Glutamatergic Preoptic Area Neurons That Express Leptin Receptors Drive Temperature-Dependent Body Weight Homeostasis

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The preoptic area (POA) regulates body temperature, but is not considered a site for body weight control. A subpopulation of POA neurons express leptin receptors (LepRb^{POA} neurons) and modulate reproductive function. However, LepRb^{POA} neurons project to sympathetic premotor neurons that control brown adipose tissue (BAT) thermogenesis, suggesting an additional role in energy homeostasis and body weight regulation. We determined the role of LepRb^{POA} neurons in energy homeostasis using *cre*-dependent viral vectors to selectively activate these neurons and analyzed functional outcomes in mice. We show that LepRb^{POA} neurons mediate homeostatic adaptations to ambient temperature changes, and their pharmacogenetic activation drives robust suppression of energy expenditure and food intake, which lowers body temperature and body weight. Surprisingly, our data show that hypothermia-inducing LepRb^{POA} neurons are glutamatergic, while GABAergic POA neurons, originally thought to mediate warm-induced inhibition of sympathetic premotor neurons, have no effect on energy expenditure. Our data suggest a new view into the neurochemical and functional properties of BAT-related POA circuits and highlight their additional role in modulating food intake and body weight.

Key words: body temperature; body weight; DREADD; energy expenditure; food intake; thermoregulation

Significance Statement

Brown adipose tissue (BAT)-induced thermogenesis is a promising therapeutic target to treat obesity and metabolic diseases. The preoptic area (POA) controls body temperature by modulating BAT activity, but its role in body weight homeostasis has not been addressed. LepRb^{POA} neurons are BAT-related neurons and we show that they are sufficient to inhibit energy expenditure. We further show that LepRb^{POA} neurons modulate food intake and body weight, which is mediated by temperature-dependent homeostatic responses. We further found that LepRb^{POA} neurons are stimulatory glutamatergic neurons, contrary to prevalent models, providing a new view on thermoregulatory neural circuits. In summary, our study significantly expands our current understanding of central circuits and mechanisms that modulate energy homeostasis.

Introduction

Adult animals and humans maintain their body weight at relatively constant levels through the intricate balance between food

intake and energy expenditure, which is largely regulated by the hypothalamus and brainstem (Gao and Horvath, 2007; Rosenbaum and Leibel, 2010). Leptin promotes a negative energy balance and weight loss by suppressing food intake and enhancing energy expenditure through leptin receptor (LepRb)-expressing neurons in the brain, while the absence of leptin action promotes

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a positive energy balance and weight gain (Münzberg and Morrison, 2015). Leptin increases energy expenditure by stimulating brown adipose tissue (BAT) thermogenesis, and leptin-deficient mice show hypothermia and cold intolerance due to reduced BAT activity (Trayhurn et al., 1977; Haynes et al., 1997). Leptin action in the dorsomedial hypothalamus/dorsal hypothalamic area (DMH/DHA) regulates BAT thermogenesis and body weight by enhancing BAT sympathetic nerve activity (SNA; Enriori et al., 2011; Rezai-Zadeh et al., 2014). Another population of BAT-related LepRb neurons is found in the preoptic area (POA; Zhang et al., 2011). However, the role of these LepRb^{POA} neurons in energy homeostasis and body weight control is unknown.

The POA responds to changes in ambient temperature. This modulates the SNA in BAT, a heat generating tissue that regulates core temperature (Nakamura, 2011). Previous studies have suggested that cold-inhibited and warm-activated GABAergic POA neurons innervate BAT-related DMH/DHA and rostral raphe pallidus (rRPa) neurons (Nakamura et al., 2002, 2005, 2009; Yoshida et al., 2009). Indeed, cold exposure potently stimulates while warm/thermoneutral exposure strongly inhibits BAT thermogenesis. These robust changes in energy expenditure typically do not affect body weight due to simultaneous stimulation or inhibition of food intake during cold or warm exposure, respectively (Ravussin et al., 2014; Xiao et al., 2015). This type of adaptation is in contrast to the negative energy balance promoted by leptin that increases energy expenditure, while decreasing food intake. In fact, leptin's involvement in POA-mediated energy homeostasis is unlikely because the selective deletion of LepRb in the POA disrupts reproductive function in female mice without affecting body weight (Bellefontaine et al., 2014).

However, the POA's well known function in BAT SNA control and the fact that LepRb^{POA} neurons multisynaptically innervate BAT through the DMH/DHA and the rRPa (Zhang et al., 2011) strongly suggest that LepRb^{POA} neurons affect BAT thermogenesis, but may be independent of leptin. Also, DMH/DHA LepRb neurons modulate energy expenditure and body weight (Rezai-Zadeh et al., 2014), further indicating that BAT-connected LepRb^{POA} neurons may similarly regulate body weight.

Employing pharmacogenetic activation of LepRb, glutamatergic, or GABAergic POA neurons, we confirmed that LepRb^{POA} neurons robustly block BAT thermogenesis, resulting in decreased core temperature. Furthermore, we uncovered their additional role in suppressing food intake, which caused significant body weight loss. In contrast to previous reports, we found no evidence that GABAergic POA neurons regulate BAT thermogenesis. To the contrary, we show compelling evidence that glutamatergic LepRb^{POA} neurons mediate adaptive changes in food intake and energy expenditure in response to ambient temperature changes. Our data suggest a new view of the neurochemical and functional properties of BAT-related POA circuits and highlight their additional role in modulating temperature-dependent food intake and body weight homeostasis.

Materials and Methods

Animals. All experiments were approved by the Institutional Animal Care and Use Committee. Mice were housed at room temperature (RT) with a 12 h light/dark cycle. Laboratory rodent diet (#5001, LabDiet) and water were available *ad libitum* unless otherwise stated. *LepRb^{EGFP}* and *LepRb^{Cre}* mice (kindly provided by Dr. Martin G. Myers, Jr., University of Michigan; Leshan et al., 2006, 2010); and *Vglut2^{Cre}*, *Vglut2^{EGFP}*, *Vgat^{Cre}*, and *Vgat^{EGFP}* mice (generously provided by Dr. Bradford B. Lowell, Beth Israel Deaconess Medical Center and Harvard Medical School; Vong et al., 2011) were obtained from in-house breeding colonies. Only male mice were used for studies of temperature-dependent cFos induction and

warm-induced change in energy expenditure and food intake (see Fig. 5). All other experiments were conducted with both sexes and no sex differences were observed.

