, animals were first allowed 48 h to acclimate to their behaviour cage while tethered to a fibre optic cord. After acclimation, mice were fasted overnight. In the morning, mice were presented with standard pre-measured pellet chow and food intake was recorded every 30 min for 2 h total either under conditions of light stimulation (5 mW, 10 ms pulses, 20 Hz, 5 s trains, 30 s intervals) or no stimulation. Trials were randomized and conducted one week apart on the same animals. Paired statistics were used to compare 'stim' and 'no-stim' conditions on these animals. As a control group, Cre-negative male littermates were injected with virus and implanted in the same way as experimental mice. Behavioural assays on these mice were done the same way under conditions of light illumination. Comparisons between mock-stimulated and experimental cohorts were done using unpaired statistics. For terminal stimulation experiments in the presence of mecamylamine (Tocris, catalogue number 2843), mice were fasted overnight and presented with a pre-measured amount of chow in the morning. Mecamylamine was administered by i.p. injection at 1 mg per kg 15 min before the start of a 2-h feeding in the presence or absence of blue light illumination. Control (sterile 1 $\times$ PBS i.p. injections) and experimental trials were conducted 1 week apart.

Electrophysiology and pharmacology of POMC-EGFP or NPY-hrGFP neurons, ChR2-expressing DBB neurons, and hM4D-expressing DBB neurons

Brain slices containing the hypothalamic arcuate nucleus were prepared from 6–8-week-old *Pomc-EGFP^{+/+}* or *Npy-hrGFP^{+/+}* transgenic mice of either gender. For ChR2 stimulation and hM4d-mediated inhibition, brain slices containing the DBB were prepared from 12–16-week-old male animals expressing either ChR2::EYFP or hM4D-EGFP in the DBB, respectively. Animals were anaesthetized with isoflurane and brains were rapidly removed and transferred into sucrose-based cutting solution, containing (in mM): 250 sucrose, 25 NaHCO₃, 1.25 NaH₂PO₄, 2.5 KCl, 1.5 MgCl₂, 2 CaCl₂, 10 glucose, and continuously bubbled with 5% CO₂ / 95% O₂. 300- μ m-thick coronal brain slices were prepared using a Leica VT 1200 vibratome and placed for recovery in a 5% CO₂ / 95% O₂ bubbled regular ACSF solution, containing (in mM): 128 NaCl, 24 NaHCO₃, 1 NaH₂PO₄, 3 KCl, 1 MgCl₂, 1.6 CaCl₂, 8 glucose. After at least 1-h recovery and 20–30 min before recording, slices were transferred into a recording chamber continuously perfused at 2 ml min⁻¹ with aforementioned ACSF at 24 °C. POMC-, NPY-, ChR2::EYFP-, and hM4D-expressing neurons were identified by transmitted light DIC and EGFP fluorescent imaging using a Slicescope Pro 6000 optical setup (Scientifica), equipped with a CoolLED pE-100 470 nm excitation light source, 49002- ET-EGFP (FITC/Cy2) emission filter (Chroma Technology), and optiMOS camera (QImaging). Electrical activity of neurons was recorded in whole-cell current clamp mode using a Multiclamp 700b amplifier and a 1440a Digidata interface (Molecular Devices). Pipette solution contained (in mM): 10 KCl, 120 K gluconate, 1 MgCl₂, 10 HEPES, 1 EGTA, 5 Na₂-ATP, 0.01 Na-GTP, pH 7.2. To test the cholinergic effects on POMC and NPY neurons, baseline neuronal activity was prerecorded for at least

