Angie C.N. Chong,[1,2] Richard A. Greendyk,[2] and Lori M. Zeltser[2,3]

Distinct Networks of Leptin- and Insulin-Sensing Neurons Regulate Thermogenic Responses to Nutritional and Cold Challenges

Diabetes 2015;64:137–146 | DOI: 10.2337/db14-0567

Defense of core body temperature (T_c) can be energetically costly; thus, it is critical that thermoregulatory circuits are modulated by signals of energy availability. Hypothalamic leptin and insulin signals relay information about energy status and are reported to promote thermogenesis, raising the possibility that they interact to direct an appropriate response to nutritional and thermal challenges. To test this idea, we used an *Nkx2.1-Cre* driver to generate conditional knockouts (KOs) in mice of leptin receptor ($L^{2.1}$KO), insulin receptor ($I^{2.1}$KO), and double KOs of both receptors ($D^{2.1}$KO). $L^{2.1}$KOs are hyperphagic and obese, whereas $I^{2.1}$KOs are similar to controls. $D^{2.1}$KOs exhibit higher body weight and adiposity than $L^{2.1}$KOs, solely due to reduced energy expenditure. At 20–22°C, fed $L^{2.1}$KOs maintain a lower baseline T_c than controls, which is further decreased in $D^{2.1}$KOs. After an overnight fast, some $L^{2.1}$KOs dramatically suppress energy expenditure and enter a torpor-like state; this behavior is markedly enhanced in $D^{2.1}$KOs. When fasted mice are exposed to 4°C, $L^{2.1}$KOs and $D^{2.1}$KOs both mount a robust thermogenic response and rapidly increase T_c. These observations support the idea that neuronal populations that integrate information about energy stores to regulate the defense of T_c set points are distinct from those required to respond to a cold challenge.

Because many bioactive proteins function within a narrow temperature range, regulation of heat production and dissipation is crucial to support basic physiological processes and survival. When energy stores are replete, a decrease in external temperature usually triggers compensatory increases in shivering and/or adaptive thermogenesis (1,2). Maintaining core body temperature (T_c) by increasing heat production can be energetically costly, particularly when living in a cold environment (1). Small animals have a larger surface area–to–volume ratio compared with large animals, and therefore, they lose heat more readily. Small mammals evolved several strategies to deal with the simultaneous challenges of low ambient temperature, heat loss, and limited nutrient availability that occur in the winter. Behavioral thermoregulation, such as nesting underground or huddling, helps to preserve heat (3). Defense of a slightly lower T_c set point over a long period of time can also conserve energy. Finally, by initiating a torpor-like state in which metabolic rate, locomotor activity, and T_c are dramatically suppressed, mammals can reduce their energy requirements by 50–90% and thus direct limited energy resources to securing more food (2,4).

At ambient temperatures below thermoneutrality, a combination of short- and long-term signals of energy status regulate the decision whether to actively defend T_c or to suppress metabolism and shut off feedback systems designed to maintain body temperature (2,4,5). Because leptin is produced in proportion to white adipose tissue (WAT) mass (6) and is sensed by neurons in key nodes of the thermoregulatory circuits (7), it is well positioned to

[1]Institute of Human Nutrition, Columbia University, New York, NY
[2]Naomi Berrie Diabetes Center, Columbia University, New York, NY
[3]Department of Pathology and Cell Biology, Columbia University, New York, NY
Corresponding author: Lori M. Zeltser, lz146@cumc.columbia.edu.

Received 7 April 2014 and accepted 5 August 2014.

ensure that sufficient energy stores are available to mount a thermogenic response. Consistent with this idea, low leptin (due to negative energy balance or genetic loss of function) acts as a permissive signal to enter torpor (8). However, low leptin levels are not sufficient to elicit torpor, and leptin treatment is not sufficient to prevent the initiation of torpor in mice that lack WAT (9). The observation that fasting-induced torpor can precede significant changes in serum leptin (4) supports the idea that complementary signals of short-term energy availability also influence thermoregulatory circuits. Because insulin levels are acutely sensitive to the prandial state and insulin is reported to act in several hypothalamic nuclei to increase brown adipose tissue (BAT) thermogenesis and T_c (10–12), insulin is well positioned to regulate the initiation of torpor-like states.

Key nodes in thermoregulatory circuits are modulated by the actions of intermingled populations of hypothalamic leptin- and insulin-sensing neurons, raising the possibility that these networks interact to influence thermoregulatory responses to external nutrient and temperature cues. To explore this hypothesis, we used the *Nkx2.1-Cre* transgene to broadly disrupt signaling through the long form of the leptin receptor (LepRb) and/or the insulin receptor (InsR) in the hypothalamus of mice, while sparing thermoregulatory circuits in the midbrain and hindbrain (13,14). In this report we provide evidence that hypothalamic leptin and insulin signals influence baselines of T_c in the fed state and oppose entry into a torpor-like state. Because *Nkx2.1-Cre*–mediated disruption of LepRb and InsR signals did not interfere with the ability to mount a thermogenic response to cold, these data support the idea that functionally distinct populations of neurons influence adaptive responses to nutritional and cold stressors.

RESEARCH DESIGN AND METHODS

Generation of Mice With InsR, LepR, and Double Knockouts of Both Receptors

To disrupt InsR and/or LepRb signaling in the hypothalamus, the *Nkx2.1-Cre* driver line [C57BL/6J-Tg(Nkx2.1-Cre)2Sand/J (provided by S. Anderson, Weill Cornell Medical College)] (13) was crossed to mice homozygous for a floxed allele of *Lepr* [FVB.BKS(D)-*Lepr*^{db}/ChuaJ (provided by S. Chua, Albert Einstein College of Medicine)] (15). The resulting F1 heterozygotes (*Nkx2.1*-Cre;*Lepr*$^{fl/+}$) were crossed to mice homozygous for a floxed allele of *Insr* [B6.129S4(FVB)-*Insr*^{tm1Khn}/J (provided by D. Acilli, Columbia University Medical Center)] (16) to generate F2 *Nkx2.1-Cre*;*Lepr*$^{fl/+}$;*Insr*$^{fl/+}$ and *Lepr*$^{fl/+}$;*Insr*$^{fl/+}$ mice. F2 *Lepr*$^{fl/+}$;*Insr*$^{fl/+}$ mice were intercrossed to generate F3 *Lepr*$^{fl/fl}$;*Insr*$^{fl/fl}$ females, which were then crossed to F2 *Nkx2.1-Cre*;*Lepr*$^{fl/+}$;*Insr*$^{fl/+}$ males to generate our experimental knockout (KO) animals: *Nkx2.1-Cre*;*Lepr*$^{fl/fl}$ ($L^{2.1}$KO), *Nkx2.1-Cre*;*Insr*$^{fl/fl}$ ($I^{2.1}$KO), *Nkx2.1-Cre*;*Lepr*$^{fl/fl}$;*Insr*$^{fl/fl}$ ($D^{2.1}$KO), and *Lepr*$^{fl/fl}$;*Insr*$^{fl/fl}$ (control) in a Mendelian ratio. Because of the low probability of achieving the desired genotypes in the offspring, two crosses were generally required to generate one $D^{2.1}$KO male. Genotypes were assessed by PCR on genomic DNA from tail tips using the following primers:

Cre 5′ GCGGTCTGGCAGTAAAAACTATC 3′ (forward)
Cre 5′ GTGAAACAGCATTGCTGTCACTT 3′ (reverse)
Lepr 5′ GTCTGATTTGATAGATGGTCTT 3′ (forward)
Lepr 5′ AGAATGAAAAAGTTGTTTTGGGA 3′ (forward)
Lepr 5′ GGCTTGAGAACATGAACAC 3′ (reverse)
Insr 5′ TGCACCCCATGTCTGGGACCC 3′ (forward)
Insr 5′ GCCTCCTGAATAGCTGAGACC 3′ (reverse)

Animal Husbandry

Mice were maintained in a temperature- and light-controlled environment (22 ± 1°C; 12-h light/12-h dark cycle). Except where noted, mice were housed in sex-matched groups of three to four, with at least one $L^{2.1}$KO, one $D^{2.1}$KO, and one control animal per cage. Pregnant and nursing mice were housed with one dam per cage. Pups were weaned on postnatal day 21. Unless otherwise noted, mice had ad libitum access to chow (9% calories from fat, 5058 Mouse Diet 20; LabDiet) and water until they were killed. For studies in singly housed animals, mice were acclimated to this condition for 2 to 3 weeks before food intake and body temperature were measured. All procedures were performed in accordance with the guidelines of the Columbia University Health Sciences Division Institutional Animal Care and Use Committee.