Stereotaxic surgery. Stereotaxic viral injection adenoassociated virus (AAV) was performed as previously described (Rezai-Zadeh et al., 2014). AAV5-hSyn-DIO-mCherry (control) and AAV5-hSyn-DIO-hM3Dq-mCherry ([DREADD-Gq (Gq-coupled Designer Receptor Exclusively Activated by Designer Drug)]) were made available by Dr. Bryan Roth and obtained through the vector core of the University of North Carolina at Chapel Hill. Deeply anesthetized 8–10-week-old *LepRb^{Cre}*, *Vglut2^{Cre}*, or *Vgat^{Cre}* mice were placed on a stereotaxic alignment system (#1900, David Kopf Instruments) to facilitate accurate, bilateral viral injections into the POA (anteroposterior, 0.55 mm; mediolateral, ± 0.4 mm; dorsoventral, -5.2 mm; 200–400 nl total, 20 nl/30 s) according to the Paxinos mouse brain atlas (Paxinos and Franklin, 2004). Guide cannula and injector remained in place for 5 min before removal to prevent backflow, and skull and incision was closed with bone wax (Lukens #901, Medline Industries) and wound clips (#203-1000, CellPoint Scientific). Mice were single-housed for experiments 2–3 weeks later. Only data from mice with correctly targeted virus injections were used for analysis. DREADD-Gq-injected mice without proper viral expression in the POA failed to show clozapine N-oxide (CNO; #C0832, Sigma-Aldrich)-induced changes in core temperature or energy expenditure. Finally, control or DREADD-Gq-injected mice received a saline or CNO (0.5 or 1.5 mg/kg, i.p.) 2–3 h before perfusions. Then brains were harvested and analyzed to confirm CNO-induced neuronal activation.

Rectal temperature measurement. Rectal temperature was measured with a thermal probe (#227-193, ThermoWorks) every 20 min for 100 min following saline or CNO injections.

Metabolic studies. Energy expenditure, locomotor activity, and respiratory exchange ratio (RER) were measured in a Comprehensive Laboratory Animal Monitoring System (CLAMS; Columbus Instruments) or a TSE system (TSE Systems). In the TSE system, food intake data were also collected. Mice were acclimated for ≥ 3 d before collection of experimental data.

Data were collected by the TSE system in response to consecutive treatments with saline or different CNO doses (0.025, 0.05, 0.1, 0.2, 0.4, 0.8, and 1.6 mg/kg, i.p.) right before the dark cycle every other day. Metabolic measurements (20 min intervals) were obtained at RT and 6 h averages were compared between saline (baseline) and CNO injections to calculate ΔVO_2 , $\Delta\text{food intake}$, $\Delta\text{activity}$, and ΔRER .

For warm (30°C) exposure-induced changes in energy expenditure and food intake, data were collected by the TSE system in male mice (22°C, $n = 6$; 30°C, $n = 6$), and 3 d mean values were normalized by body weight.

For measurements during changing ambient temperatures (*LepRb^{POA}*, *Vglut2^{POA}*, and *Vgat^{POA}* DREADD-Gq mice, $n = 4$ for each group), mice were initially kept at RT (22°C). At circadian time (CT) 4, ambient temperature was changed to 30°C, 10°C, or maintained at RT. At CT5, mice received consecutive treatments with saline or CNO (0.5 mg/kg, i.p.) injections and, at CT12, ambient temperature was returned to RT (see Fig. 6A). ΔVO_2 , $\Delta\text{activity}$, and ΔRER were calculated from baselines (1 h mean before the onset of ambient temperature change) and 4 h means after injections, and data were compared between saline and CNO injections.

To test the interaction between LepRb^{POA} neurons and $\beta 3$ adrenergic receptor ($\beta 3\text{AR}$) signaling, VO_2 , locomotor activity, and RER were measured in a group of *LepRb^{POA}* DREADD-Gq mice ($n = 4$) by CLAMS at RT. After 3 d of acclimation, saline, CNO (0.5 mg/kg, i.p.), CL316,243 (1.0 mg/kg, i.p.), or CNO + CL316,243 was injected at CT5 on 4 consecutive days.

The effect of chronic CNO injection on body weight was tested by injecting saline (3 d) or CNO (6 d; 0.3 mg/kg, i.p.) twice daily, once at CT2.5 and another at CT11 in *LepRb^{POA}* control ($n = 5$) and *LepRb^{POA}* DREADD-Gq ($n = 5$) mice while they were housed in the TSE system at RT (see Fig. 3A). Body weight was measured every day just before a morning injection with *ad libitum* food and water. VO_2 , locomotor activity, RER, and food intake were monitored every 25 min.

Restricted feeding. To measure pharmacogenetically induced food-intake changes, a group of *LepRb^{POA}* control ($n = 5$) and *LepRb^{POA}*

Figure 1. *LepRb^{POA}* neurons decrease core temperature and energy expenditure. **A**, Distribution of LepRb-expressing neurons in the POA of *LepRb^{EGFP}* reporter mice. Right panels show corresponding regions in the mouse brain atlas (Paxinos and Franklin, 2004). 3V, Third ventricle; MPA, medial preoptic area; LPO, lateral preoptic area; VOLT, vascular organ of the lamina terminalis; AVPe, anteroventral periventricular nucleus; VMPO, ventromedial preoptic nucleus; VLPO, ventrolateral preoptic nucleus; MnPO, median preoptic nucleus; ac, anterior commissure. **B**, Representative images showing virus-infected neurons (mCherry, red) and cFos (green) in the POA in *LepRb^{POA}* control and *LepRb^{POA}* DREADD-Gq mice. CNO (0.5 mg/kg, i.p.) was injected 2–3 h before perfusion. Insets show magnified images of the indicated areas (dotted line boxes). **C**, Rectal temperature of *LepRb^{POA}* control (n = 7) and *LepRb^{POA}* DREADD-Gq (n = 9) mice after CNO injection (1.5 mg/kg, i.p.) at RT. **D**, VO_2 measurement in *LepRb^{POA}* DREADD-Gq mice (n = 4) during saline (black) or CNO (0.5 mg/kg, i.p.; red) injections at RT. **E**, Locomotor activity in the same *LepRb^{POA}* DREADD-Gq mice shown in **D** during the same period. Locomotor activity from 12:00 to 6:00 P.M. is enlarged in the box. **F**, Change in VO_2 (percentage) in *LepRb^{POA}* control (n = 4) and *LepRb^{POA}* DREADD-Gq (n = 4) mice at different CNO concentrations at RT. **G**, Photos capturing postural extension 70 min after CNO injection (1.5 mg/kg, i.p.) in a *LepRb^{POA}* DREADD-Gq mouse compared with a control mouse at RT in their home cages. Data are represented as mean $\pm$ SEM. *p < 0.05, **p < 0.01, and ***p < 0.0001 (two-way repeated-measures ANOVA and Bonferroni's pairwise comparisons).