10 min to assure a stable firing rate, after which acetylcholine (Sigma-Aldrich, A6625) was added to the bath perfusion at 100 μ M. For ChR2 stimulation, 12 consecutive trains of blue light were given at 30 s intervals. Each train lasted for 5 s with 10 ms light pulses delivered at 20 Hz. For recordings from hM4D-expressing cells, we recorded 90 s baseline sweeps and delivered 5 s current injections at 2 pA and 10 pA, spaced 25 s apart. This protocol was repeated again 6 min after CNO bath application. For all recordings, neuronal firing activity was analysed offline using the event detection feature of Clampfit 10.3 software (Molecular Devices). Repeated measures ANOVA with Holm–Sidak multiple comparison, and Sigma Plot 11.0 software (Systat Software) were used for statistical analyses of data, where applicable. For acute acetylcholine responses, a localized 2 s application of acetylcholine (100 mM, Sigma-Aldrich, A6625) was applied near a patched POMC-EGFP neuron (held at -70 mV) using a FemtoJet (Eppendorf). Each recording was performed using a 20-s sweep with an inter-trial interval of 1 min and repeated for 5 sweeps each for baseline, synaptic blockers (10 μ M CNQX (Tocris), 20 μ M APV (Tocris), 50 μ M GABAzine (Tocris), and nicotinic blockers (10 μ M mecamylamine (Tocris), 0.1 μ M methyllycaconitine citrate (Tocris), and 10 μ M dihydro- β -erythroidine hydrobromide (Tocris).

CAV-Cre tracing experiments

R26^{LSL-tdTomato} animals were stereotactically injected bilaterally with 70 nl of CAV-Cre virus (purchased from the vector core at the Institut de Génétique Moléculaire de Montpellier) targeted to the arcuate nucleus (from bregma: AP = -1.70 , DV = -5.80 , ML = ± 0.20). Sections through the DBB were obtained and stained for ChAT using the identical ChAT IHC protocol detailed previously. All sections were mounted using DAPI Fluoromount-G (Southern Biotech, 0100-20). Detection of fluorescent expression was performed using a Leica TCS SPE confocal microscope under a 10 $\times$ or 20 $\times$ objective.

hM4D-mediated feeding assays

12-week-old, male *Chat-cre^{+/-}* mice were stereotactically injected bilaterally into the DBB (500 nl per hemisphere) with an hM4D-EGFP-expressing AAV (*AAV-CBA-FLEX-hM4Di-P2A-EGFP-WPRE-sv40pA*, serotype 2/9, Addgene, plasmid number 52536). After a two-week recovery, mice were fasted overnight in their home cage, and food was presented in the morning. Food intake was measured every 30 min for 2 h total, in the presence or absence of CNO. For control experiments, mice were injected i.p. with sterile saline and allowed to wait 15 min before food presentation. For experimental conditions, mice were injected i.p. with CNO (5 mg per kg) and allowed to wait 15 min before food presentation and measurement, which was recorded for 2 h in total. Trials were randomized and conducted one week apart on the same animals. Paired statistics were used to compare ‘saline’ and ‘CNO’ conditions on these animals.

Transcript analyses

For *Agrp* and *Pomc* transcript analysis from the arcuate nucleus, 4 DBB-ablated mice and 4 non-ablated mice were euthanized in the morning and their brains were immediately dissected. For AChR transcript analysis, 4 wild-type male mice were taken and brains dissected and processed identically. Small sections of the ventral hypothalamus containing the arcuate nucleus were dissected out and RNA was isolated following the TRIzol (Life

