Effects of GABA and Leptin Receptor-Expressing Neurons in the Lateral Hypothalamus on Feeding, Locomotion, and Thermogenesis

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Objective: The lateral hypothalamus (LH) is known for its role in feeding, and it also regulates other aspects of energy homeostasis. How genetically defined LH neuronal subpopulations mediate LH effects on energy homeostasis remains poorly understood. The behavioral effects of chemogenetically activating LH gamma-aminobutyric acid (GABA) and the more selective population of LH GABA neurons that coexpress the leptin receptor (LepR) were compared.

Methods: LepR-cre and VGAT-cre mice were injected with AAV5-hSyn-DIO-hM3DGq-mCherry in the LH. The behavioral effects of LH GABA or LH LepR neuronal activation on feeding, locomotion, thermogenesis, and body weight were assessed.

Results: The activation of LH GABA neurons increased body temperature ($P \leq 0.008$) and decreased body weight ($P \leq 0.01$) despite decreased locomotor activity ($P = 0.03$) and transiently increased chow intake ($P \leq 0.009$). Also, similar to other studies, this study found that activation of LH GABA neurons induced gnawing on both food and nonfood ($P = 0.001$) items. Activation of LH LepR neurons decreased body weight ($P \leq 0.01$) and chow intake when presented on the cage floor ($P \leq 0.04$) but not when presented in the cage top and increased locomotor activity ($P = 0.002$) and body temperature ($P = 0.03$).

Conclusions: LH LepR neurons are a subset of LH GABA neurons, and LH LepR activation more specifically regulates energy homeostasis to promote a negative energy balance.

Introduction

The lateral hypothalamus (LH) has emerged as the feeding center because lesions of the LH in rats and cats attenuate feeding and even result in starvation (1-3). The LH has also been associated with other aspects of body weight regulation, such as physical activity and thermogenesis (4,5). Although the LH has gained attention for its role in body weight regulation, there is still a poor understanding of the role of specific LH neuronal subpopulations in this process.

The LH contains a heterogeneous assembly of cell populations, in which gamma-aminobutyric acid (GABA)ergic neurons predominate (6,7). LH GABA neurons are known to mediate multiple behaviors important for body weight regulation, such as promoting consumption of chow (8-13) and palatable solutions (9,10) and altering energy expenditure (10,13). LH GABA neurons differ in expression of neurochemical markers, such as neuropeptide Y, galanin, and the leptin receptor (LepR). How different neuronal subgroups contribute to LH GABA-mediated behaviors remains largely unknown.

LH LepR neurons were shown to colocalize with LH GABA neurons (14). Leptin is a white adipose tissue-derived hormone well known for decreasing food intake (15) and increasing energy expenditure (16,17) via LepR signaling in the brain. In previous studies, intra-LH leptin injections resulted in decreased feeding and body weight (14), leptin decreased the rewarding effects of LH stimulation (18), and deletion of LepR from specific LH neuronal subpopulations modulated energy expenditure and nutrient preference (19,20).

We hypothesized that LH LepR neurons are a distinct group of LH GABA neurons and that these neurons more specifically affect body weight homeostasis and promote a negative energy balance compared...
with all LH GABA neurons. To test this hypothesis, we compared the effects of chemogenetically activating LH GABA and LH LepR neurons on multiple aspects of energy homeostasis: consumption of chow and palatable foods, locomotion, body temperature, and body weight.

**Methods**

All experiments were approved by the Animal Experimentation Committee of the University Utrecht and were carried out in agreement with Dutch law (Herzien Wet op Dierproeven, Art 10.a2, 2014) and European regulations (Guideline 2010/63/EU).

**Animals**

In-house-bred, adult, male, homozygote LepRb-cre and VGAT-cre transgenic mice (stock 008320 [B6.129-Leprtm2(cre)Rck/J] and stock 028862 [B6J129S6(Cg)-Slc32a1tm2(cre)Jowl/MwarJ]; The Jackson Laboratory) were used for experiments. Animals were housed individually in plastic cages (Type II L, 365 × 207 × 140 mm, 530 cm²; Tecniplast) and maintained in a temperature (21°C ± 2°C)- and humidity (60%-70%)-controlled room on a 12-hour reversed light/dark cycle (lights on at 9 pm) with ad libitum access to chow (3.1 kcal/g; Standard Diet Service) and water.