Analysis of Body Composition

Beginning at 6 weeks of age, mice were weighed weekly. To determine body composition, mice underwent nuclear magnetic resonance imaging (minispec; Bruker) at 6, 8, 10, 12, and 14 weeks of age.

Preservation of WAT Depots

Animals were anesthetized with 2.5% Avertin (0.02 mL/g i.p.) before cervical dislocation. Inguinal and gonadal depots were fixed overnight and embedded in paraffin. Sections (5-μm thick) were stained with hematoxylin and eosin, and images were acquired at original magnification ×200 using a Nikon Eclipse 80i equipped with a Retiga EXi camera and an X-Cite 120 fluorescent illumination system. Images were converted into a binary format and analyzed with ImageJ software with an adapted analysis method. At least four images per adipose tissue sample and three to four animals per group were analyzed.

Measurement of Glucose, Insulin, Leptin, and Acylated Ghrelin Levels

All blood samples were collected between 10:00 A.M. and noon. Fasting blood samples were taken after 14–16 h of fasting with ad libitum access to drinking water. Blood for hormone measurements was collected from an orbital sinus puncture of isoflurane-anesthetized animals, clotted

at room temperature for 1 h, and centrifuged. Serum was decanted and stored at –20°C until used in leptin (Millipore) or insulin (Millipore) ELISA, per the manufacturer's protocol. Plasma acylated ghrelin levels were assayed by ELISA (Bertin Pharma). Whole blood for glucose levels was taken from a tail nick and assayed using a glucometer with disposable test strips (Abbott). The upper limit of measurement was 500 mg/dL; any "HI" readings were recorded as 501 mg/dL.

Indirect Calorimetry and Food Intake

Eight-week-old male mice were acclimated to respiratory chambers for 24 h before measurements. VO_2, CO_2 production, food intake, and locomotor activity were measured simultaneously over a 72-h period using a 16-cage, indirect calorimetry system combined with feeding monitor and TSE ActiMot system (TSE Systems). The VO_2 and VCO_2 were measured using a paramagnetic oxygen sensor and a spectrophotometric CO_2 sensor over a 24-h period.

Measurement of Food Intake in Group-Housed Animals

Two to three mice of the same genotype were placed in a cage with a hopper filled with preweighed food at 5 and 10 weeks. The amount of food remaining in the hopper was measured every 2–3 days. Average daily food intake was calculated based on the number of days between measurements and the number of mice per cage.

Measurement of T_c

Readings from a rectal temperature probe thermometer (ThermoWorks) were obtained between 10:00 A.M. and noon in mice >8 weeks old. Baseline measurements were obtained under ad libitum feeding conditions or after an overnight fast of 12–16 h. In the acute cold challenge, mice fasted overnight were individually placed in a glass beaker submerged in ice for 75 min without food, and body temperature was recorded every 15 min.

Statistics

Data are presented as group mean ± SEM. Statistical comparisons were performed between sex- and age-matched groups using the two-tailed, unpaired Student *t* test or one-way ANOVA with Bonferroni post hoc analysis. A *P* value of ≤0.05 was considered to be statistically significant.

RESULTS

Nkx2.1-Cre–Mediated Loss of InsR Signaling Exacerbates Body Weight and Adiposity Phenotypes of $L^{2.1}$KOs

To explore the possibility that interactions between hypothalamic leptin- and insulin-sensing networks regulate energy homeostasis, we sought to identify changes in physiological parameters in mice lacking both signals ($D^{2.1}$KOs) that are more severe than would be predicted by the additive effects of disrupting leptin ($L^{2.1}$KOs) and insulin signals ($I^{2.1}$KOs). Consistent with observations in mice lacking neuronal InsR signaling (NIRKO mice) (17), body weight and adiposity in $I^{2.1}$KO males were not different from controls (Fig. 1*A* and *B*). Body weight and adiposity were significantly higher in $L^{2.1}$KO and $D^{2.1}$KO males compared with $I^{2.1}$KO and controls starting from 6 weeks, and $D^{2.1}$KOs diverged from $L^{2.1}$KO at 10 weeks (Fig. 1*A* and *B*).

Female $I^{2.1}$KO mice weighed the same as the controls but exhibited a mild, but significant, increase in adiposity starting from 10 weeks of age (Fig. 1*E* and *F*), similar to observations in NIRKO females (17). $L^{2.1}$KO and $D^{2.1}$KO females both had significantly higher body weight and adiposity than $I^{2.1}$KOs and controls from 6 weeks of age (Fig. 1*E* and *F*). $L^{2.1}$KO and $D^{2.1}$KO females had similar body weights throughout the study (Fig. 1*E*); however, adiposity of $D^{2.1}$KOs was slightly but significantly higher from 10 weeks (Fig. 1*F*). Because gonadal fat depots are preferentially affected in NIRKO females (17), we performed histological analyses of inguinal and gonadal fat depots in $L^{2.1}$KO and $D^{2.1}$KO females. Gonadal adiposity was increased by 34–40% in $D^{2.1}$KO females compared with $L^{2.1}$KOs at 9 and 19 weeks, partly due to a 14–24% increase in adipocyte cell size (Fig. 1*C*, *D*, *G*, and *H*). In contrast, fat mass and adipocyte size in inguinal depots were similar. A similar trend of increased gonadal fat pad weights was also observed in $D^{2.1}$KO males but did not reach significance (data not shown).

Nkx2.1-Cre–Mediated Loss of InsR Signaling in $L^{2.1}$KOs Increases Leptin and Ghrelin Levels

Because serum levels of leptin and acylated ghrelin are dramatically altered in leptin-deficient models (18), we measured these hormones in all groups. Consistent with severe obesity in $D^{2.1}$KO and $L^{2.1}$KO males and females at 8 and 12 weeks, serum leptin levels were more than 20-fold higher in these groups than in controls and $I^{2.1}$KOs, whereas levels in lean $I^{2.1}$KOs were similar to controls (Fig. 2*A* and *B*). Serum leptin was increased by 30% at 8 weeks and by 47% at 12 weeks in male $D^{2.1}$KOs compared with $L^{2.1}$KOs (Fig. 2*A*). Female $D^{2.1}$KOs had higher adiposity than $L^{2.1}$KOs, but this was not reflected in increased leptin levels (Fig. 2*B*).