Technologies, 15596-018) protocol for tissue homogenization and RNA isolation. In brief, tissue was placed in 1 ml of TRIzol reagent and homogenized using a 1.5 ml pre-sterilized pestle. Homogenized samples were allowed to incubate at room temperature for 5 min. 0.2 ml of chloroform was added, and the tube was shaken vigorously by hand for 15 s. The sample was again incubated at room temperature for 2 min and centrifuged at 12,000g for 15 min at 4 °C. The upper aqueous phase was pipetted into a new tube, 0.5 ml of isopropanol was added, and the mixture was incubated at room temperature for 10 min. The tube was centrifuged at 12,000g for 10 min at 4 °C. The supernatant was removed and the RNA pellet was washed with 1 ml of 75% ethanol. The sample was vortexed and centrifuged at 7,500g for 5 min at room temperature. The pellet was air-dried for 15 min and resuspended in 40 µl RNase-free water at 60 °C. RNA was DNase-digested using the manufacturer's protocol (Promega). DNase was inactivated via phenol-chloroform extraction. Purified RNA was quantified using a NanoDrop (Wilmington, DE), and first-strand cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Negative controls did not contain reverse transcriptase. Transcripts were amplified using standard PCR conditions (95 °C for 120 s, 95 °C for 20 s, 60 °C for 20 s, 72 °C for 20 s, 34 cycles, 7 °C for 300 s, 4 °C until storage at -20 °C). Amplified products were run on 2% agarose gels, imaged, and quantified using ImageJ software. AChR primer sequences were as follows (forward primer first, reverse primer second, in 5'-3' orientation), CHRNA1: GTCCAATAACGCCGCTGAGG, CTAGCGATGGCTATGGCTGG; CHRNA2: GACTCTTCGGTGAAGGAAGATTG, AGAGCAGAAGA TGGTTGTCCAG; CHRNA3: GCCAAAGAGATTCAAGATGATTGG, TCTGGGGCTATTGAGAAAGTGC; CHRNA4: GACTTCTCGGTGAAG GAGGAC, GGAAGATGTGGGTGACTGACG; CHRNA5: CGTCCGCG AGGTTGTTGAAG, AGCTGCTTGACTGCTCACTAAG; CHRNA6: CAAACGAGGTATAAGACGACTG, TCTTGTGGGGCTAGCTCGG; CHRNA7: CCTAAGTGACCAGGATCATTC, ATGTAGAGCAGGTTGCCATTGC; CHRNA9: GTCCCTCTGATAGGAAAATACTAC, CTAAGGCAGC TCTCACCCAC; CHRNA10: ACTCATCGGAAAGTACTATATGGC, GACTCTAATGGCTTGGACTGTC; CHRNA1: ACCAGATGCAGGAGAGA AGATG, GAGCGATGATGCAGGTTGAGG; CHRNA2: TGACCAGAGTG TGAGGGAGG, AGCTGCAAATGAGAGACCTCAC; CHRNA3: ACTTCATC AGTCAGGTTGTTCAAG, CTAGGTGGGATTCTCTCTATGTG; CHRNA4: ATCAGAGTGTATCGAGGACTG, CACTAGGCTGCTCATATCATCC; CHRM1: GCCAAGGTGATGCCCTTACTC, TGCCTGTCACTGTAGCCAGAG; CHRM2: AGAGCCCTGAAGTCGCAGATC, CTCCTGGATCTGGCTT TCAG; CHRM3: GGCTTCCTGGCATTGGTGAC, GCCAGAGGTCACAGG CTAAG; CHRM4: TGAAGTGTTCCTGAGCCTG, AGTAGCCCTTGATGAT GTATAAGG; CHRM5: ACTATTACCTGCTCAGCTTGGC, GTAACGATCAA AGCTAATCACCAG.

AgRP products were quantified by qPCR using the following primer pair: GCGGAGGTGCTAGATCCACAGAA and AGGACTCGTGAGCCTTACAC. POMC products were quantified by qPCR using the following primer pair: AGAACGCCATCATCAAGAAC and AAGAGGCTAGAGGTCATCAG. Actin was used as a reference control using the following primer pair: GCAAGCAGGAGTACGATGAG and TAACAGTCCGCCTAGAAGCA. For quantitative transcript analysis, all reactions were done in triplicates with no reverse transcriptase negative control samples. Samples were

prepared according to the BIO-RAD iQ SYBR Green Supermix instructions. Briefly, cDNA was diluted to 1 ng μl^{-1} and primers were diluted to 2.5 μM stock of combined forward and reverse primers. Reactions were set up in 15 μl total volume per sample (7.5 μl SYBR Green Supermix, 5 μl cDNA, 1.5 μl forward and reverse primer mix, 1 μl water) in Applied Biosystems Advanced Studio 3 compatible 96-well plates. Plates were sealed with adhesive film and mixed thoroughly before amplification. Amplification occurred on the Applied Biosystems Advanced Studio 3 qPCR machine on its standard amplification protocol. All transcript analysis was standardized to the amplification curve of actin for each sample, and a Student's t-test was performed to analyse differences in transcript expression among samples.