DREADD-Gq (n = 4) mice were trained to consume their daily food within 4 h during the light phase (CT3–CT7). After 3 d, mice reached steady-state body weight ($-13 \sim 14\%$ compared with initial body weight) and were injected with saline or CNO (0.2 mg/kg, i.p.) on alternating days, 30 min before the feeding session. Food intake was recorded and compared between groups and treatments for the initial 2 h (CT3–CT5). Food was removed at CT7 and mice were moved to new cages. Average 2 h food intake was calculated from day 3 and 5 for saline and from day 4 and 6 for CNO injections (see Fig. 4C).

cFos induction in *LepRb^{EGFP}* mice at different ambient temperatures. Four-month-old male *LepRb^{EGFP}* mice were single-housed and acclimated to environmental chambers at RT over 4 d, and they were exposed to either 4°C (n = 6), 30°C (n = 6), or maintained at RT (n = 9) for 3 h. Mice were perfused and brains were further processed for immunohistochemical analysis of cFos induction in *LepRb^{POA}* EGFP neurons.

Leptin-induced phosphorylation of signal transducer and activator of transcription 3 in *Vglut2^{EGFP}* and *Vgat^{EGFP}* mice. Functional LepRb neurons were visualized by immunohistological detection of leptin-induced

Figure 2. LepRb^{POA} neurons inhibit β 3 adrenergic receptor-dependent BAT thermogenesis. **A**, 24 h VO₂ measurement in LepRb^{POA} DREADD-Gq mice ($n = 4$) after injections of saline (black), CNO (0.5 mg/kg, i.p.; red), CL316,243 (1.0 mg/kg, i.p.; blue), or CNO + CL316,243 (green). **B**, The average VO₂ during 6 h of postinjection was compared between injections. Values inside columns represent percentages compared with the saline injection. **C**, The average locomotor activity during 6 h after injection was compared between injections. Values inside columns represent percentages compared with the saline injection. Data are represented as mean $\pm$ SEM. Bars with different letters denote significant differences at $p < 0.05$ (one-way repeated-measures ANOVA and Fisher's least significant difference pairwise comparisons).

phosphorylation of signal transducer and activator of transcription 3 (pSTAT3; Faouzi et al., 2007; Laque et al., 2015). To identify whether LepRb^{POA} neurons are excitatory glutamatergic or inhibitory GABAergic, male *Vglut2^{EYFP}* and *Vgat^{EYFP}* mice ($n = 3$ each) received a bolus injection of murine leptin (5 mg/kg, i.p.; National Hormone and Peptide Program, <http://www.humc.edu/hormones>) and were perfused 1 h later. Brains were further processed for immunohistochemical detection of pSTAT3 and enhanced yellow fluorescent protein (EYFP).

Perfusion and immunohistochemistry. Perfusions and immunohistochemistry were performed as previously described (Zhang et al., 2011).

Briefly, deeply anesthetized mice were transcardially perfused with ice-cold physiological saline, followed by 10% formalin. Brains were removed and postfixed in 10% formalin overnight at 4°C and cryoprotected in 30% sucrose. Brains were sliced at 30 μ m thickness and processed for free-floating immunohistochemistry. pSTAT3 and occasionally cFos were visualized by diaminobenzene (DAB; #34065, Thermo Fisher Scientific) following treatment with Vectastain ABC (#PK-6100, Vector Laboratories) after incubation with biotinylated secondary antibodies. All other proteins were visualized by fluorophore-labeled secondary antibodies. Primary antibodies used are goat anti-cFos (1:200; #sc-52-G, Santa Cruz Biotechnology), rabbit anti-cFos (1:3000; #PC38, EMD Millipore), rabbit anti-pSTAT3 (Tyr705; 1:500; #9131, Cell Signaling Technology), rabbit anti-dsRed (1:500; #632496, Clontech Laboratories), and chicken anti-GFP (1:1000; #ab13970, Abcam). Secondary antibodies used were donkey anti-goat IgG-Alexa594 (#A11058, Life Technologies), donkey anti-goat IgG-Alexa488 (#A11055, Life Technologies), donkey anti-rabbit IgG-Alexa594 (#A12107, Life Technologies), donkey anti-chicken IgY-DyLight488 (#703-486-155, Jackson ImmunoResearch Laboratories), donkey anti-goat IgG-biotin (#705-065-003, Jackson ImmunoResearch Laboratories), and donkey anti-rabbit IgG-biotin (#711-065-152, Jackson ImmunoResearch Laboratories).

Microscopy and estimates of cell counts. Immunohistochemistry staining was visualized with a fluorescent microscope (#BX51, Olympus), and images were taken with a digital camera (#DP30BW, Olympus) using appropriate filters for different fluorophores or bright-field illumination for DAB stains. Images from identical areas were taken for double immunohistochemistry, overlaid, and pseudocolored using Adobe Photoshop CS6 (Adobe Systems). Contrast and brightness were adjusted with identical settings for all images within an experiment using Adobe Photoshop CS6 for better visualization of signals.

To determine the percentage of LepRb^{POA} neurons that were activated by different temperature exposures, total LepRb^{POA}-EGFP⁺ neurons and LepRb^{POA}-EGFP/cFos double-positive cells were counted to calculate the percentage of cFos⁺ LepRb^{POA} neurons. The anatomical extent of the POA area subjected for cell counts was defined by the expression of LepRb-EGFP⁺ neurons, which are found within 2–3 sections (from bregma +0.62 to +0.26 mm along the rostrocaudal axis) using one of four series of 30- μ m-thick coronal sections per animal. Thus, the total number of LepRb^{POA}-EGFP⁺ neurons was estimated by cell counts and the coexpression with cFos was assessed within these identified LepRb^{POA}-EGFP⁺ neurons. Cell counts were done manually and positive cells were identified individually by an investigator for the appearance of cytoplasmic (EGFP⁺) or nuclear (cFos) fluorescent stain. Any stain that could not be easily identified as a cytoplasmic or nuclear stain was omitted from cell counts.

To quantify leptin-induced pSTAT3 in *Vglut2^{EYFP}* and *Vgat^{EYFP}* mice, total numbers of pSTAT3⁺ neurons in the POA and pSTAT3/EYFP double-positive cells were counted to calculate the percentage of glutamatergic or GABAergic LepRb^{POA} neurons ($n = 3$ for each group). Similar to the method mentioned above, we defined the POA area by the appearance of LepRb neurons. Here LepRb neurons were identified by the expression of leptin-induced pSTAT3 (DAB stain) as a surrogate for LepRb neurons. Again we consistently identified 2–3 sections per animal for cell counts and the total number of pSTAT3⁺ neurons was estimated by cell counts and the coexpression with EGFP⁺ vesicular GABA transporter (Vgat) or vesicular glutamate transporter 2 (*Vglut2*) neurons was further assessed within these identified pSTAT3⁺ neurons. Cell counts were done manually by an investigator as noted above.

Statistical analysis. All data were statistically analyzed by SPSS 22 (IBM). See results and figure legends for individual statistical test. $p < 0.05$ was considered statistically significant in all experiments.

Results

Pharmacogenetic activation of LepRb^{POA} neurons decreases core temperature and energy expenditure

LepRb in the POA is mainly expressed in the median preoptic nucleus (MnPO; Fig. 1A). Because LepRb^{POA} neurons are connected to sympathetic premotor neurons and multisynaptically

project to BAT (Zhang et al., 2011), we hypothesized that $\text{LepRb}^{\text{POA}}$ neurons would likely modulate energy expenditure by controlling BAT activity.