$I^{2.1}$KO males and controls had similar serum levels of acylated ghrelin in fed and fasted conditions (Fig. 2*C*). Similar to observations in $Lep^{ob/ob}$ mice (18), ghrelin levels in $L^{2.1}$KO males were ~65% lower than in controls at baseline, and this difference was amplified during fasting (Fig. 2*C*). $D^{2.1}$KOs exhibited ghrelin levels that were intermediate between $L^{2.1}$KOs and $I^{2.1}$KOs, although this difference did not reach significance in the fed state (Fig. 2*C*). Expression of neuropeptide Y (*Npy*) and Agouti-related peptide (*Agrp*) was similarly elevated in fasted $L^{2.1}$KOs and $D^{2.1}$KOs compared with controls and $I^{2.1}$KOs (Fig. 2*D*). In summary, although adiposity-related phenotypes in $L^{2.1}$KOs are exacerbated in $D^{2.1}$KOs, ghrelin levels are normalized.

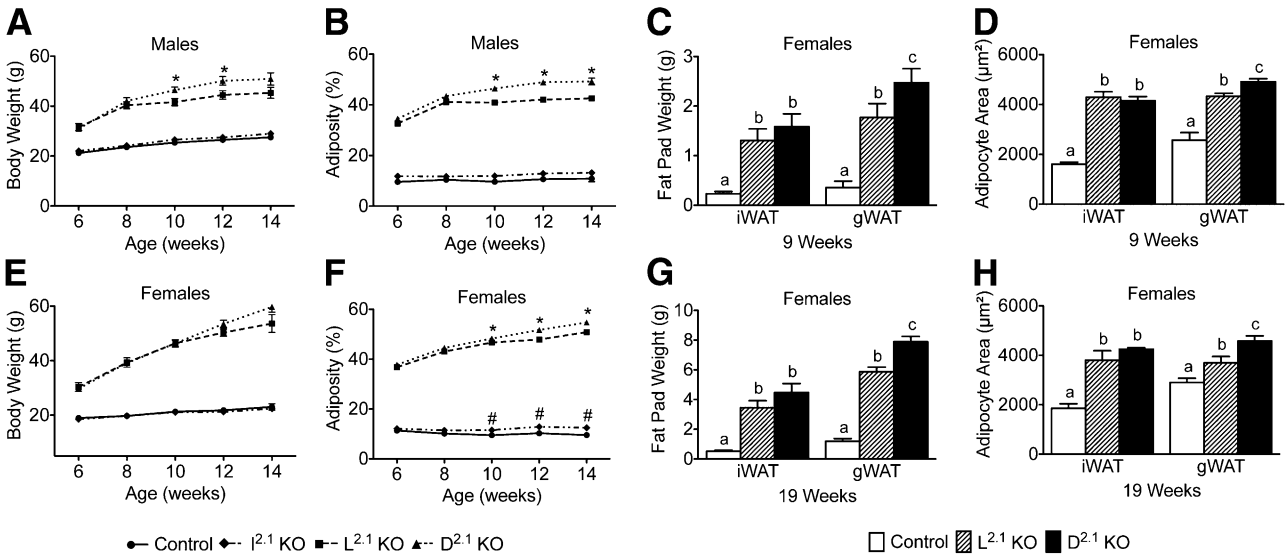

Figure 1—*Nkx2.1-Cre*–mediated disruption of InsR signals exacerbates obesity of $L^{2.1}$KOs. Body weight (*A* and *E*) and adiposity (*B* and *F*) of male and female controls, $I^{2.1}$KOs, $L^{2.1}$KOs, and $D^{2.1}$KOs ($n \geq 10$ per group). *C* and *G*: Gonadal and inguinal fat pad weights of control, $L^{2.1}$KO, and $D^{2.1}$KO females ($n \geq 3$ per group at 9 weeks and $n \geq 7$ per group at 19 weeks). *D* and *H*: Average cross-sectional areas of gonadal (g)WAT and inguinal (i)WAT from histological sections of control, $L^{2.1}$KO, and $D^{2.1}$KO females ($n \geq 3$ per group at 9 weeks of age; $n \geq 4$ per group at 19 weeks of age). All data are mean ± SEM. *P* values were calculated between age-matched groups. *A*, *B*, *E*, and *F*: $^*P < 0.05$ $L^{2.1}$KO vs. $D^{2.1}$KO; $\#P < 0.05$ $I^{2.1}$KO vs. control. *C*, *D*, *G*, and *H*: The lowercase letters above the bars denote statistically similar ($P > 0.05$) groups.

Nkx2.1-Cre–Mediated Loss of InsR Signaling Does Not Exacerbate Blood Glucose Levels in $L^{2.1}$KOs

We also assessed phenotypes related to glucose homeostasis. Whereas $I^{2.1}$KO males and females had the same blood glucose and serum insulin levels as controls, $L^{2.1}$KO and $D^{2.1}$KO males and females were hyperglycemic and hyperinsulinemic from 6 weeks of age (Fig. 3*A*–*D*). Despite the elevated adiposity in $D^{2.1}$KO versus $L^{2.1}$KO males, fasting or fed blood glucose (Fig. 3*A*) and serum insulin (Fig. 3*B*) were similar in both groups. Fed blood glucose was mildly increased (Fig. 3*C*) and serum insulin trended higher (Fig. 3*D*) in $D^{2.1}$KO females compared with $L^{2.1}$KO at 12 weeks.

Energy Expenditure Is Decreased in $D^{2.1}$KOs Versus $L^{2.1}$KOs, Whereas Food Intake Is Similar

To investigate whether elevated adiposity in $D^{2.1}$KOs compared with $L^{2.1}$KOs is due to effects on energy intake and/or expenditure, we measured food intake, VO_2 and locomotor activity at 8 weeks. Males in both groups were singly housed in their home cages with food hoppers or in metabolic cages. In either type of cage environment, ~30% of singly housed $D^{2.1}$KO males were aphagic and died within 3–5 days (Fig. 4*A*, bottom curve). The remaining $D^{2.1}$KOs consumed the same amount of food in a 24-h period as $L^{2.1}$KOs (Fig. 4*A*). To overcome the high mortality rate of single housing in

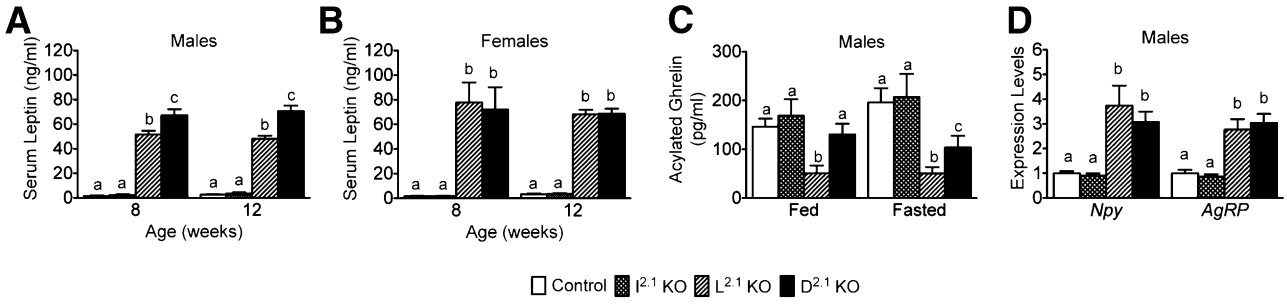

Figure 2—Disruption of hypothalamic InsR signals in male $L^{2.1}$KOs increases leptin and ghrelin levels. Male (*A*) and female (*B*) serum leptin, as measured by ELISA, at 8 and 12 weeks of fed controls, $I^{2.1}$KOs, $L^{2.1}$KOs, and $D^{2.1}$KOs. *C*: Plasma acylated ghrelin levels at baseline (fed) and after an overnight fast of 20-week-old adult male controls, $I^{2.1}$KOs, $L^{2.1}$KOs, and $D^{2.1}$KOs. *D*: *Npy* and *Agrp* expression, as measured by quantitative PCR, of fasted controls, $I^{2.1}$KOs, $L^{2.1}$KOs, and $D^{2.1}$KOs ($n \geq 4$). All data are mean ± SEM. *P* values were calculated between age- and sex-matched groups. The lowercase letters above the bars denote statistically similar ($P > 0.05$) groups.