Statistical analyses

With the exception of electrophysiological-based experiments (previously described), all other statistical analyses were performed using GraphPad Prism 6 software (GraphPad), accounting for appropriate distribution and variance to ensure proper statistical parameters were applied. Experimental sample sizes were chosen according to minimal accepted norms within the field. With regards to experimental randomization for cell ablation, *Chat* conditional knockout, and optogenetic behaviour assays, mice were randomly separated into two groups before manipulation by an independent, blinded assistant not involved in experimentation or experimental design. For quantification of *Chat* conditional knockout in the DBB, DBB images were acquired by an independent assistant not involved in the experimentation and cell counts were then objectively tallied by a second assistant without knowledge of the experimental groups. Statistical methods used are described in figure legends for the respective experiments.

Extended Data

Extended Data Figure 1. Anatomy of the diagonal band of Broca

a–c, The DBB is situated in the basal forebrain and consists of an anteriorly located vertical limb (VDB), which branches posteriorly into separate bilateral horizontal limbs (HDB). **d**, *AAV-FLEX-tdTomato* (pseudocoloured in green) injected bilaterally into the HDB to show expression throughout the full extent of the diagonal band (VDB and HDBs). **e–g**, Bilateral viral injections into the HDB (*AAV-FLEX-Syn::EGFP* shown as an example) are sufficient to target the full extent of the diagonal band (VDB and HDB). Scale bars, 100 μ m. Schematic in **a** was adapted from images from the Allen Brain Institute Reference Atlas³⁰.

Extended Data Figure 2. Time-dependent changes in body content and metabolic measures between non-ablated and DBB-ablated mice

a, Oxygen consumption, represented as VO_2 , at various stages post DBB-ablation. Data are represented as mean $\pm$ s.e.m. * $P < 0.05$ by two-sided, unpaired Student's t -test. **b, c**, Average total lean mass (**b**) or body fat (**c**) content at various stages post DBB-ablation. Data are represented as mean $\pm$ s.e.m. * $P < 0.05$ by two-sided, unpaired Student's t -test. **d–f**, Blood cholesterol (**d**), leptin (**e**), insulin (**f**), and blood glucose concentration (**g**) at various stages post DBB-ablation. Data are represented as mean $\pm$ s.e.m. * $P < 0.05$ by two-sided, unpaired Student's t -test. **h, i**, Representative non-ablated (**h**) or DBB-ablated (**i**) animals ($n = 3$ mice) dissected to show abdominal fat pads and enlarged fatty livers. **j, k**, Representative haematoxylin and eosin (H&E) stains showing fat deposition of the liver in non-ablated (**j**) or DBB-ablated (**k**) animals. Scale bar, 100 μ m.

Extended Data Figure 3. Cholinergic DBB neurons are VGAT-positive

a–c, *Vgat-cre^{+/−}* mouse injected with AAV-FLEX-tdTomato into the HDB and stained with anti-ChAT antibody (green channel). Data suggest a high percentage of co-localization between VGAT-positive neurons and ChAT. Scale bar, 100 μ m.

Extended Data Figure 4. hM4D-mediated inhibition of the cholinergic DBB increases food intake

a, Whole-cell electrophysiological recordings from a representative cholinergic neuron expressing the hM4D inhibitory DREADD receptor before (top) and after (bottom) CNO treatment. **b**, Total two-hour food intake after an overnight fast ($n=11$ mice). ‘Saline’ and ‘CNO’ groups represent separate trials of the same animals in the absence or presence of CNO, respectively. Data are represented as mean $\pm$ s.e.m. $*P < 0.05$ by two-sided, paired Student’s t -test.