To test this idea, DREADD-Gq was selectively expressed in $\text{LepRb}^{\text{POA}}$ neurons of $\text{LepRb}^{\text{Cre}}$ mice ($\text{LepRb}^{\text{POA}}$ DREADD-Gq). Subsequent CNO injection strongly induced cFos (a neuronal activation marker) in virus-infected cells (indicated by mCherry⁺ neurons) in $\text{LepRb}^{\text{POA}}$ DREADD-Gq mice, but not in mice injected with control virus (Fig. 1B). CNO injection induced a robust drop of rectal temperature that reached the nadir ~1 h after the CNO injection in $\text{LepRb}^{\text{POA}}$ DREADD-Gq, but not in control mice (Fig. 1C; repeated-measures ANOVA for the interaction between time and group: $F_{(6,84)} = 34.99$, $p < 0.001$). Only mice with correct DREADD-Gq expression in $\text{LepRb}^{\text{POA}}$ neurons decreased rectal temperature with CNO (data not shown).

Consistent with the reduced core temperature phenotype, CNO-induced activation of $\text{LepRb}^{\text{POA}}$ neurons significantly decreased energy expenditure (VO_2) by 80% (Fig. 1D; repeated-measures ANOVA for the effect of treatment during 6 h postinjection: $F_{(1,3)} = 189.55$, $p < 0.01$). During the same period, locomotor activity and RER also significantly decreased (Fig. 1E; repeated-measures ANOVA for the effect of treatment during 6 h postinjection: $F_{(1,3)} = 15.06$, $p < 0.05$; data not shown), indicating that part of the reduction in energy expenditure was due to decreased activity. The degree and duration of VO_2 decrease was CNO dose dependent (Fig. 1F; repeated-measures ANOVA for the interaction between concentration and group: $F_{(7,42)} = 7.85$, $p < 0.001$), while locomotor activity and RER only showed trends of CNO dose dependency (data not shown), suggesting direct CNO effects on VO_2 , while locomotor activity and RER may passively follow the direct changes in VO_2 . Activation of $\text{LepRb}^{\text{POA}}$ neurons also promoted a postural extension (Fig. 1G), a typical behavioral response in mice exposed to higher ambient temperature (Roberts, 1988). $\text{LepRb}^{\text{POA}}$ DREADD-Gq mice with CNO injection were mostly immobile but awake and alert (Fig. 1E; data not shown).

$\text{LepRb}^{\text{POA}}$ neurons inhibit β_3 adrenergic receptor-dependent BAT thermogenesis

Next, we aimed to verify that $\text{LepRb}^{\text{POA}}$ neurons act via sympathetic outputs to BAT. The $\beta_3\text{AR}$ is predominantly expressed in BAT and white adipose tissue, and mediates adaptive thermogenesis by promoting expression of uncoupling protein 1 and lipolysis (Susulic et al., 1995; Grujic et al., 1997; Collins et al., 2010).

Figure 3. Chronic activation of $\text{LepRb}^{\text{POA}}$ neurons. **A**, The experimental scheme showing timings of body weight measurement and injections for chronic CNO treatment. CNO was injected intraperitoneally twice daily at 0.3 mg/kg for 6 consecutive days following 3 d of saline injections, and no injection was made during 3 d of recovery. **B**, Body weight of $\text{LepRb}^{\text{POA}}$ control ($n = 5$) and $\text{LepRb}^{\text{POA}}$ DREADD-Gq ($n = 5$) mice during chronic CNO treatment. **C**, CNO reduced energy expenditure during 6 d of CNO treatment in $\text{LepRb}^{\text{POA}}$ DREADD-Gq ($n = 5$) but not in control ($n = 5$) mice. **D**, Average daily energy expenditure (kcal/g/d) was compared between groups and treatments. **E**, Chronic CNO reduced daily food intake in $\text{LepRb}^{\text{POA}}$ DREADD-Gq ($n = 5$) but not in control ($n = 5$) mice. **F**, Average daily food intake (kcal/g/d) was compared between groups and treatments. **G**, Change in food intake (percentage) in $\text{LepRb}^{\text{POA}}$ control ($n = 4$) and $\text{LepRb}^{\text{POA}}$ DREADD-Gq ($n = 4$) mice at different CNO concentrations at RT. Data are represented as mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, and bars with different letters denote significant differences at $p < 0.05$ (two-way repeated-measures ANOVA and Bonferroni's pairwise comparisons).

As expected, a systemic injection of CL316,243, a $\beta_3\text{AR}$ agonist, increased VO_2 (Fig. 2A,B; repeated-measures ANOVA for the effect of treatment for 6 h mean of postinjection in B: $F_{(3,9)} = 121.89$, $p < 0.001$) in $\text{LepRb}^{\text{POA}}$ DREADD-Gq mice without affecting locomotor activity (Fig. 2C; repeated-measures ANOVA for the effect of treatment for 6 h mean of postinjection: $F_{(3,9)} = 8.77$, $p < 0.01$). The combined injection of CL316,243 with CNO prevented the CNO-mediated VO_2 decrease (Fig. 2A,B), which is consistent with a model hypothesizing that activation of $\text{LepRb}^{\text{POA}}$ neurons suppresses $\beta_3\text{AR}$ -induced adaptive thermogenesis.

The coinjection of CL316,243 and CNO modestly recovered CNO-decreased locomotor activity even though CL316,243

Figure 4. *LepRb^{POA}* neurons modulate food intake. **A**, Average daily locomotor activity was compared between groups and treatments during chronic CNO treatment. **B**, Total travel distance during 5 min was compared between groups (control, $n = 5$; DREADD-Gq, $n = 4$) and treatments 30 min after injections. **C**, The experimental scheme for restricted feeding. **D**, The first 2 h food intake during restricted feeding was measured in *LepRb^{POA}* control ($n = 5$) and *LepRb^{POA}* DREADD-Gq ($n = 4$) mice. Only *LepRb^{POA}* DREADD-Gq mice reduced food intake by CNO injection. **E**, Average 2 h food intake during the restricted feeding (control, $n = 5$; DREADD-Gq, $n = 4$). Saline data are from days 3 and 5, and CNO data are from days 4 and 6. Data are represented as mean $\pm$ SEM. * $p < 0.01$ (paired t test). Data with different letters denote significant differences at $p < 0.05$ (two-way repeated-measures ANOVA and Bonferroni's pairwise comparisons).

alone did not affect locomotor activity (Fig. 2C). Thus, the increased locomotor activity from the coinjection may not be a direct consequence of the activation of the $\beta 3$ AR, but rather a secondary effect of elevated core temperature.