**Surgeries**

Mice were anesthetized with an intraperitoneal injection of ketamine (75 mg/kg, Narketan; Vetoquinol BV) and medetomidine (1 mg/kg, SedaStart; Ast Farma BV). Mice were given eye cream (CAF; CEVA Sante Animale BW) and placed in a stereotactic frame (Kopf Instruments). An incision was made along the midline of the skull, and additional analgesia was applied by spraying Xylocaine (lidocaine 100 mg/mL; AstraZeneca BV) on the skull. Microinjections of AAV5-hSyn-DIO-hM3DGq-mCherry (0.3 µL/side, 3.0 × 10⁹ genomic copies/µL; UNC Vector Core) or AAV-Elf1a-DIO-hChR2-eYFP (0.3 µL/side, 3.0 × 10⁹ genomic copies/µL; UNC Vector Core) were performed bilaterally in the LH (−1.2 mm anterior/posterior, +2.0 mm medial/lateral, −5.4 mm dorsal/ventral, 10° angle) at a rate of 0.1 µL/min per side, followed by a 10-minute waiting period before retracting the needles. Following surgery, mice were given carprofen (5 mg/kg, subcutaneous, Carpofal; Ast Farma BV), atipamezole (2.5 mg/kg, intraperitoneal, SedaStop; Ast Farma BV), and saline for rehydration. During the following 2 days, mice were given carprofen (5 mg/kg, subcutaneous) and were allowed to recover for at least 1 week. To ensure viral expression, testing commenced 3 weeks after virus injection.

**Drugs**

Clozapine N-oxide (CNO 99%; AK Scientific, Inc.) was dissolved in 0.9% saline and injected intraperitoneally with a dose of 1.0 mg/kg. For all experiments, each animal received saline and CNO injections in a Latin square design.

**Experimental procedures**

*Feeding experiments.* To simplify the search for food pieces during measurements, all animals were habituated to a second cage (Type II L, 365 × 207 × 140 mm, 530 cm²; Tecniplast), in which no bedding but only three tissues (to retain comfort and environmental enrichment in the form of nest building) and a water bottle were present, henceforth referred to as the “feeding cage.” All feeding tests were done in these cages, were initiated 30 minutes prior to onset of the dark phase, and lasted 7 hours. Tests were done with standard rodent chow (3.1 kcal/g; Standard Diet Service), sugar cubes (4 kcal/g, Van Gilse), lard (9.1 kcal/g; Oseweit/Blanc de Boeuf), or a combination. Mice were habituated to novel foods overnight at least 3 days prior to testing to prevent neophobia. On test days, directly after saline/CNO injections, mice were placed in the feeding cage with a preweighed amount of food present and a water bottle. Chow or sugar cubes were present on the cage floor, and lard was presented on a suspended spoon (Walking Dinner Amuse Spoon, Yong). Food intake was measured 1, 2, 3, 5, and 7 hours after injections. If food was wet, food was replaced and wet food was dried before weighing.

*Intake assessment of 20% sucrose.* Mice were habituated to a bottle with 20% (weight per volume) granulated sugar solution (4 kcal/g; Jumbo) prior to testing. Tests were commenced 3 hours into the dark phase and last 2 hours, during which animals were placed in the feeding cage with only the sucrose bottle present.

*Wood block exposure.* During feeding experiments, we observed gnawing behavior after LH GABA stimulation. To test whether this was nonspecific (nonfood directed) behavior, we determined the extent of gnawing on a nonfood item, a wood block. Mice were habituated to a wood block prior to testing. Tests started 4 hours into the dark phase. Mice were placed in their feeding cage with a preweighed block of wood (~20 g) for 2 hours, after which wood blocks were dried and weighed. We also simultaneously presented mice with chow and a wood block, during which we more accurately separated intake from spilling by weighing pieces of chow and chow dust separately, making sure to remove all pieces of wood and feces.

*Locomotion assessment.* All animals were habituated to an empty cage (Type III H, 425 × 266 × 185 mm, 800 cm²; Tecniplast) for 1 hour on 2 separate days. Locomotion tests were performed 3 to 4 hours into the dark phase and lasted 1 hour. Thirty minutes prior to testing, animals were injected with either saline or CNO and placed in the behavioral testing room. Mice were placed in their own locomotion cage, and horizontal movement was tracked using a camera placed above the cages that was coupled to a computer running EthoVision 7 (Noldus).