Extended Data Figure 5. Representative cholinergic DBB-innervating projection sites in the brain

a, b, Schematic (**a**) and representative image (**b**) showing injection site in the HDBs of a *Chat-cre*^{+/-} mouse with *AAV-FLEX-Syn::EGFP*. Scale bar, 1 mm. **c–e**, *Chat-cre*^{+/-} mouse injected bilaterally into the HDB with *AAV-FLEX-Syn::EGFP* and stained with an anti-ChAT antibody. Staining shows high co-localization of ChAT-positive neurons with the Cre-dependent AAV, demonstrating the efficacy of the *Chat-cre* mouse line. Of the neurons infected with virus, only a small fraction do not express ChAT. Scale bars, 100 μ m (**c–e**) and 50 μ m (**c'–e'**). **f–h**, Cholinergic projections from the DBB can be validated by known projection sites including the olfactory bulb. Scale bar, 200 μ m; GL, glomerular layer; EPL, external plexiform layer; MCL, mitral cell layer; GCL, granule cell layer. **i–k**, Hippocampus. Scale bar, 200 μ m. **l–n**, Amygdala (basolateral amygdala shown here). Scale bar, 100 μ m. **o–u**, Also shown are areas of the ventral hypothalamus including, Median eminence (**o–q**), and arcuate nucleus (**r–u**). Scale bars, 100 μ m. Schematic in **a** was adapted from an image from the Allen Brain Institute Reference Atlas³⁰.

Extended Data Figure 6. *Pomc* expression is reduced in pair-fed DBB-ablated animals

a, Relative *Agrp* and *Pomc* expression levels in arcuate nuclei between pair-fed non-ablated and DBB-ablated mice ($n=4$ mice per group). Data are represented as mean $\pm$ s.e.m. $*P < 0.05$ by two-sided, unpaired Student's *t*-test. **b**, Acetylcholine does not significantly alter arcuate NPY neuron firing. Data shown as mean $\pm$ s.e.m. of spike frequency in 8 acetylcholine-treated cells (1 recorded neuron per slice, 1–2 slices per mouse, $n=6$ mice), comparisons made versus baseline values (1–4 min) by repeated measures ANOVA with Holm–Sidak multiple comparison. **c**, **d**, Acetylcholine significantly increases arcuate POMC neuron firing. Data shown as mean $\pm$ s.e.m. of spike frequency in 7 acetylcholine-treated cells (1 recorded neuron per slice, 1–2 slices per mouse, $n=4$ mice), $*P < 0.001$ versus baseline values (1–4 min) by repeated measures ANOVA with Holm–Sidak multiple comparison. **e**, Representative voltage clamp recording from POMC-EGFP neuron after local administration of acetylcholine and after pharmacological manipulation of fast synaptic transmission, as well as nicotinic acetylcholine receptor blockade (20-s sweep with an inter-trial interval of 1 min and repeated for 5 sweeps each for baseline, synaptic blockers (CNQX, 2-amino-5-phosphonopentanoic acid (APV), GABA_A and tetrodotoxin (TTX)), and nicotinic blockers (mecamylamine (MEC), methyllycaconitine (MLA) and dihydro- β -erythroidine hydrobromide (DBHE)).

Extended Data Figure 7. Diffuse cholinergic DBB projections into the hypothalamus

a, Site of *AAV-FLEX-Syn::mRuby2* injections in the DBB. **b–d**, Representative images of cholinergic terminals (red) from the DBB into the hypothalamus. Scale bar, 100 μ m. ME, median eminence. **b'–d'**, Higher magnification images from the section shown in the dotted squares in **b–d**. Scale bar, 50 μ m. Closed arrowheads indicate close apposition or co-localization between cholinergic terminals and POMC (green) cell bodies, whereas open arrowheads show apposition or co-localization on or near POMC neuronal processes. Of note, cholinergic terminals also appear on non-POMC neurons. **e, f**, DAPI (**e**), anti- β -endorphin (**f**) (POMC neuron marker), **g, h**, *AAV-FLEX-Syn::mRuby2* (injected into the DBB) (**g**), and merged (**h**) channels from **f** and **g** show innervation of the hypothalamus from cholinergic neurons in the DBB. Scale bar, 200 μ m. Schematic in **a** was adapted from an image from the Allen Brain Institute Reference Atlas³⁰.