LepRb^{POA} neurons modulate food intake and body weight

We further tested how the dramatic decrease in energy expenditure would influence body weight during chronic activation of *LepRb^{POA}* neurons. A group of *LepRb^{POA}* control and *LepRb^{POA}* DREADD-Gq mice were injected with low-dose CNO (0.3 mg/kg, i.p.) twice daily for 6 d. Body weight, food intake, energy expenditure, and locomotor activity were monitored (Fig. 3A).

Chronic activation of *LepRb^{POA}* was expected to cause a positive energy balance and body weight gain. Unexpectedly, body weight of *LepRb^{POA}* DREADD-Gq mice gradually decreased during daily CNO treatment and started to return to preinjection levels once the CNO injection stopped (Fig. 3B; repeated-measures ANOVA for the interaction between day and group: $F_{(10,80)} = 3.07$, $p < 0.01$). Twenty-four hour energy expenditure decreased during daily CNO injections as expected (Fig. 3C,D;

repeated-measures ANOVA for the interaction between treatment and group in D: $F_{(2,16)} = 4.61$, $p < 0.05$), implying a simultaneous reduction of food intake. Indeed, daily food intake decreased significantly during CNO injections and recovered to normal levels without any signs of compensatory overfeeding after CNO injections stopped (Fig. 3E,F; repeated-measures ANOVA for the interaction between day and group in E: $F_{(10,80)} = 3.01$, $p < 0.01$; between treatment and group in F: $F_{(2,16)} = 3.93$, $p < 0.05$). We also found that food intake decreased CNO dose-dependently (Fig. 3G; repeated-measures ANOVA for the interaction between concentration and group: $F_{(7,42)} = 6.62$, $p < 0.001$), similar to the decrease in energy expenditure (Fig. 1F).

Although we used a relatively low CNO dose for the daily treatment, we worried that the effects of low core temperature on locomotor activity may have physically limited normal feeding behavior even though locomotor activity of *LepRb^{POA}* DREADD-Gq mice did not significantly decrease during CNO treatment (Fig. 4A; repeated-measures ANOVA for the interaction between treatment and group: $F_{(2,16)} = 1.91$, $p > 0.05$). In a new cohort of *LepRb^{POA}* DREADD-Gq mice, we found that a CNO dose of 0.2 mg/kg did not affect locomotor activity (Fig. 4B; repeated-measures ANOVA for the effect of treatment: $F_{(1,7)} = 0.002$, $p > 0.05$). Furthermore, we trained this cohort of control and *LepRb^{POA}* DREADD-Gq mice to consume their daily food in a restricted 4 h time window, during which the mice would be maximally motivated to consume food. Saline or CNO was injected 30 min before the feeding session and the initial 2 h food intake was evaluated (Fig. 4C). CNO still reduced 2 h food intake in *LepRb^{POA}* DREADD-Gq mice (Fig. 4D; repeated-measures ANOVA for the interaction between day and group: $F_{(5,35)} = 2.89$, $p < 0.05$). The comparison of average 2 h food intake between saline (days 3 and 5) and CNO (days 4 and 6) injections also showed significant difference (-33% compared with saline treatment) only in *LepRb^{POA}* DREADD-Gq mice (Fig. 4E; paired t test for DREADD-Gq: $t_{(3)} = 8.00$, $p < 0.01$). Thus, these data overall further corroborate the finding that *LepRb^{POA}* neurons indeed modulate food intake.

LepRb^{POA} neurons are activated by warm, but not by cold exposure

It has been suggested that POA neurons are inhibited by cold exposure, which leads to activation of BAT adaptive thermogenesis. Conversely, the adaptation to thermoneutral, warm ambient temperature causes a reduction in energy expenditure and inhibition of BAT adaptive thermogenesis (Cannon and Nedergaard, 2011). Thus, we hypothesized that *LepRb^{POA}* neurons may be similarly affected by ambient temperature. We initially

Figure 5. LepRb^{POA} neurons are activated by warm exposure, but not by cold exposure. **A**, Comparisons of energy expenditure and food intake between mice housed at 22°C ($n = 6$) and 30°C ($n = 6$). **B**, Representative immunohistochemical images showing the distribution of cFos (red, top panels) and cFos/LepRb^{POA}-EGFP (bottom panels) after 3 h of cold (4°C; $n = 6$), RT (22°C; $n = 9$), and warm (30°C; $n = 6$) exposure in LepRb^{EGFP} mice. Insets show magnified images of the indicated areas (dotted line boxes). **C**, The percentage of LepRb^{POA}-EGFP cells that express cFos was calculated for each temperature condition. **D**, **E**, Total number of EGFP⁺ (**D**) and EGFP⁺/cFos⁺ (**E**) cells in the POA counted in each temperature condition. * $p < 0.001$ (independent t test). Data with different letters denote significant differences at $p < 0.01$ for **C** and $p < 0.05$ for **E** (one-way ANOVA and Fisher's least significant difference pairwise comparisons). n.s., Not significant.

tested whether the physiological adaptation to warm temperature (30°C) results in similar changes of energy expenditure and food intake as observed after pharmacogenetic activation of LepRb^{POA} neurons. Indeed, energy expenditure and food intake dramatically decreased at 30°C compared with RT (Fig. 5A; independent t test: for energy expenditure, $t_{(10)} = 15.32$, $p < 0.001$; for food intake, $t_{(10)} = 7.72$, $p < 0.001$), thus reflecting the same physiological changes as observed by pharmacogenetic activation of LepRb^{POA} neurons.

We next tested how changes in ambient temperature affect activity of LepRb^{POA} neurons. LepRb^{EGFP} mice were exposed to cold temperature (4°C), RT (22°C), or warm temperature (30°C) for 3 h, and cFos induction in LepRb^{POA}-EGFP neurons was analyzed. Only warm exposure increased the number of cFos⁺ LepRb^{POA} neurons by approximately twofold (Fig. 5B,C; ANOVA in C: $F_{(2,18)} = 8.67$, $p < 0.01$). While the total number of LepRb^{POA}-EGFP cells was similar in all groups (Fig. 5D; ANOVA: $F_{(2,18)} = 1.50$, $p > 0.05$), the number of LepRb^{POA}-EGFP and cFos double-positive cells was only increased at 30°C (Fig. 5E; ANOVA: $F_{(2,18)} = 3.82$, $p < 0.05$), suggesting that pharmacogenetic activation of LepRb^{POA} neurons mimics the physiological response to warm temperature, except that their activation at RT resulted in hypothermia (Fig. 1C).

Based on these results, we predicted that the effect of pharmacogenetic LepRb^{POA} neuronal activation should be minimal at thermoneutral temperature, because LepRb^{POA} neurons are already activated and adaptive thermogenesis is minimal. Conversely, pharmacogenetic activation of LepRb^{POA} neurons should have maximal effects at cold ambient temperature be-

cause LepRb^{POA} neurons should be naturally inhibited to promote adaptive thermogenesis.