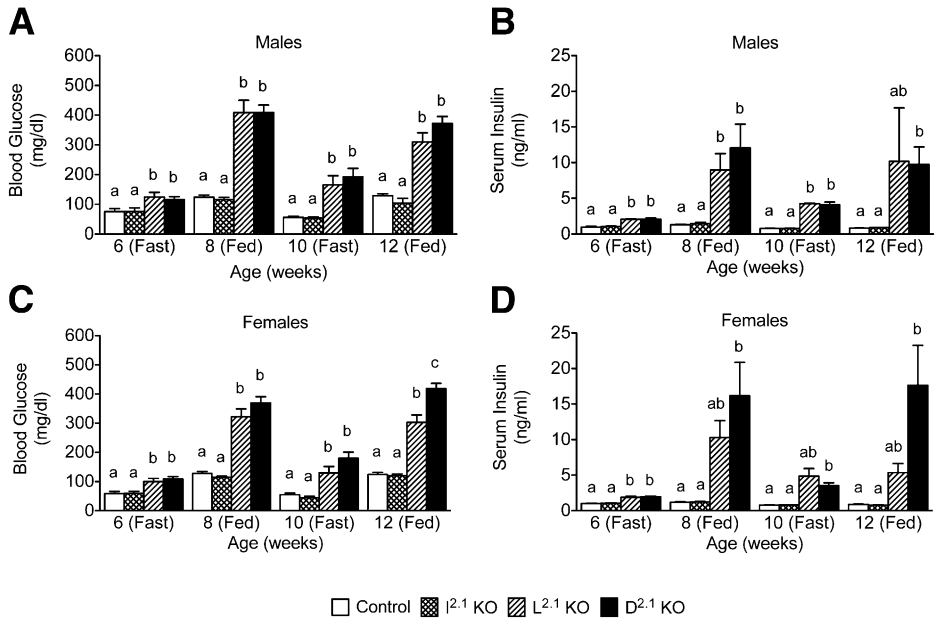

Figure 3—Disruption of hypothalamic InsR signals does not exacerbate glucose impairment in male $L^{2.1}$KOs. Blood glucose at 6 (fasted), 8 (random-fed), 10 (fasted), and 12 (random-fed) weeks of age in controls, $I^{2.1}$KOs, $L^{2.1}$KOs, and $D^{2.1}$KOs males ($n \geq 4$ per group) (*A*) and females ($n \geq 7$ per group) (*C*). Serum insulin, as measured by ELISA, at 6 (fasted), 8 (random-fed), 10 (fasted), and 12 (random-fed) weeks of age in control, $I^{2.1}$KO, $L^{2.1}$KO, and $D^{2.1}$KO males (*B*) and females (*D*) ($n \geq 3$ per group). All data are mean ± SEM. *P* values were calculated between age- and sex-matched groups. The lowercase letters above the bars denote statistically similar ($P > 0.05$) groups.

$D^{2.1}$KO males, intake from food hoppers was also assessed under group-housing conditions, with two to three mice of the same genotype in a cage. $I^{2.1}$KO males and females consumed the same amount of food as controls (Fig. 4*B*). $L^{2.1}$KO and $D^{2.1}$KO males and females were hyperphagic at 5 and 10 weeks of age compared with controls and $I^{2.1}$KOs but did not differ from each other (Fig. 4*B*).

To avoid difficulties in comparing energy expenditure between groups with disparate body composition (19), V_{O_2} was measured at 8 weeks of age, before body weight and adiposity diverge in $D^{2.1}$KOs (Fig. 1*A* and *B*). Average V_{O_2} during the light and the dark cycles was decreased by ~16% in $D^{2.1}$KOs compared with weight-matched $L^{2.1}$KOs, although this difference did not reach significance (Fig. 5*A*). Because of the high mortality rate of singly housed $D^{2.1}$KOs, we did not attempt to further increase numbers to achieve significance. Together, these observations are consistent with the idea that changes in energy expenditure, and not food intake,

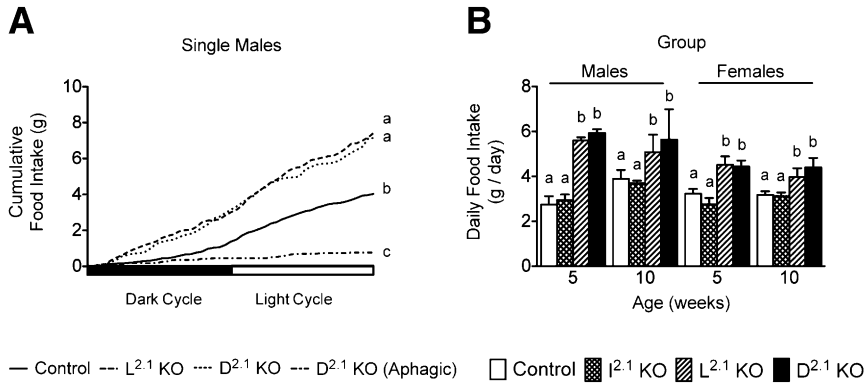

Figure 4—Increased adiposity in $D^{2.1}$KOs is not due to effects on food intake. *A*: Cumulative food intake over a 24-h period, as measured in metabolic cages, of adult singly housed male controls, $L^{2.1}$KOs, $D^{2.1}$KOs, and $D^{2.1}$KOs (aphagic). *B*: Averaged daily food intake at 5 and 10 weeks of group-housed male and female controls, $I^{2.1}$KOs, $L^{2.1}$KOs, and $D^{2.1}$KOs ($n \geq 3$–4 per group). All data are mean ± SEM. *P* values were calculated between age- and sex-matched groups. The lowercase letters above the bars denote statistically similar ($P > 0.05$) groups.