Extended Data Figure 8. ChR2-mediated decrease in feeding is partially suppressed by nAChR blockade

a, b, Total (a) and normalized (with respect to the mean 'No-stim' value; b) food intake after a two-hour re-feeding after an overnight fast under specified conditions. Comparisons between 'No-Stim,' 'Stim,' 'No-Stim + Mec,' and 'Stim + Mec' refer to separate trials conducted on the same animals ($n = 5$ mice). Data are represented as mean $\pm$ s.e.m. $*P < 0.05$ by two-sided, paired Student's t -test.

Extended Data Figure 9. Arcuate acetylcholine receptor (AChR) expression profile

a, Region-specific transcript expression profile of nicotinic and muscarinic AChRs in the arcuate nucleus, relative to the housekeeping gene, *Gapdh* ($n = 4$ mice). Data are represented as mean $\pm$ s.e.m.

Acknowledgments

This study was supported by NIH grants 5F31NS089411 to A.M.H., R01NS078294 to B.R.A., R01DK109934 to B.R.A. and Q.T., P30DK079638 to the BCM Mouse Metabolic Core, and U54HD083092 to the BCM IDRC. Support was also provided to B.R.A. from the Klarman Family Foundation, the Klingenstein-Simons Fellowship Award, the Brain and Behavior Research Foundation, the Charif Souki Fund, and the McNair Medical Institute. We thank H. Zoghbi, H. Bellen, M. Wang, and M. Krashes for input on this manuscript.

References

1. Aponte Y, Atasoy D, Sternson SM. AGRP neurons are sufficient to orchestrate feeding behavior rapidly and without training. *Nat Neurosci.* 2011; 14:351–355. [PubMed: 21209617]
2. Chen Y, Lin YC, Kuo TW, Knight ZA. Sensory detection of food rapidly modulates arcuate feeding circuits. *Cell.* 2015; 160:829–841. [PubMed: 25703096]
3. Luquet S, Perez FA, Hnasko TS, Palmiter RD. NPY/AgRP neurons are essential for feeding in adult mice but can be ablated in neonates. *Science.* 2005; 310:683–685. [PubMed: 16254186]
4. Pinto S, et al. Rapid rewiring of arcuate nucleus feeding circuits by leptin. *Science.* 2004; 304:110–115. [PubMed: 15064421]
5. Garfield AS, et al. A neural basis for melanocortin-4 receptor-regulated appetite. *Nat Neurosci.* 2015; 18:863–871. [PubMed: 25915476]
6. Morton GJ, Cummings DE, Baskin DG, Barsh GS, Schwartz MW. Central nervous system control of food intake and body weight. *Nature.* 2006; 443:289–295. [PubMed: 16988703]
7. Elmquist JK, Elias CF, Saper CB. From lesions to leptin: hypothalamic control of food intake and body weight. *Neuron.* 1999; 22:221–232. [PubMed: 10069329]
8. Pistelli F, Aquilini F, Carrozzi L. Weight gain after smoking cessation. *Monaldi Arch Chest Dis.* 2009; 71:81–87. [PubMed: 19719041]
9. Fulkerson JA, French SA. Cigarette smoking for weight loss or control among adolescents: gender and racial/ethnic differences. *J Adolesc Health.* 2003; 32:306–313. [PubMed: 12667735]
10. Voorhees CC, Schreiber GB, Schumann BC, Biro F, Crawford PB. Early predictors of daily smoking in young women: The National Heart, Lung, And Blood Institute growth and health study. *Prev Med.* 2002; 34:616–624. [PubMed: 12052022]
11. Zaborszky, L., van den Pol, A., Gyengesi, E. The Mouse Nervous System. Watson, C.Paxinos, G., Puelles, L., editors. 2012. p. 684–718.
12. Wang D, et al. Whole-brain mapping of the direct inputs and axonal projections of POMC and AgRP neurons. *Front Neuroanat.* 2015; 9:40. [PubMed: 25870542]
13. Sakurai T, et al. Input of orexin/hypocretin neurons revealed by a genetically encoded tracer in mice. *Neuron.* 2005; 46:297–308. [PubMed: 15848807]
14. Atasoy D, Aponte Y, Su HH, Sternson SM. A FLEX switch targets Channelrhodopsin-2 to multiple cell types for imaging and long-range circuit mapping. *J Neurosci.* 2008; 28:7025–7030. [PubMed: 18614669]
15. Saunders A, Granger AJ, Sabatini BL. Corelease of acetylcholine and GABA from cholinergic forebrain neurons. *eLife.* 2015; 4:e06412.
16. Ichikawa T, Ajiki K, Matsuura J, Misawa H. Localization of two cholinergic markers, choline acetyltransferase and vesicular acetylcholine transporter in the central nervous system of the rat: in situ hybridization histochemistry and immunohistochemistry. *J Chem Neuroanat.* 1997; 13:23–39. [PubMed: 9271193]
17. Gropp E, et al. Agouti-related peptide-expressing neurons are mandatory for feeding. *Nat Neurosci.* 2005; 8:1289–1291. [PubMed: 16158063]
18. Mineur YS, et al. Nicotine decreases food intake through activation of POMC neurons. *Science.* 2011; 332:1330–1332. [PubMed: 21659607]
19. Soudais C, Laplace-Builhe C, Kissa K, Kremer EJ. Preferential transduction of neurons by canine adenovirus vectors and their efficient retrograde transport *in vivo*. *FASEB J.* 2001; 15:2283–2285. [PubMed: 11511531]