*Temperature measurements.* Temperature measurements started 4 hours into the dark phase. Prior to baseline temperature measurements, food, water, and tissues were removed from the home cage. To measure temperature, mice were placed in a smaller cage, and a movie was made using a thermal camera (Flir T420; FLIR Systems, Inc.) and the accompanying software program ResearchIR (version 4.40.6.24, 64 bit; FLIR Systems). Fifteen and thirty minutes after removal, baseline temperature was recorded. Mice were then injected with either saline or CNO, and temperature was recorded 30, 60, and 90 minutes after injections. In between movies, mice were placed back in their home cage. ResearchIR was used to determine eye temperature by directing a 3 × 3-sized pixel toward the region of the eyes with the least standard deviation with mice standing on their hind legs and looking upward. Temperature is displayed as a change in temperature from baseline (temperature – average of baseline temperatures).
Repetitive injections. Mice were injected with saline or CNO at 8:30 AM and at 3:30 PM for three consecutive days. Directly after injections, the mice, chow in the cage top, and water bottles were weighed. On the fourth day, no injections were given, but mice, food, and water were weighed at 8:30 AM. Before repeating this task, animals were allowed to recover for at least 1 week.

Immunohistochemistry

Animals were anesthetized with an overdose of sodium pentobarbital (Euthanatal; Alfasan BV) and transcardially perfused with 1x phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in 1xPBS. Brains were dissected and kept in 4% paraformaldehyde for 24 hours at 4°C and then in 30% sucrose in 1xPBS for at least 48 hours at 4°C. Brain slices (40 μm) were 3 x 10 minutes in 1xPBS and then blocked for 1 hour in 1xPBS containing 10% normal goat serum (NGS) and 0.25% Triton-X100. Slices were then placed in 1xPBS containing primary antibody (rabbit anti-dsRed 1:500; number 632496; Clontech, Takara Bio USA Inc) and 2% NGS overnight at 4°C. At room temperature, slices were washed 3 x 10 minutes in 1xPBS and then placed in 1xPBS containing secondary antibody (goat anti-rabbit 569, 1:500; number ab75471; Abcam Plc) and 2% NGS for 2 hours. Finally, slices were washed in 1xPBS, mounted onto glass slides, and covered using FluorSave (EMD Millipore Corp.). For overlays of viral expression, microscopic images of the LH at bregma −1.3 mm were analyzed using “analyze particles” in Fiji (21). Next, images were overlaid using Sum Slices in the Z Project option.

Data analysis and statistics

Behavioral data were analyzed using Microsoft Excel, Graphpad Prism (version 7.05, Graphpad Software Inc.) and SPSS Statistics software version 23 (IBM Corp.). Animals that ate <1 g of chow over 7 hours after injections were removed from analyses (average chow intake after saline injections: 2.2±0.1 g). One animal from the LH LepR group was removed because of a lack of viral expression. Paired t tests and two-way repeated-measures ANOVA tests were used where applicable with Bonferroni adjusted post hoc tests. On cumulative consumption and locomotion data, main effects of time are not reported because time will, by definition, increase when analyzing cumulative data. A significance criterion of P<0.05, two tailed, was adopted in all statistical analyses.

Results

We injected VGAT-cre and LepR-cre mice with AAV5-hSyn-DIO-hM3Dq-mCherry targeted at the LH region. All LH VGAT (n=6) and LH LepR (n=8) mice had prominent viral expression in the LH (Figure 1A and Figure 2A). Because of viral spread, expression of hM3Dq-mCherry extended to a region near the LH, the zona incerta (ZI). As expected, expression in LH VGAT mice was clearly higher than in LH LepR mice, reflecting lower numbers of LepR neurons.

Feeding experiments

To test for effects of chemogenetically activating LH GABA and LH LepR neurons on feeding, we measured intake of regular chow and palatable foods over 7 hours. We pilot tested the effect of three doses of CNO (0.3 mg/kg, 1.0 mg/kg, and 3.0 mg/kg) on chow intake. Based on these experiments, we decided to continue using CNO 1.0 mg/kg because CNO 1.0 mg/kg gave the largest behavioral effect, and there were no nonspecific effects in control mice (data not shown). Chemogenetically activating LH GABA neurons increased chow intake at 1 hour and 2 hours, but not at other time points (Figure 1B; interaction of Time × Injection: F_{5,25}=5.651, P=0.0013; 1 hour: P=0.0092, 2 hours: P=0.0086). Because previous studies have reported increased consumption of palatable liquids (9,10), we also presented mice with 20% sucrose solution injections, which revealed that five out of six mice increased the weight change of sucrose solution; however, this did not reach statistical significance (Figure 1C; t(5)=−1.714, P=0.15). Next, we tested intake of nonliquid palatable foods: sugar cubes and lard. LH VGAT mice did not alter intake of sugar cubes after CNO injections (Figure 1D). Lard intake was decreased at 3, 5, and 7 hours upon CNO compared with vehicle injections (Figure 1E; interaction of Time × Injection: F_{5,25}=5.722, P=0.0012; 3 hours: P=0.0031, 5 hours: P=0.000017, 7 hours: P=0.000077). Finally, we presented mice with a combination of chow, sugar cubes, and lard. CNO-treated VGAT animals did not consume more calories over 7 hours (“Total” in Figure 1F). When taking the separate foods into account, paired t tests revealed increased chow (t(5)=−2.661, P=0.045) and decreased lard (t(5)=3.100, P=0.027) intake, but when corrected for multiple testing, these values did not reach statistical significance. Preference for palatable foods (calculated as preference ratio: caloric intake of sugar cubes + lard / total caloric intake) was decreased in LH VGAT animals after CNO injections compared with vehicle injections (Figure 1G; t(5)=5.248, P=0.003).