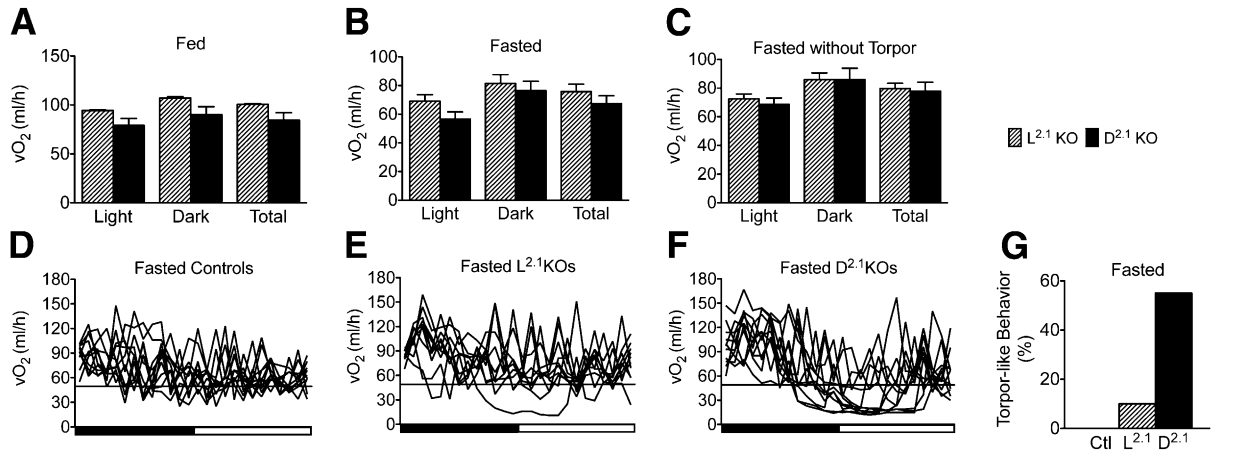

Figure 5—Increased torpor-like behavior after an overnight fast in $D^{2.1}$KOs. Daily V_{O_2}, as measured in metabolic cages, of adult singly housed weight-matched fed (*A*) and fasted (*B*) $L^{2.1}$KO and $D^{2.1}$KO males (including all mice) and in fasted $L^{2.1}$KO and $D^{2.1}$KO males (*C*) after excluding mice that exhibited torpor-like behavior ($n \geq 5$ per group). Data are presented for light cycle, dark cycle, or 24 h total. V_{O_2} in individual mice over a 24-h period in adult singly housed male controls (*D*), $L^{2.1}$KOs (there was no difference in body weight of $L^{2.1}$KOs that entered into torpor [38.25 ± 1.55 g] vs. those that did not [40.75 ± 0.66 g]) (*E*), and $D^{2.1}$KOs (there was no difference in body weight of $D^{2.1}$KOs that entered into torpor [51.17 ± 1.32 g] vs. those that did not [43.33 ± 6.12 g]) (*F*) ($n \geq 10$ per group). *G*: Percentage of mice exhibiting torpor-like behavior when fasted in the metabolic cages. *A–C* and *G*: All data are mean ± SEM. *P* values were calculated between age- and sex-matched groups.

contribute to increased body weights and adiposity in $D^{2.1}$KO mice.

Nkx2.1-Cre–Mediated Disruption of InsR Signals From $L^{2.1}$KOs Promotes Torpor-Like Behavior in Response to Fasting

Because leptin and insulin actions in the hypothalamus are reported to regulate BAT thermogenesis (7,10,12,20), a major component of energy expenditure in the rodent (21), we explored whether disruption of these signaling pathways would have synergistic effects on thermogenic responses to nutritional and cold challenges. After 12 h of fasting, all of the controls maintained consistent but slightly reduced (~12%) levels of energy expenditure (Fig. 5*D*). Consistent with reports in leptin-deficient mice (22), 10% of fasted $L^{2.1}$KOs dramatically reduced V_{O_2} by more than 40% within 1 h and entered a torpor-like state (4) (Fig. 5*E* and *G*). The incidence of torpor-like behavior was markedly higher in fasted $D^{2.1}$KOs, reaching 55% (Fig. 5*F* and *G*). Apparent reductions in V_{O_2} in fasted $D^{2.1}$KOs versus $L^{2.1}$KOs observed when all readings were averaged (Fig. 5*B*) were largely lost when mice that exhibited bouts of torpor were excluded from the analysis (Fig. 5*C*).

The initiation of a torpor-like state is characterized by a rapid suppression of metabolic rate, followed by a decrease in T_c (4); therefore, we assessed the effect of an overnight fast on T_c under group- and single-housing conditions in all experimental groups. Because we previously determined that $L^{2.1}$KOs acquire the capability to thermoregulate in response to a short-term cold challenge by 6 weeks of age (14), these studies were performed in males older than 8 weeks. Mice in all groups decreased T_c in response to fasting (Fig. 6*A* and *C*). When mice were group-housed, fasting induced a larger decrease in T_c in $D^{2.1}$KOs compared with all other groups (controls, −1.07 ± 0.39°C; $I^{2.1}$KO, −0.80 ± 0.74°C; $L^{2.1}$KO, −1.70 ± 0.33°C; $D^{2.1}$KO, −2.90 ± 0.20°C; $P < 0.05$; Fig. 6*A*).

Social thermoregulation (huddling) can reduce the requirement for thermogenesis (23) and thus obscure deficits in thermoregulation in a group-housed setting. Therefore, we also measured changes in T_c after an overnight fast in singly housed mice. Fasting-induced losses in T_c were the same in group-housed and singly housed controls (Fig. 6*A*). Although fasting-induced decreases in T_c were greater in singly housed than group-housed $I^{2.1}$KOs and $L^{2.1}$KOs, this difference did not reach significance (Fig. 6*A*). Preventing social thermoregulation in $D^{2.1}$KOs increased the loss of T_c by ~69% (group, −2.9 ± 0.2°C vs. single, −4.9 ± 0.6°C; $P < 0.05$; Fig. 6*A*).

Under single-housing conditions, the average change in T_c in all fasted $L^{2.1}$KOs was significantly higher than controls; this difference was further amplified in $D^{2.1}$KOs (Fig. 6*A*). However, as we observed in the metabolic cages, there was a bimodal distribution of T_c phenotypes in fasted animals, and some defended a T_c over 32°C, whereas others did not (Fig. 6*C*). Although all singly housed controls maintained a T_c over 32°C, 6% of $I^{2.1}$KOs (1 of 18), 22% of $L^{2.1}$KOs (4 of 18), and 68% of $D^{2.1}$KOs (22 of 32) had a T_c lower than 32°C after the overnight fast, consistent with the initiation of a torpor-like state (Fig. 6*B*). When mice that exhibited a fasting-induced T_c below 32°C were excluded from the analysis, the data were nearly identical to the fed data (Fig. 6*C* and *D*). These observations are consistent with the idea that leptin and insulin signals disrupted by *Nkx2.1-Cre*–mediated

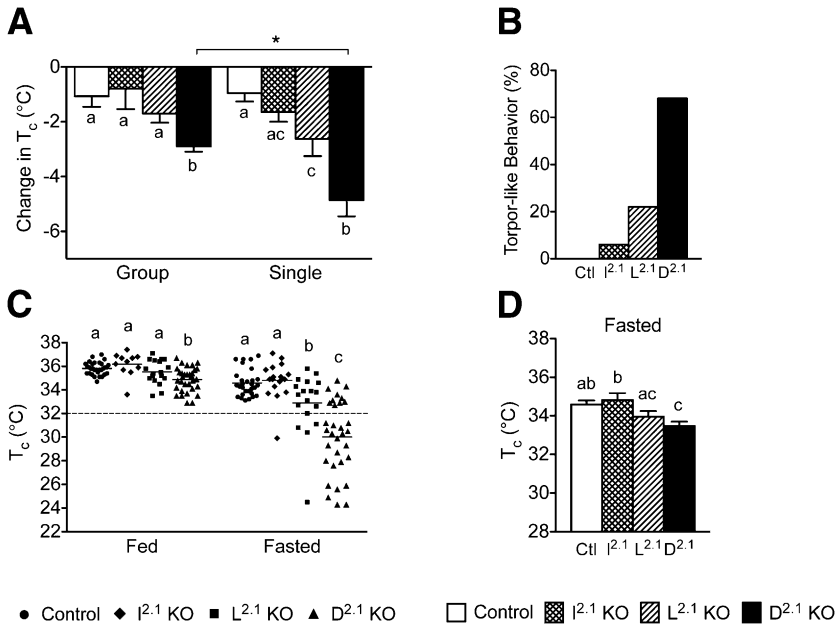

Figure 6—Disruption of hypothalamic InsR signals promotes torpor-like behavior in fasted $L^{2.1}$KOs. *A*: Change in T_c of group-housed and singly housed control, $I^{2.1}$KO, $L^{2.1}$KO, and $D^{2.1}$KO adult males in response to an overnight fast ($n \geq 4$ per group). *$P < 0.05$ for statistical difference of animals with the same genotype under fed vs. fasted condition. *B*: Percent of mice in each group that initiated a torpor-like state (T_c <32°C) when fasted overnight. *C*: Fed and fasted T_c of singly housed control, $I^{2.1}$KO, $L^{2.1}$KO, and $D^{2.1}$KO adult males ($n \geq 10$ per group). *D*: T_c of singly housed fasted control, $I^{2.1}$KO, $L^{2.1}$KO, and $D^{2.1}$KO adult males, after excluding mice that exhibited torpor-like behavior (i.e., those with T_c >32°C; $n \geq 4$ per group). All data are mean ± SEM. The lowercase letters above the bars denote statistically similar ($P > 0.05$) groups under the same condition.

recombination provide critical signals to maintain thermogenesis and prevent torpor under conditions of negative energy balance.