To test this idea, we injected saline or CNO into LepRb^{POA} DREADD-Gq mice 1 h after the temperature change from RT to cold (10°C) or warm (30°C; Fig. 6A). As predicted, at 30°C, pharmacogenetic activation of LepRb^{POA} neurons resulted in a minimal, albeit significant, decrease in VO₂ (Fig. 6B; repeated-measures ANOVA for the effect of treatment during 6 h postinjection, $F_{(1,3)} = 129.51$, $p < 0.01$); conversely, at 10°C, CNO injection vastly attenuated cold-induced VO₂ increase (Fig. 6C; repeated-measures ANOVA for the effect of treatment during 6 h postinjection: $F_{(1,3)} = 38.45$, $p < 0.01$). The VO₂ difference (Δ VO₂) between saline and CNO injections at different temperatures was largest at 10°C and smallest at 30°C (Fig. 6D,E; repeated-measures ANOVA for the interaction between temperature and treatment in D: $F_{(2,6)} = 39.69$, $p < 0.001$; for the effect of temperature in E: $F_{(2,6)} = 36.69$, $p < 0.001$), thus confirming our initial prediction that the effect of pharmacogenetic LepRb^{POA} neuronal activation should be minimal at 30°C because LepRb^{POA} neurons are already activated and adaptive thermogenesis is minimal. Locomotor activity and RER showed a similar trend as VO₂ (Fig. 6F,G; repeated-measures ANOVA for the effect of treatment during 6 h postinjection in F: $F_{(1,3)} = 4.85$, $p > 0.05$; in G: $F_{(1,3)} = 66.27$, $p < 0.01$; data not shown). Of note, the minimal VO₂ level reached at RT was lower than that at 10°C, implying that at 10°C, other cold defense response(s), such as shivering, may have contributed to overall energy expenditure. Indeed,

Figure 6. Pharmacogenetic activation of $LepRb^{POA}$ neurons at warm and cold ambient temperature. **A**, The experimental scheme showing timings of temperature changes and injections for indirect calorimetry experiment. **B**, At 30°C, $LepRb^{POA}$ DREADD-Gq mice further decreased VO_2 by CNO injection compared with saline injection. **C**, At 10°C, CNO injection greatly attenuated cold-induced increase in VO_2 in $LepRb^{POA}$ DREADD-Gq mice. **D**, Average VO_2 during 4 h postinjection was calculated and the change from baseline (ΔVO_2) was compared between saline and CNO in each temperature condition. RT value is calculated from Figure 1D. **E**, ΔVO_2 Saline – ΔVO_2 CNO was calculated for each temperature condition and normalized to 30°C. **F**, **G**, Locomotor activity in the same $LepRb^{POA}$ DREADD-Gq mice shown in **B** and **C** during the same period. Locomotor activity from 12:00 to 6:00 P.M. is enlarged in the box. Data are represented as mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ (two-way repeated-measures ANOVA and Bonferroni's pairwise comparisons for **B–D**, **F**, and **G**, and one-way repeated-measures ANOVA and Fisher's least significant difference pairwise comparisons for **E**).

$LepRb^{POA}$ DREADD-Gq mice injected with CNO at 10°C shivered noticeably (visual observation), revealing that $LepRb^{POA}$ neurons do not affect shivering thermogenesis.

Glutamatergic, not GABAergic, POA neurons mediate adaptations to ambient temperature

Previous studies suggested that GABAergic inhibitory inputs from the POA to the DMH and rostral medulla suppress BAT thermogenesis, and cold-induced inhibition of these signals pro-

motes BAT thermogenesis (Nakamura, 2011). We hypothesized that $LepRb^{POA}$ neurons represent those GABAergic POA neurons that inhibit BAT thermogenesis. Leptin-induced pSTAT3 is a reliable marker of $LepRb$ -expressing neurons (Faouzi et al., 2007; Laque et al., 2015). Using reporter mice for $Vglut2^{EYFP}$ mice and $Vgat^{EYFP}$ mice to visualize glutamatergic and GABAergic neurons, respectively (Vong et al., 2011; Xu et al., 2013), we analyzed their colocalization with leptin-induced pSTAT3 (Fig. 7).

Glutamatergic neurons are restricted to the MnPO and anteroventral periventricular nucleus, closely matching the expression pattern of $LepRb$ in this area (Fig. 7A–C), while GABAergic neurons are more dispersed (Fig. 7D). We found that $58.42 \pm 6.49\%$ of leptin-induced pSTAT3⁺ neurons were glutamatergic while only $18.93 \pm 2.64\%$ were GABAergic ($n = 3$ each), and colocalized cells were mostly located in the ventrolateral regions of the MnPO (Fig. 7E,F). This was surprising because of a discrepancy between our own data showing that $LepRb^{POA}$ neurons innervate the DMH and rostral medulla (Zhang et al., 2011) and literature that consistently suggests the GABAergic nature of warm-sensitive POA neurons that inhibit neurons in the DMH and rostral medulla (Nakamura, 2011).

To determine whether stimulation of glutamatergic versus GABAergic POA neurons reproduce the effect of pharmacogenetic activation of $LepRb^{POA}$ neurons, we injected adeno-associated virus expressing DREADD-Gq into the POA of $Vglut2^{Cre}$ ($Vglut2^{POA}$ DREADD-Gq) and $Vgat^{Cre}$ ($Vgat^{POA}$ DREADD-Gq) mice. Subsequent CNO injection decreased core temperature in $Vglut2^{POA}$ DREADD-Gq mice (Fig. 7G; repeated-measures ANOVA for the interaction between time and group: $F_{(6,42)} = 25.98$, $p < 0.001$), but not in $Vgat^{POA}$ DREADD-Gq mice (Fig. 7H; repeated-measures ANOVA for the interaction between time and group: $F_{(6,84)} = 0.85$, $p > 0.05$). Similarly, reduction in VO_2 and postural extension were only observed in $Vglut2^{POA}$ DREADD-Gq mice at RT (Fig. 8A,D; repeated-measures ANOVA for the effect of treatment during 6 h postinjection in **A**: $F_{(1,3)} = 22.04$, $p < 0.05$; in **D**: $F_{(1,3)} = 3.79$, $p > 0.05$; data not shown), suggesting that adaptations to warm temperature elicited by $LepRb^{POA}$ neurons are likely mediated by glutamatergic neurons. We further investigated whether ambient temperature challenges would reveal a role of GABAergic neurons in energy expenditure. However, also at cold and warm ambient temperature, CNO only decreased energy expenditure in $Vglut2^{POA}$, but not in $Vgat^{POA}$, DREADD-Gq mice (Fig. 8B,C,E,F; repeated-measures ANOVA for the effect of treatment during 6 h postinjection in **B**: $F_{(1,3)} = 55.33$,

$p < 0.01$; in C: $F_{(1,3)} = 9.76$, $p = 0.052$; in E: $F_{(1,3)} = 2.51$, $p > 0.05$; in F: $F_{(1,3)} = 0.037$, $p > 0.05$), further corroborating the finding that glutamatergic POA neurons regulate BAT thermogenesis, while GABAergic POA neurons play no obvious role in the control of energy expenditure. Furthermore, as observed with CNO-activated $LepR^b^{POA}$ neurons, pharmacogenetic activation of $Vglut2^{POA}$ neurons showed the same ambient temperature-dependent changes in VO_2 (Fig. 8G–I; repeated-measures ANOVA for the interaction between temperature and treatment in G: $F_{(2,6)} = 8.73$, $p < 0.05$; in H: $F_{(2,6)} = 1.88$, $p > 0.05$; for the effect of temperature for $Vglut2^{POA}$ DREADD-Gq in I: $F_{(2,6)} = 8.73$, $p < 0.05$).