Throughout the feeding experiments, we anecdotally observed torn tissues, wet cages, chow dust, and bite marks on plastic petri dishes by CNO- but not saline-injected VGAT mice. We tested whether this urge to chew was nonspecific (nonfood directed) gnawing behavior. Therefore, we presented the animals with a block of wood similar to Navarro et al. (10). Indeed, CNO-injected LH VGAT mice shredded the wood and decreased the wood weight (t(5)=6.651, P=0.001; Figure 1H). To further differentiate feeding from nonspecific gnawing, we simultaneously presented a wood block and chow. This revealed that gnawing on the wood block was increased irrespective of whether chow was presented or not (Supporting Information Figure S1A; main effect of Injection: F_{1,5}=96.18, P=0.00019). We also more accurately assessed the consumption of chow by measuring both the total weight change of chow pieces and the weight of chow dust formed on the cage floor. This revealed that total weight change of chow was not affected by CNO (Supporting Information Figure SIB). However, the amount of chow dust was increased upon CNO injections (Supporting Information Figure SIC), but this did not result in a decrease in the amount of chow actually consumed (Supporting Information Figure SID).

We performed the same feeding experiments in LH LepR mice. Activating LH LepR neurons decreased intake of chow at all time points measured (Figure 2B; interaction of Time × Injection: F_{3,30}=7.875, P=0.000078; 1 hour: P=0.041, 2 hours: P=0.0018, 3 hours: P=0.000036, 5 hours: P=0.000013, 7 hours: P=0.000000053). Injecting CNO in LH LepR mice had no effect on the consumption of 20% sucrose solution, sugar cubes, or lard (Figure 2C-2E). Finally, when LH LepR mice were given free choice of chow, sugar cubes, or lard, there was no effect of CNO on total caloric intake or on total caloric intake of the separate foods (Figure 2F). The preference for palatable foods over chow was not affected by CNO (Figure 2G).
We also assessed whether the more specific group of LH GABA-LepR neurons contribute to LH GABA-activation-induced gnawing. We did not notice any urge to chew in CNO- or vehicle-treated LH LepR mice. Similarly, LH LepR mice did not gnaw on wood after either vehicle or CNO injections (Figure 2H).

Locomotion and body temperature

To test two aspects of energy expenditure, we assessed both horizontal locomotor activity and body temperature. As a proxy for body temperature, we used the temperature of the eye, which we found to be the highest temperature spot detectable on images produced by a thermosensitive camera. Moreover, temperature within the eye is more stable across different voxels compared with other regions used to detect temperature, such as brown adipose tissue.

LH VGAT mice decreased 1-hour locomotor activity after CNO injections (Figure 3A; \( t[5] = 3.031, P = 0.029 \)). Furthermore, CNO injections increased eye temperature at all time points measured (Figure 3C; interaction of Time \( \times \) Injection: \( F_{3,15} = 9.043, P = 0.0012 \); 0-30 minutes: \( P = 0.0057 \), 30-60 minutes: \( P = 0.000009 \), 60-90 minutes: \( P = 0.0078 \); Figure 3D; \( t[5] = 4.397, P = 0.0070 \)).
Activation of LH LepR neurons increased both aspects of energy expenditure: LH LepR mice increased locomotor activity (Figure 4A; \( t[7]=-4.820, P=0.002 \)) and increased eye temperature after CNO compared with vehicle injections (Figure 4C; main effect of Injection \( F_{1,7}=7.495, P=0.029 \); Figure 4D; \( t[7]=2.505, P=0.041 \)).