$D^{2.1}$KOs Can Mount a Robust Thermogenic Response to an Acute Cold Challenge

Because leptin and insulin have been implicated in thermoregulatory responses to cold exposure (12,22), we assessed the ability of fasted $L^{2.1}$KOs and $D^{2.1}$KOs to maintain T_c during an acute 4°C challenge. Although T_c was significantly lower in singly housed fasted $L^{2.1}$KOs and $D^{2.1}$KOs at an ambient temperature of 20–22°C, within 15 min of exposure to 4°C, mice in both groups increased their T_c by 2.5°C and 4.5°C, respectively (Fig. 7). At 15 min postexposure, the T_c of $D^{2.1}$KOs and $L^{2.1}$KOs was similar (Fig. 7), but both groups had significantly lower (3–5%) T_c compared with controls throughout the experiment (Fig. 7).

DISCUSSION

Merits and Limitations of the $D^{2.1}$KO Model

Whereas the *Nkx2.1-Cre* transgene broadly extinguishes STAT3-mediated signaling throughout the hypothalamus (14), PCR-based analyses reveal that this driver line achieves only a partial (30%) reduction in the expression of targeted alleles in the hypothalamus (data not shown) (24). The relatively mild decrease in *Insr* expression is likely due to a combination of expression in hypothalamic regions that do not express the transgene (i.e., SCN) (13) as well as in astrocytes and other nonneuronal cells in the hypothalamus (25). Thus, the failure to observe a particular phenotype in *Nkx2.1-Cre*–mediated loss of function mutants (i.e., body weight, blood glucose, etc.) does not exclude a role for hypothalamic insulin signaling. Because we did not detect changes in any metabolic parameters in $I^{2.1}$KO males, a strength of the $D^{2.1}$KO model is that reductions in V_{O_2} and T_c compared with $L^{2.1}$KOs can be attributed to interactions between

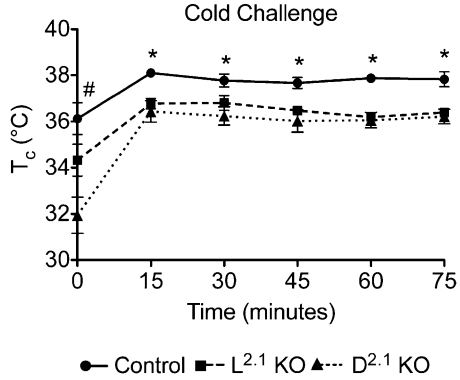

Figure 7—$L^{2.1}$KO and $D^{2.1}$KO males can mount a thermogenic response to an acute cold challenge. T_c during a 75-min cold challenge in overnight-fasted control, $L^{2.1}$KO, and $D^{2.1}$KO adult males at 8–10 weeks ($n \geq 4$ per group). All data are mean ± SEM. *$P < 0.05$ $L^{2.1}$KO and $D^{2.1}$KO vs. control; #$P < 0.05$ $D^{2.1}$KO vs. control.

leptin- and insulin-signaling networks and not to secondary consequences of metabolic dysregulation in $I^{2.1}$KOs.

Our observations support the hypothesis that interactions between leptin and insulin signals exert a mild but significant influence on body temperature set points (and thus energy expenditure) in the fed state but exert a major effect on the decision to enter a torpor-like state to conserve energy when food is scarce.

Leptin-Independent Signals That Influence Torpor-Like Behavior in Response to Negative Energy Balance

Torpor is necessarily associated with limited productivity and increased vulnerability to predation; thus, robust mechanisms to limit the initiation of torpor-like states to periods when it is absolutely essential to conserve energy would promote survival. Reduced serum leptin is permissive for entry into torpor, but other fasting-related signals are required (9,26,27). Levels of NPY in the hypothalamus increase in response to fasting (28) and have been implicated in the initiation and maintenance of torpor bouts (29–31), raising the possibility that fasting-related signals are also relayed through effects on neurons expressing NPY and AgRP in the arcuate nucleus of the hypothalamus (ARH).

Ghrelin and insulin are hormones that are highly sensitive to the prandial state and modulate the activity of arcuate NPY neurons and thus are well positioned to influence the initiation of torpor. The stomach-derived hormone ghrelin is upregulated during fasting (32) and can enhance the torpor state in fasted mice but is not sufficient to induce torpor in fed mice (30). Ghrelin's influence on torpor bouts is thought to be mediated through the activation of NPY/AgRP neurons (30,33,34). Circulating levels of insulin and glucose decrease during hibernation (35), and reduced central glucose sensing is reported to increase the susceptibility of mice to initiate torpor (36). Central glucose and insulin are reported to inhibit the activity of NPY/AgRP neurons (37–39), and reduced central glucose sensing increases susceptibility of mice to enter into torpor (36). In theory, reduced insulin and glucose signaling and/or increased ghrelin signaling in arcuate NPY/AgRP neurons (Fig. 2) could underlie the increased likelihood of entry into a torpor-like state in fasted $D^{2.1}$KOs relative to $L^{2.1}$KOs (Fig. 5). However, the observation that fasted $D^{2.1}$KOs can defend body temperature when subjected to a cold challenge (Fig. 7) supports the idea that the initiation of torpor in response to circulating signals of negative energy balance can be overridden by cues from the external environment.

Increased Mortality Rate in $D^{2.1}$KOs When Singly Housed at Room Temperature

It is very difficult to parse contributions of fasting, inability to huddle for warmth, and psychological stressors associated with single housing to the high rates of torpor and mortality in $D^{2.1}$KO mice. Although the $D^{2.1}$KOs that died during single housing exhibited aphagia, starvation per se was not likely the proximal cause of death because they died within 48 h of aphagia, much sooner than is observed when lean or obese mice are fasted (40). Thermogenic stress due to the inability of singly housed $D^{2.1}$KOs to huddle for warmth would be predicted to increase the likelihood of torpor under fasting conditions (22). Torpor induced by fasting does not necessarily culminate in death, because all singly housed $D^{2.1}$KOs subjected to an investigator-initiated fast exited from torpor (Fig. 5*F*). Moreover, when fasted singly housed $D^{2.1}$KOs were exposed to an acute thermogenic stress (4°C), they all responded by increasing body temperature and did not enter torpor or die (Fig. 7). These observations support the idea that thermogenic stress of perceived cold is not sufficient to initiate torpor or trigger death.

Some of our observations support the idea that psychological factors associated with social isolation stress contribute to the high mortality of singly housed $D^{2.1}$KOs. Because all singly housed $D^{2.1}$KOs subjected to an investigator-initiated fast exited from torpor (Fig. 5*F*), psychological factors responsible for the aphagic behavior also likely contributed to the failure to exit torpor. In addition, the observation that ~45% of fasted $D^{2.1}$KOs did not initiate torpor (Fig. 5*G*) is reminiscent of reports of "resilient" populations in other stress-induced outcomes (41). Taken together, the most parsimonious explanation of the high mortality rates of $D^{2.1}$KOs is that they have increased susceptibility to enter torpor in response to nutrient deprivation and a reduced likelihood to exit this state.