20. Henry FE, Sugino K, Tozer A, Branco T, Sternson SM. Cell type-specific transcriptomics of hypothalamic energy-sensing neuron responses to weight-loss. *eLife*. 2015; 4:e09800.
21. Ren J, et al. Habenula “cholinergic” neurons co-release glutamate and acetylcholine and activate postsynaptic neurons via distinct transmission modes. *Neuron*. 2011; 69:445–452. [PubMed: 21315256]
22. Saunders A, et al. A direct GABAergic output from the basal ganglia to frontal cortex. *Nature*. 2015; 521:85–89. [PubMed: 25739505]
23. Saper CB, Chou TC, Elmquist JK. The need to feed: homeostatic and hedonic control of eating. *Neuron*. 2002; 36:199–211. [PubMed: 12383777]
24. Liu C, Lee S, Elmquist JK. Circuits controlling energy balance and mood: inherently intertwined or just complicated intersections? *Cell Metab*. 2014; 19:902–909. [PubMed: 24630814]
25. Wise RA. Brain reward circuitry: insights from unsensed incentives. *Neuron*. 2002; 36:229–240. [PubMed: 12383779]
26. Picciotto MR, Higley MJ, Mineur YS. Acetylcholine as a neuromodulator: cholinergic signaling shapes nervous system function and behavior. *Neuron*. 2012; 76:116–129. [PubMed: 23040810]
27. Miwa JM, Freedman R, Lester HA. Neural systems governed by nicotinic acetylcholine receptors: emerging hypotheses. *Neuron*. 2011; 70:20–33. [PubMed: 21482353]
28. Hangya B, Ranade SP, Lorenc M, Kepecs A. Central cholinergic neurons are rapidly recruited by reinforcement feedback. *Cell*. 2015; 162:1155–1168. [PubMed: 26317475]
29. Hoebel BG, Avena NM, Rada P. Accumbens dopamine-acetylcholine balance in approach and avoidance. *Curr Opin Pharmacol*. 2007; 7:617–627. [PubMed: 18023617]
30. Website: © 2015 Allen Institute for Brain Science. Allen Mouse Brain Atlas [Internet]. Available from: <http://mouse.brain-map.org>