Repeated CNO injections in LH VGAT mice resulted in a larger decrease in body weight compared with vehicle injections (Figure 5A; interaction effect of Injection: \( F_{5,12}=5.353, P=0.0104 \); day 1: \( P=0.0032 \), day 2: \( P=0.0199 \), day 3: \( P=0.00029 \)). Intake of chow was not affected (Figure 5B), but repeated CNO administration increased the weight change of water compared with vehicle (Figure 5C; interaction of Time × Injection: \( F_{6,30}=7.097, P=0.000089 \); day 0.5: \( P=0.029 \), day 1: \( P=0.00053 \), day 1.5: \( P=0.000029 \), day 2: \( P=0.000071 \), day 2.5: \( P=0.00000051 \), day 3: \( P=0.00000069 \)).

Repeated CNO injections in LH LepR mice decreased body weight to a greater extent than vehicle injections (Figure 6A; interaction of Time × Injection: \( F_{3.21}=3.74, P=0.0269 \); day
To control for nonspecific effects of CNO itself and of its reverse-metabolized parent compound clozapine (22), we injected LepR-cre and VGAT-cre mice with a control virus that induces expression of channelrhodopsin, which does not respond to CNO or clozapine. Because we detected no differences in behavioral outcome between the mice, we combined them to form one control group. CNO injections in control mice had no effect on any parameter tested (Supporting Information Figure S2; n=4). Therefore, we conclude that behavioral effects observed in hM3Dq-injected mice are the result of enhanced neuronal activation in LH VGAT and LH LepR mice.

Discussion

Activating LH GABA neurons acutely and transiently increased feeding and decreased locomotion. In contrast, activating LH LepR neurons decreased feeding and increased locomotion. Both LH GABA and LH LepR activation increased body temperature, and repeated stimulation led to body weight loss. Our results showed that all behavioral effects mediated by LH LepR neurons promoted a negative energy balance, which was not the case for LH GABA neurons. Therefore, our data suggest that LH LepR neurons more specifically regulated parameters of energy balance compared with LH GABA neurons.

Recently, a study reported that LH GABA stimulation induced eating by low-frequency optogenetic stimulation but induced gnawing at higher frequencies that did not elicit feeding (8). These results imply that LH GABA neurons modulate feeding and gnawing depending on the extent of stimulation and that we and others who have reported gnawing behavior did not examine true feeding effects. We observed that solid chow and water bottle sippers robustly induced gnawing behavior upon LH GABA activation, but porous (sugar cubes) or soft (lard) foods did not. The extent of the weight change of chow was almost identical to previous studies that have assessed the effect of LH GABA activation on chow intake in the dark phase (9,10). Gnawing increased the weight change of food or water, which suggests that consumption of chow and water was increased. However, we observed that gnawing also resulted in spillage of water and food. Even more, when we directly measured the amount of chow actually consumed, we found that the amount consumed remained the same, even though gnawing of chow and wood occurred. These results suggest that the increased weight change in our original chow experiments did not represent an increase in consumption per se but was most likely the result of gnawing on the chow. Therefore, we suspect that previous reports on ingestive behaviors (reporting intake of chow and palatable solution) upon activation of LH GABA neurons may be similarly overestimated because of spillage as a result of gnawing.
We observed decreased consumption of chow when activating LH LepR neurons, which is in line with previous intra-LH leptin injections (14). However, the LH LepR stimulation decrease in feeding was observed only when chow was presented on the floor and not during repeated CNO injections, when chow was in the cage top. Interestingly, after vehicle injections, mice ate more when chow was more easily accessible on the cage floor (2.0 ± 0.1 g) compared with chow in the cage top, which requires that they stand up or climb to consume chow (1.5 ± 0.1 g). Thus, activation of LH LepR neurons decreased consumption of chow only when food was easily accessible.

To our knowledge, we have provided the first evidence that stimulating LH VGAT and LH LepR neurons increases body temperature. In LH LepR mice, increased locomotor activity may have contributed to the increase in body temperature. However, LH VGAT mice decreased locomotion, indicating an effect of LH GABA activation on thermogenesis independent of locomotor activity. LH lesions have been shown to decrease body temperature (23-25), and disinhibition of neurons in the LH have been shown to increase thermogenesis (4). Furthermore, the periaqueductal grey (PAG) was shown to be involved in thermogenesis (26), and both LH LepR and GABAergic neurons are known to substantially project to the PAG (14,27). Our data implicate both LH GABA and the more specific LH GABA-LepR neurons in LH-mediated thermogenesis, perhaps via projections to the PAG.