Locomotor activity of both $Vglut2^{POA}$ and $Vgat^{POA}$ DREADD-Gq mice was not significantly affected by CNO in any temperature condition (Fig. 8J,K; data not shown for 30 and 10°C), which might explain the smaller reduction in core temperature and VO_2 in $Vglut2^{POA}$ DREADD-Gq mice compared with $LepR^b^{POA}$ DREADD-Gq mice. However, CNO decreased RER similarly in $Vglut2^{POA}$ and $LepR^b^{POA}$ DREADD-Gq mice but not in $Vgat^{POA}$ DREADD-Gq mice (data not shown).

Discussion

$LepR^b^{POA}$ neurons inhibit energy expenditure

Consistent with the overall role of POA neurons in thermoregulation, our data confirm that $LepR^b^{POA}$ neurons robustly modulate energy expenditure and body temperature and that this likely involves $\beta 3AR$ -dependent activation of BAT thermogenesis. Our data define a distinct population of glutamatergic $LepR^b^{POA}$ neurons that inhibit the sympathetic outflow to BAT and blunt adaptive thermogenesis. This is also in line with extensive studies in rats suggesting that POA neurons are mostly warm-sensitive and control cold or LPS-induced BAT thermogenesis (Morrison et al., 2014). Reduced BAT activity contributes to decreased energy expenditure and promotes weight gain, and the amount of BAT inversely correlates with the body mass index in humans (Cypess et al., 2009; van Marken Lichtenbelt et al., 2009). Therefore, our study contributes to a better understanding of how BAT activity is regulated for a potential therapeutic strategy to treat obesity.

We further speculate that $LepR^b^{POA}$ neurons also modulate basal metabolic rate because pharmacogenetic activation of $LepR^b^{POA}$ neurons still caused a significant decrease in energy expenditure at thermoneutrality (30°C), at which adaptive thermogenesis does not contribute to energy expenditure (Cannon and Nedergaard, 2011), and CL316,243 was not able to block the

Figure 7. $LepR^b^{POA}$ neurons are glutamatergic. **A, B**, Representative immunohistochemical images showing leptin-induced pSTAT3 (black, DAB staining) in the POA of $Vglut2^{EYFP}$ (**A**, $n = 3$) and $Vgat^{EYFP}$ (**B**, $n = 3$) mice. Areas within dotted line indicate areas used for pSTAT3 cell counting. **C, D**, Representative immunohistochemical images showing leptin-induced pSTAT3 (red) and EYFP (green) in the POA of $Vglut2^{EYFP}$ (**C**) and $Vgat^{EYFP}$ (**D**) mice. Insets show magnified images of the indicated areas (dotted-line boxes) to show colocalization of pSTAT3 and EYFP. **E, F**, Schematic drawings for the distribution of pSTAT3 and EYFP double-positive cells in the POA in $Vglut2^{EYFP}$ and $Vgat^{EYFP}$ mice. **G, H**, At RT, CNO injection (1.5 mg/kg, i.p.) decreased rectal temperature in $Vglut2^{POA}$ DREADD-Gq but not in $Vgat^{POA}$ DREADD-Gq mice. Data are represented as mean $\pm$ SEM. * $p < 0.01$ and ** $p < 0.001$ (two-way repeated-measures ANOVA and Bonferroni's pairwise comparisons).

full CNO effect on VO_2 in $LepR^b^{POA}$ DREADD-Gq mice. This additional suppression cannot be solely explained by decreased locomotor activity because activation of glutamatergic POA neurons in $Vglut2^{POA}$ DREADD-Gq mice still reduced energy expenditure at 30°C in the absence of locomotor activity changes. In contrast to the highly regulated adaptive thermogenesis, basal metabolic rate is not subject to much variation, even though the thyroid hormone is one of the few modulators of basal metabolic rate (Kim, 2008). Indeed, temperature-sensitive POA neurons

Figure 8. Glutamatergic, not GABAergic, POA neurons mediate adaptations to ambient temperature changes. **A–F**, VO_2 was measured in *Vglut2^{POA}* and *Vgat^{POA}* DREADD-Gq mice ($n = 4$ each) at RT, 30°C, and 10°C. Saline (black) or CNO (0.5 mg/kg, i.p.; red) was injected at 11:00 A.M. (CT5, arrows; Fig. 6A). Orange and blue boxes indicate temperature change to 30 and 10°C, respectively. Only *Vglut2^{POA}* DREADD-Gq mice, but not *Vgat^{POA}* DREADD-Gq mice, further decreased VO_2 by CNO injection compared with saline injection in all temperature conditions. **G, H**, Average VO_2 during 4 h after injection was calculated and the change from baseline (ΔVO_2) was compared between saline and CNO in each temperature condition. **I**, ΔVO_2 Saline – ΔVO_2 CNO was calculated for each temperature condition and normalized to 30°C. Data for *LepRb^{POA}* DREADD-Gq mice are from Figure 6E. **J, K**, Locomotor activity in the same mice shown in **A** and **D** during the same period at RT. Locomotor activity was not affected by CNO (0.5 mg/kg, i.p.) in either *Vglut2^{POA}* ($n = 4$) or *Vgat^{POA}* ($n = 4$) DREADD-Gq mice. Data are represented as mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ (two-way repeated-measures ANOVA and Bonferroni's pairwise comparisons for **A–H, J**, and **K**, and one-way repeated-measures ANOVA and Fisher's least significant difference pairwise comparisons for **I**).

regulate circulating thyroid-stimulating hormone levels and likely modulate thyroid-related changes in energy expenditure (Andersson et al., 1963; Martelli et al., 2014). Future studies will directly address whether and to what extent *LepRb^{POA}* neurons require the thyroid axis to modulate basal metabolic rate.