Figure 1. DBB-specific cholinergic cell death results in hyperphagia and obesity
a–f, c-Fos and ChAT immunohistochemistry in the HDB. **g–n**, DBB of saline-treated (**g–j**) or diphtheria toxin treated (**k–n**) animals. Scale bars, 100 μ m. **o**, Food intake between DBB-ablated ($n=15$ mice) and non-ablated animals ($n=13$ mice). Time points represented as mean $\pm$ s.e.m., $P=0.0002$, $F(1, 26) = 18.06$ by two-way ANOVA with repeated measures. **p**, Body weight between DBB-ablated ($n=15$) and non-ablated animals ($n=13$). Time points represented as mean $\pm$ s.e.m., $P<0.0001$, $F(1, 26) = 143.1$ by two-way ANOVA with repeated measures. **q**, Representative DBB-ablated and non-ablated mice. **r, s**, Average daily food intake (**r**) and body mass (normalized with respect to the day-0 mass; **s**) during paired feeding ($n=5$ mice per group), pink shading represents restrictive period. **t**, Total time active per day before obesity, and during early and late stages of obesity. Data represented as mean $\pm$ s.e.m., $*P<0.05$ by two-sided, unpaired Student's t -test.

Figure 2. *Chat* conditional knockout from the DBB leads to obesity and hyperphagia
a, Strategy for *Chat* conditional knockout. **b–k**, ChAT immunostains of DBB in EGFP-expressing (**b–f**), or EGFP-Cre-expressing (**g–k**) *Chat*^{loxP/loxP} brains. Scale bars represent 1 mm (**c**, **h**) or 100 μm (**f**, **k**). **l**, Percentage of DBB neurons expressing ChAT ($n = 3$ per gender). Data represented as mean $\pm$ s.e.m., * $P < 0.05$, ** $P < 0.01$ by two-sided, unpaired Student's *t*-test. **m**, **n**, Daily average food intake (**m**) and average body mass (**n**) (12 weeks after the conditional knockout, $n = 7$ females, 5 males for EGFP-Cre or EGFP mice, $n = 6$ females, 6 males for wild type). Data represented as mean $\pm$ s.e.m., * $P < 0.05$, ** $P < 0.01$ by two-sided, unpaired Student's *t*-test. Schematic in **a** was adapted from an image from the Allen Brain Institute Reference Atlas³⁰.

Figure 3. *In vivo* ChR2-mediated DBB stimulation decreases food intake

a, ChR2::EYFP in cell bodies of the DBB. Scale bar, 1 mm. Membrane-bound ChR2::EYFP is not restricted to cell bodies and shows neuronal projections. **b–d**, ChR2::EYFP in DBB neurons after stimulation and stained to identify c-Fos. **e**, **f**, Strategy for ChR2-assisted feeding assay. **g**, Representative recordings from a ChR2-expressing cholinergic DBB neuron (10 ms pulses, 20 Hz, 5 s trains, 30 s intervals). **h**, **i**, Raw (**h**) and normalized (with respect to the mean pre-stimulation value; **i**) food intake values before, during, and post-stimulation ($n=7$ mice). **j**, **k**, Raw (**j**) and normalized (with respect to the mean no-stimulation value; **k**) food intake values in the absence or presence of stimulation ($n=5$ mice). Data represented as mean $\pm$ s.e.m., $*P < 0.05$ by two-sided, paired Student's *t*-test. Schematic in **e** was adapted from an image from the Allen Brain Institute Reference Atlas³⁰.

Figure 4. Stimulation of cholinergic DBB terminals in the hypothalamus suppresses food intake
a, Strategy for CAV-Cre-mediated circuit mapping. **b**, tdTomato expression in the hypothalamus. **c**, ChAT immunohistochemistry, **d**, **e**, tdTomato expression (**d**) and merged (**e**) images in the DBB. Scale bars, 200 μ m (top row). Respective zoomed images from the dotted boxes in **c–e** are shown in the bottom row. Scale bar, 100 μ m. White arrows point to neurons showing ChAT and tdTomato co-localization. **f**, Strategy for ChR2-assisted *in vivo* behaviour. **g**, **h**, Total (**g**) and normalized (with respect to the mean no-stimulation value; **h**) food intake ($n = 8$ mice) after two-hour re-feeding after overnight fast. Data represented as mean $\pm$ s.e.m. * $P < 0.05$ by two-sided, paired Student's *t*-test. Schematics in figures **a** and **f** were adapted from images from the Allen Brain Institute Reference Atlas³⁰.