Deep brain stimulation of the LH in humans and rats has been shown to promote weight loss (21,29-31). Our data reveal that repeated stimulation of both LH GABA and LH LepR neurons enhanced body weight loss and suggest that LH-induced weight loss by deep brain stimulation was likely mediated by LH GABA neurons. Weight loss in LH LepR mice may have been the consequence of both increased locomotion and thermogenesis, whereas weight loss in LH VGAT mice seemed to be primarily driven by thermogenesis because locomotion was decreased.

LH LepR neurons have been shown to mainly coexpress neuropeptide Y (Nts) and galanin (Gal) (32,33). Our results that LH LepR neurons increased locomotion, but did not affect acute feeding, are in line with behavioral effects of LH Gal or LH Nts activation (13,34-36). Furthermore, body weight loss was also observed upon repeated LH Nts neuronal activation, which was similarly mainly the result of increased energy expenditure (36). LH Nts neurons, however, increased consumption of liquids, such as water and sucrose solution, but also quinine solution (34,36), whereas LH Gal neurons had no effect on ensure intake (13). Perhaps the simultaneous activation of LH Gal neurons suppressed the increase in intake of liquid induced by LH Nts neurons.

Several limitations to this study exist. First, expression of hM3Dq was not exclusive to the LH. In LH VGAT mice, there was copious expression of the DREADDGq in the ZI, which is similar to other LH GABA-targeted...
Figure 5: Body weight regulation upon repeated chemogenetic activation of LH GABA neurons. CNO (1.0 mg/kg) injections compared with saline injections in VGAT-cre animals (n=6) (A) increased body weight loss, (B) had no effect on food intake, and (C) increased water intake during a 3-day twice-daily injection scheme. Mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

Figure 6: Body weight regulation upon repeated chemogenetic activation of LH LepR neurons. CNO (1.0 mg/kg) injections compared with saline injections in LepR-cre animals (n=8) (A) increased body weight loss and (B) had no effect on food intake or (C) water intake during a 3-day twice-daily injection scheme. Mean ± SEM. *P < 0.05, **P < 0.01.
experiments (9,10,13). ZI expression was also observed in LH LepR animals but to a far lesser degree. Activation of ZI GABA neurons was shown to increase body weight and feeding, including of palatable foods, and to not affect locomotion (37). In our study, activation of LH GABA neurons increased feeding but decreased lord intake, locomotion, and body weight. This suggests that it is unlikely that activation of ZI GABA neurons had a major effect on the parameters that we assessed here.

Secondly, leptin was shown to depolarize 33% and hyperpolarize 22% of LH LepR neurons (14). Changes in chow intake by LH LepR were observed after intra-LH leptin injections (14), but not after LH LepR deletion. LepR deletion in neuronal subpopulations of the LH (19,20,38), or when we chemogenetically activated the majority of LH LepR neurons. This suggests that leptin-induced feeding in the LH was regulated by the intricate activation and inhibition of LH LepR neurons, and perhaps effects on feeding were masked when increasing the activity of all LH LepR neurons.

Third, the group size of LH VGAT mice was low, and negative data should therefore be interpreted with caution. For instance, the weight change of sugar solution was increased in five out of six CNO-injected LH VGAT mice but did not reach statistical significance. However, the findings that reached statistical significance were observed in all LH VGAT mice. Therefore, even though we used a low number of mice, the behavioral output was rather consistent between animals.

Fourth, to assess locomotor behavior, we tracked horizontal locomotion. LH VGAT activation decreased locomotion, which is line with findings reported by Navarro et al. (10), who also measured horizontal locomotion. However, this in contrast to findings by Qualls-Creekmore et al. (13), who made use of beam breaks and subcategorically implanted telemetric transmitters. A previous study showed that telemetry was more sensitive for detecting hyperactivity than a home cage monitoring system, let alone video tracking of horizontal movement (39). In our study, activation of LH GABA neurons was more sensitive for detecting hyperactivity than a home cage monitoring system, let alone video tracking of horizontal movement (39).

Conclusion
Our results reveal that LH LepR neurons more specifically regulate aspects of energy balance to promote a negative energy balance compared with LH GABA neurons. Furthermore, we have provided the first evidence that activation of LH GABA and LH LepR neurons modulates body temperature and, when stimulated repeatedly, enhances body weight loss. The latter is especially interesting in light of the obesity epidemic and may aid in the discovery of new treatment strategies to combat obesity.

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