The Role of Leptin in Thermoregulatory Circuits That Regulate the Response to Cold Stress

To promote survival during the winter, thermoregulatory circuits must match the energy required to maintain temperature at a level that supports optimal biological functions with the available energy stores. Although leptin can clearly act as a permissive factor to support thermogenesis in lean mice under fasted conditions (26,27,42,43), its function in thermoregulation in the fed state is less clear. For example, leptin administration in the periphery or in the dorsomedial nucleus of the hypothalamus (DMH) can increase sympathetic tone and temperature in BAT (20,44) but does not appear to exert a major effect on T_c (20,45).

Dramatic differences in the nature of the response to fasting versus cold in $L^{2.1}$KOs and $D^{2.1}$KOs strongly support the idea that leptin- and insulin-sensing populations that influence T_c set points in the fed state are distinct from those that regulate the thermogenic response to cold. Leptin is not necessary for thermoregulation at thermoneutrality (46), consistent with the idea that leptin relays information about energy resources to thermoregulatory circuits under conditions of cold stress. In response to the mild-cold stress associated with an ambient temperature of 20–22°C, we observed that the average T_c in $L^{2.1}$KOs is ~0.5°C lower than in controls and that T_c in $D^{2.1}$KOs is further reduced by ~1°C (Fig. 6). These findings

are consistent with the hypothesis that reduced insulin and leptin signals in neuronal populations that express the *Nkx2.1-Cre* transgene convey a state of negative energy balance to key nodes in the thermoregulatory circuits, which decreases the defended T_c set point to conserve energy. Leptin-sensing populations in the ARH, DMH, and preoptic area (POA), and insulin-sensing populations in the POA that express the *Nkx2.1-Cre* transgene, are well positioned to mediate this function (7,12–14,20,47,48).

Neuronal LepRb is sufficient to support a thermogenic response to acute cold stress (8,49). Even though fasting T_c was 5°C lower in $L^{2.1}$KOs and 12°C lower in $D^{2.1}$KOs compared with controls, mice in both groups mounted a robust thermogenic response to an acute cold stress. These observations support the idea that regions expressing the *Nkx2.1-Cre* transgene are not required to mount a thermogenic response to acute cold. Although *Nkx2.1-Cre* is broadly expressed in the ARH, DMH, and POA (13,14), it is possible that a small subpopulation within one of these nuclei that escapes Cre-mediated recombination is involved. Another explanation is that the relevant subpopulation is in another hypothalamic region that projects to BAT in which *Nkx2.1-Cre* is expressed poorly, such as the retrochiasmatic area (7). However, reports that disrupting LepRb signaling in forebrain neurons that express *CamKII-Cre* or *Rip-Cre* are also able to mount a cold response (50,51) raise the possibility that extrahypothalamic regions are involved. Observations that chronic decerebrate rats are able to initiate a thermogenic response to an acute cold challenge support a role for extrahypothalamic populations (52). Leptin action in the hindbrain has been reported to potentiate sympathetic activation (42,44), consistent with the hypothesis that these circuits are engaged during a cold challenge in hyperleptinemic $L^{2.1}$KOs. Leptin-sensing neurons in the midbrain and brainstem that form polysynaptic connections with BAT are strong candidates (7).

Future Directions

Suppression of T_c in response to reduced energy availability is well documented in small mammals but is rarely discussed in the context of human physiology. It has been observed that patients with diabetes are more likely to be hospitalized for hypothermia (53), particularly in association with hypoglycemia (54) and diabetic ketoacidosis (54). Although it has been postulated that autonomic neuropathy is responsible for the diminished capacity for thermoregulation, our findings raise the possibility that reduced signaling through central insulin-sensing networks could contribute as well. Identification of discrete neuronal subpopulations that influence different aspects of thermoregulation is an important area for future research.

Acknowledgments. The authors thank the Columbia Diabetes and Endocrinology Research Center Pathology Core for cryosectioning and the Mouse Phenotyping Core for help with metabolic cages, D. Accili (Columbia University Medical Center) for *Insr* floxed mice, S. Anderson (Weill Cornell Medical College) for *Nkx2.1-Cre* mice, S. Chua (Albert Einstein College of Medicine) for *Lepr* floxed mice.

Funding. This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases R01-DK-089038 (L.M.Z.). The Pathology Core is funded by P30-DK-63608.

Duality of Interest. No potential conflicts of interest relevant to this article were reported.

Author Contributions. A.C.N.C. and L.M.Z. designed the experiments. A.C.N.C. analyzed data and performed experiments. R.A.G. performed experiments (Fig. 1*C*, *D*, *G*, and *H*). A.C.N.C. and L.M.Z. wrote the manuscript. L.M.Z. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

References

1. Griggio MA. Thermogenic mechanisms in cold-acclimated animals. Braz J Med Biol Res 1988;21:171–176

2. Humphries MM, Thomas DW, Kramer DL. The role of energy availability in Mammalian hibernation: a cost-benefit approach. Physiol Biochem Zool 2003;76:165–179

3. Himms-Hagen J, Villemure C. Number of mice per cage influences uncoupling protein content of brown adipose tissue. Proc Soc Exp Biol Med 1992;200:502–506

4. Swoap SJ. The pharmacology and molecular mechanisms underlying temperature regulation and torpor. Biochem Pharmacol 2008;76:817–824

5. Heller HC, Hammel HT. CNS control of body temperature during hibernation. Comp Biochem Physiol A Comp Physiol 1972;41:349–359

6. Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM. Positional cloning of the mouse obese gene and its human homologue. Nature 1994;372:425–432

7. Zhang Y, Kerman IA, Laque A, et al. Leptin-receptor-expressing neurons in the dorsomedial hypothalamus and median preoptic area regulate sympathetic brown adipose tissue circuits. J Neurosci 2011;31:1873–1884

8. Trayhurn P, Thurlby PL, James WP. Thermogenic defect in pre-obese ob/ob mice. Nature 1977;266:60–62

9. Gavrilova O, Leon LR, Marcus-Samuels B, et al. Torpor in mice is induced by both leptin-dependent and -independent mechanisms. Proc Natl Acad Sci U S A 1999;96:14623–14628

10. Menéndez JA, Atrens DM. Insulin and the paraventricular hypothalamus: modulation of energy balance. Brain Res 1991;555:193–201

11. McGowan MK, Andrews KM, Grossman SP. Role of intrahypothalamic insulin in circadian patterns of food intake, activity, and body temperature. Behav Neurosci 1992;106:380–385

12. Sanchez-Alavez M, Tabarean IV, Osborn O, et al. Insulin causes hyperthermia by direct inhibition of warm-sensitive neurons. Diabetes 2010;59:43–50

13. Xu Q, Tam M, Anderson SA. Fate mapping Nkx2.1-lineage cells in the mouse telencephalon. J Comp Neurol 2008;506:16–29

14. Ring LE, Zeltser LM. Disruption of hypothalamic leptin signaling in mice leads to early-onset obesity, but physiological adaptations in mature animals stabilize adiposity levels. J Clin Invest 2010;120:2931–2941

15. McMinn JE, Liu SM, Dragatsis I, et al. An allelic series for the leptin receptor gene generated by CRE and FLP recombinase. Mamm Genome 2004;15:677–685

16. Kulkarni RN, Brüning JC, Winnay JN, Postic C, Magnuson MA, Kahn CR. Tissue-specific knockout of the insulin receptor in pancreatic beta cells creates an insulin secretory defect similar to that in type 2 diabetes. Cell 1999;96:329–339