LepRb^{POA} neurons inhibit food intake and decrease body weight

Surprisingly, stimulation of *LepRb^{POA}* neurons decreased body weight despite the robust hypometabolism and reduced locomotor

activity, which is explained by the simultaneous decrease in food intake. We show here that the concurrent decrease in energy expenditure and food intake is a typical homeostatic adaptation to warm ambient temperature, which is meant to maintain constant body temperature and body weight. However, when warm-sensing glutamatergic *LepRb^{POA}* neurons are activated in a nonthermoneutral environment (i.e., RT) to decrease energy expenditure and food intake as done in this study, body temperature and body weight are potently modulated as a result. Similarly, an adaptation to cold ambient temperature causes

simultaneous increase in energy expenditure and food intake (Ravussin et al., 2014), and activation of cold-adaptive responses at warm temperature can be predicted to similarly modulate body temperature and weight.

We show that thermoneutral ambient temperature, but not cold temperature, strongly stimulates LepRb^{POA} neurons, suggesting that pharmacogenetic activation of LepRb^{POA} neurons mimics the physiological and behavioral responses to warm/thermoneutral temperature exposure. Our data are consistent with studies showing that local warming of the POA reduces food intake (Andersson and Larsson, 1961) and that POA lesions disrupt proper food intake adjustment in response to changing ambient temperatures (Hamilton and Brobeck, 1964). Thus, our data support a major effect of LepRb^{POA} neurons on feeding. Interestingly, after cessation of chronic LepRb^{POA} neuronal stimulation, we did not observe compensatory overfeeding, which is typically observed after a state of negative energy balance, such as fasting or food restriction (Harris et al., 1986; Dulloo et al., 1997). This result suggests that the negative energy balance obtained by LepRb^{POA} neuronal activation was not sensed as a physiological need state that requires compensation. It further argues against a physical locomotor limitation as a reason for the reduced food intake, which should have resulted in a compensatory overfeeding behavior once CNO injections were terminated.

Feeding behavior is centrally controlled by the hypothalamus. The arcuate nucleus (ARC) and the paraventricular nucleus of the hypothalamus (PVH) have been intensively studied for their potent effects on food intake, where orexigenic and anorexigenic neuronal populations and their neuropeptides mediate changes in food intake (Joly-Amado et al., 2014; Sutton et al., 2014; Münzberg and Morrison, 2015). It is unclear whether LepRb^{POA} neurons modulate feeding by connecting with those known feeding circuits in the PVH or the ARC. The POA neurons were shown to innervate feeding behavior-related sites, such as the PVH, ventral tegmental area, and lateral hypothalamic area, but not the ARC or ventromedial nucleus of the hypothalamus (Swanson, 1976; Thompson and Swanson, 2003; McKinley et al., 2015). Future studies will specifically address how LepRb^{POA} neurons mediate changes in food intake.

A new view for homeostatic POA circuits

POA neurons are mainly warm-sensing (Boulant and Dean, 1986), and in line with previous reports, we show that LepRb^{POA} neurons represent at least a subpopulation of warm-sensitive neurons. Several studies in rats show that temperature-sensing POA neurons innervate the DMH/DHA and rRPa, which was recently confirmed in mice, in which LepRb^{POA} neurons spec-

A Original View

B New View

Figure 9. A new view for BAT-related POA circuits. **A**, The original view suggests direct GABAergic innervation from POA neurons to the DMH and RPa. Here, warm-sensitive GABAergic POA neurons directly inhibit BAT-related neurons in the DMH/DHA and RPa. Upon cold exposure, POA GABAergic neurons are inhibited and DMH and RPa neurons are disinhibited, which causes activation of SNA to BAT to counteract heat loss from cold exposure. **B**, The new view proposes glutamatergic inputs to the DMH and RPa, which must be indirect to BAT-activating DMH and RPa neurons. Alternatively, LepRb^{POA} neurons might directly innervate warm-sensitive DMH neurons that inhibit BAT-activating RPa neurons via acetylcholine (ACh; Jeong et al., 2015). Here, warm-sensitive glutamatergic LepRb^{POA} neurons modulate both energy expenditure and food intake in a temperature-dependent manner to regulate body temperature and body weight. Solid lines indicate direct neuronal connections and dashed lines indicate indirect connections. Connections with question marks require further verification. cs, Cold-sensitive neurons; EE, energy expenditure; FI, food intake; Glut, glutamate; NE, norepinephrine; ws, warm-sensitive neurons.

ically innervate the DMH/DHA and rRPa (Zhang et al., 2011). The DMH/DHA is a key regulator of BAT thermogenesis (Zaretskaia et al., 2002, 2003; Cao et al., 2004; Madden and Morrison, 2004) and leptin maintains normal energy expenditure by directly stimulating DMH/DHA LepRb neurons (Enriori et al., 2011; Zhang et al., 2011; Rezai-Zadeh et al., 2014).

POA warm-sensitive neurons are proposed as predominantly GABAergic neurons that are inhibited by cold exposure or febrile stimuli, which results in the disinhibition of their target neurons in the DMH/DHA and rRPa (Morrison et al., 2014). In our study, pharmacogenetic activation of GABAergic Vgat^{POA} neurons had no effect on thermoregulatory responses, contradicting previous reports. The exact reasons for this discrepancy are unclear but several possibilities can be postulated. First, DREADD-Gq virus injections may not have optimally targeted warm-sensing GABAergic POA neurons. Second, species-specific difference or different experimental conditions may have contributed to inconsistent results. Most previous studies were conducted in anesthetized rats in which cooling or febrile responses were altered with chemical drugs to modulate neuronal activity, without direct manipulation of GABA or glutamatergic neurons. In contrast, our studies are the first to test the activation of genetically defined neuronal subpopulations at different ambient tempera-

tures in freely moving mice. Third, separate GABAergic neurons may represent cold-sensitive and warm-sensitive neurons in the POA (Nakamura and Morrison, 2008), and stimulating both populations simultaneously may offset each other's function.

In any case, our data make a strong case that DMH/DHA-projecting and RPa-projecting LepRb^{POA} neurons are mainly glutamatergic and robustly modulate energy expenditure and food intake in mice, even though it should be noted that activation of glutamatergic POA neurons also includes non-LepRb^{POA} neurons that may have contributed to the modulation of energy expenditure. Consequently, our data argue against a direct stimulation of BAT-activating LepRb^{DMH/DHA} or RPa neurons by LepRb^{POA} neurons (Cao et al., 2004; Cao and Morrison, 2006; Nakamura and Morrison, 2007; Rezai-Zadeh et al., 2014). Therefore, we propose a new view for the neurochemical and functional properties of BAT-related POA circuits (Fig. 9). Here, glutamatergic LepRb^{POA} neurons are stimulated by warm ambient temperature and communicate this stimulatory signal to the DMH/DHA and RPa. For example, warm-sensitive cholinergic neurons in the DMH decrease energy expenditure by inhibiting rRPa neurons (Jeong et al., 2015). Thus, LepRb^{POA} neurons may directly innervate these neurons, and future studies will have to identify which LepRb^{POA} neuronal targets mediate the observed warm-adaptive responses. Most importantly, LepRb^{POA} neurons regulate energy expenditure and food intake, and are critical for maintaining homeostasis for body weight and body temperature.

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