17. Brüning JC, Gautam D, Burks DJ, et al. Role of brain insulin receptor in control of body weight and reproduction. Science 2000;289:2122–2125

18. Ueno N, Asakawa A, Inui A. Blunted metabolic response to fasting in obese mice. Endocrine 2007;32:192–196

19. Kaiyala KJ, Morton GJ, Leroux BG, Ogimoto K, Wisse B, Schwartz MW. Identification of body fat mass as a major determinant of metabolic rate in mice. Diabetes 2010;59:1657–1666

20. Enriori PJ, Sinnayah P, Simonds SE, Garcia Rudaz C, Cowley MA. Leptin action in the dorsomedial hypothalamus increases sympathetic tone to brown adipose tissue in spite of systemic leptin resistance. J Neurosci 2011;31:12189–12197
21. Cannon B, Nedergaard J. Brown adipose tissue: function and physiological significance. Physiol Rev 2004;84:277–359
22. Himms-Hagen J. Food restriction increases torpor and improves brown adipose tissue thermogenesis in ob/ob mice. Am J Physiol 1985;248:E531–E539
23. Vinter J, Hull D, Elphick MC. Onset of thermogenesis in response to cold in newborn mice. Biol Neonate 1982;42:145–151
24. Tsou RC, Rak KS, Zimmer DJ, Bence KK. Improved metabolic phenotype of hypothalamic PTP1B-deficiency is dependent upon the leptin receptor. Mol Metab 2014;3:301–312
25. Schechter R, Yanovitch T. Insulin effects on extracellular signal regulated kinase cascade in fetal rat astrocyte cell cultures. Neurosci Lett 1999;268:1–4
26. Döring H, Schwarzer K, Nuesslein-Hildesheim B, Schmidt I. Leptin selectively increases energy expenditure of food-restricted lean mice. Int J Obes Relat Metab Disord 1998;22:83–88
27. Eiden S, Doering H, Schmidt I. Developmental and food-access-dependent changes in effector systems activated by leptin. Pflugers Arch 2002;445:366–374
28. Kalra SP, Dube MG, Sahu A, Phelps CP, Kalra PS. Neuropeptide Y secretion increases in the paraventricular nucleus in association with increased appetite for food. Proc Natl Acad Sci U S A 1991;88:10931–10935
29. Paul MJ, Freeman DA, Park JH, Dark J. Neuropeptide Y induces torpor-like hypothermia in Siberian hamsters. Brain Res 2005;1055:83–92
30. Gluck EF, Stephens N, Swoap SJ. Peripheral ghrelin deepens torpor bouts in mice through the arcuate nucleus neuropeptide Y signaling pathway. Am J Physiol Regul Integr Comp Physiol 2006;291:R1303–R1309
31. Dark J, Pelz KM. NPY Y1 receptor antagonist prevents NPY-induced torpor-like hypothermia in cold-acclimated Siberian hamsters. Am J Physiol Regul Integr Comp Physiol 2008;294:R236–R245
32. Tschöp M, Smiley DL, Heiman ML. Ghrelin induces adiposity in rodents. Nature 2000;407:908–913
33. Nakazato M, Murakami N, Date Y, et al. A role for ghrelin in the central regulation of feeding. Nature 2001;409:194–198
34. Cowley MA, Smith RG, Diano S, et al. The distribution and mechanism of action of ghrelin in the CNS demonstrates a novel hypothalamic circuit regulating energy homeostasis. Neuron 2003;37:649–661
35. Laurila M, Suomalainen P. Studies in the physiology of the hibernating hedgehog. 19. The changes in the insulin level induced by seasons and hibernation cycle. Ann Acad Sci Fenn [Biol] 1974;0:1–40
36. Mounien L, Marty N, Tarussio D, et al. Glut2-dependent glucose-sensing controls thermoregulation by enhancing the leptin sensitivity of NPY and POMC neurons. FASEB J 2010;24:1747–1758
37. Schwartz MW, Marks JL, Sipols AJ, et al. Central insulin administration reduces neuropeptide Y mRNA expression in the arcuate nucleus of food-deprived lean (Fa/Fa) but not obese (fa/fa) Zucker rats. Endocrinology 1991;128:2645–2647
38. Sato I, Arima H, Ozaki N, et al. Insulin inhibits neuropeptide Y gene expression in the arcuate nucleus through GABAergic systems. J Neurosci 2005;25:8657–8664
39. Fioramonti X, Contié S, Song Z, Routh VH, Lorsignol A, Pénicaud L. Characterization of glucosensing neuron subpopulations in the arcuate nucleus: integration in neuropeptide Y and pro-opio melanocortin networks? Diabetes 2007;56:1219–1227
40. Cuendet GS, Loten EG, Cameron DP, Renold AE, Marliss EB. Hormone-substrate responses to total fasting in lean and obese mice. Am J Physiol 1975;228:276–283
41. Krishnan V, Han MH, Graham DL, et al. Molecular adaptations underlying susceptibility and resistance to social defeat in brain reward regions. Cell 2007;131:391–404
42. Hermann GE, Barnes MJ, Rogers RC. Leptin and thyrotropin-releasing hormone: cooperative action in the hindbrain to activate brown adipose thermogenesis. Brain Res 2006;1117:118–124
43. Rogers RC, Barnes MJ, Hermann GE. Leptin "gates" thermogenic action of thyrotropin-releasing hormone in the hindbrain. Brain Res 2009;1295:135–141
44. Haynes WG, Morgan DA, Walsh SA, Mark AL, Sivitz WI. Receptor-mediated regional sympathetic nerve activation by leptin. J Clin Invest 1997;100:270–278
45. Pelleymounter MA, Cullen MJ, Baker MB, et al. Effects of the obese gene product on body weight regulation in ob/ob mice. Science 1995;269:540–543
46. Trayhurn P. Thermoregulation in the diabetic-obese (db/db) mouse. The role of non-shivering thermogenesis in energy balance. Pflugers Arch 1979;380:227–232
47. Kong D, Tong Q, Ye C, et al. GABAergic RIP-Cre neurons in the arcuate nucleus selectively regulate energy expenditure. Cell 2012;151:645–657
48. Shi YC, Lau J, Lin Z, et al. Arcuate NPY controls sympathetic output and BAT function via a relay of tyrosine hydroxylase neurons in the PVN. Cell Metab 2013;17:236–248
49. Chua SC Jr, Liu SM, Li Q, et al. Transgenic complementation of leptin receptor deficiency. II. Increased leptin receptor transgene dose effects on obesity/diabetes and fertility/lactation in lepr-db/db mice. Am J Physiol Endocrinol Metab 2004;286:E384–E392
50. McMinn JE, Liu SM, Liu H, et al. Neuronal deletion of Lepr elicits diabesity in mice without affecting cold tolerance or fertility. Am J Physiol Endocrinol Metab 2005;289:E403–E411
51. Covey SD, Wideman RD, McDonald C, et al. The pancreatic beta cell is a key site for mediating the effects of leptin on glucose homeostasis. Cell Metab 2006;4:291–302
52. Nautiyal KM, Dailey M, Brito N, et al. Energetic responses to cold temperatures in rats lacking forebrain-caudal brain stem connections. Am J Physiol Regul Integr Comp Physiol 2008;295:R789–R798
53. Neil HA, Dawson JA, Baker JE. Risk of hypothermia in elderly patients with diabetes. Br Med J (Clin Res Ed) 1986;293:416–418
54. Field JB. Hypoglycemia. Definition, clinical presentations, classification, and laboratory tests. Endocrinol Metab Clin North Am 1989;18:27–43