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 (L2.1KO), insulin receptor (I2.1KO), and double KOs of both receptors (D2.1KO). L2.1KOs are hyperphagic and obese, whereas I2.1KOs are similar to controls. D2.1KOs exhibit higher body weight and adiposity than L2.1KOs, solely due to reduced energy expenditure. At 20–22°C, fed L2.1KOs maintain a lower baseline $T_c$ than controls, which is further decreased in D2.1KOs. After an overnight fast, some L2.1KOs dramatically suppress energy expenditure and enter a torpor-like state; this behavior is markedly enhanced in D2.1KOs. When fasted mice are exposed to 4°C, L2.1KOs and D2.1KOs 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...
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). 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 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\( ^{fl/fl}\)/ChuaJ (provided by D. Acilli, Columbia University Medical Center)] (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: \( Nkx2.1-Cre;Lepr^{fl/fl} \) (\( L^2.1KO \)), \( Nkx2.1-Cre;Insr^{fl/fl} \) (\( D^2.1KO \)), \( Nkx2.1-Cre;Lepr^{fl/fl};Insr^{fl/fl} \) (\( D^2.1KO \)), 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 \( D^2.1KO \) male. Genotypes were assessed by PCR on genomic DNA from tail tips using the following primers:

\[
\begin{align*}
\text{Cre} & \quad 5’\ GCGGTCTGGCAGTAAAAACTATC 3’ \quad \text{(forward)} \\
\text{Cre} & \quad 5’\ GTGAAACAGCATTTGCTGACCT 3’ \quad \text{(reverse)} \\
\text{Lepr} & \quad 5’\ GTCTGATTGGATGATGCTTCT 3’ \quad \text{(forward)} \\
\text{Lepr} & \quad 5’\ AGAATGAAAGTGTGGGGGA 3’ \quad \text{(forward)} \\
\text{Lepr} & \quad 5’\ GGCCTGAAACATGAACAC 3’ \quad \text{(reverse)} \\
\text{Insr} & \quad 5’\ TGACCCCCATGTCGGGACC 3’ \quad \text{(forward)} \\
\text{Insr} & \quad 5’\ GCCTCTGAATAGCTGAGACC 3’ \quad \text{(reverse)}
\end{align*}
\]

**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 \( L^2.1KO \), one \( D^2.1KO \), 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 \( \times200 \) 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. $V_{O2}$, $CO_2$ 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 $V_{O2}$ and $V_{CO2}$ 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 $T_e$**

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 D2.1KOs**

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 (D2.1KOs) that are more severe than would be predicted by the additive effects of disrupting leptin (L2.1KOs) and insulin signals (D2.1KOs). Consistent with observations in mice lacking neuronal InsR signaling (NIRKO mice) (17), body weight and adiposity in D2.1KOs were not different from controls (Fig. 1A and B). Body weight and adiposity were significantly higher in L2.1KO and D2.1KO males compared with L2.1KO and controls starting from 6 weeks, and D2.1KOs diverged from L2.1KO at 10 weeks (Fig. 1A and B).

Female I2.1KO mice weighed the same as the controls but exhibited a mild, but significant, increase in adiposity starting from 10 weeks of age (Fig. 1E and F), similar to observations in NIRKO females (17). I2.1KO and D2.1KO females both had significantly higher body weight and adiposity than L2.1KOs and controls from 6 weeks of age (Fig. 1E and F). L2.1KO and D2.1KO females had similar body weights throughout the study (Fig. 1E); however, adiposity of D2.1KOs was slightly but significantly higher from 10 weeks (Fig. 1F). Because gonadal fat depots are preferentially affected in NIRKO females (17), we performed histological analyses of inguinal and gonadal fat depots in L2.1KO and D2.1KO females. Gonadal adiposity was increased by 34–40% in D2.1KO females compared with L2.1KOs at 9 and 19 weeks, partly due to a 14–24% increase in adipocyte cell size (Fig. 1C, 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 D2.1KO males but did not reach significance (data not shown).

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 D2.1KO and L2.1KO males and females at 8 and 12 weeks, serum leptin levels were more than 20-fold higher in these groups than in controls and L2.1KOs, whereas levels in lean I2.1KOs were similar to controls (Fig. 2A and B). Serum leptin was increased by 30% at 8 weeks and by 47% at 12 weeks in male D2.1KOs compared with L2.1KOs (Fig. 2A). Female D2.1KOs had higher adiposity than L2.1KOs, but this was not reflected in increased leptin levels (Fig. 2B).

L2.1KO males and controls had similar serum levels of acylated ghrelin in fed and fasted conditions (Fig. 2C). Similar to observations in Lepob/ob mice (18), ghrelin levels in L2.1KO males were ~65% lower than in controls at baseline, and this difference was amplified during fasting (Fig. 2C). D2.1KOs exhibited ghrelin levels that were intermediate between L2.1KOs and I2.1KOs, although this difference did not reach significance in the fed state (Fig. 2C). Expression of neuropeptide Y (Npy) and Agouti-related peptide (Agrp) was similarly elevated in fasted L2.1KOs and D2.1KOs compared with controls and I2.1KOs (Fig. 2D). In summary, although adiposity-related phenotypes in L2.1KOs are exacerbated in D2.1KOs, ghrelin levels are normalized.
We also assessed phenotypes related to glucose homeostasis. Whereas I2.1KO males and females had the same blood glucose and serum insulin levels as controls, L2.1KO and D2.1KO males and females were hyperglycemic and hyperinsulinemic from 6 weeks of age (Fig. 3A–D). Despite the elevated adiposity in D2.1KO versus L2.1KO males, fasting or fed blood glucose (Fig. 3A) and serum insulin (Fig. 3B) were similar in both groups. Fed blood glucose was mildly increased (Fig. 3C) and serum insulin trended higher (Fig. 3D) in D2.1KO females compared with L2.1KO at 12 weeks.

Energy Expenditure Is Decreased in D2.1KOs Versus L2.1KOs, Whereas Food Intake Is Similar

To investigate whether elevated adiposity in D2.1KOs compared with L2.1KOs is due to effects on energy intake and/or expenditure, we measured food intake, VO2 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 D2.1KO males were aphagic and died within 3–5 days (Fig. 4A, bottom curve). The remaining D2.1KOs consumed the same amount of food in a 24-h period as L2.1KOs (Fig. 4A).

To overcome the high mortality rate of single housing in
D2.1KO males, intake from food hoppers was also assessed under group-housing conditions, with two to three mice of the same genotype in a cage. L2.1KO males and females consumed the same amount of food as controls (Fig. 4B). L2.1KO and D2.1KO males and females were hyperphagic at 5 and 10 weeks of age compared with controls and L2.1KOs but did not differ from each other (Fig. 4B).

To avoid difficulties in comparing energy expenditure between groups with disparate body composition (19), VO2 was measured at 8 weeks of age, before body weight and adiposity diverge in D2.1KOs (Fig. 1A and B). Average VO2 during the light and the dark cycles was decreased by ∼16% in D2.1KOs compared with weight-matched L2.1KOs, although this difference did not reach significance (Fig. 5A). Because of the high mortality rate of singly housed D2.1KOs, 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,
contribute to increased body weights and adiposity in D2.1KO mice.

*Nkx2.1-Cre–Mediated Disruption of InsR Signals From L2.1KOs 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. 5D). Consistent with reports in leptin-deficient mice (22), 10% of fasted L2.1KOs dramatically reduced VO2 by more than 40% within 1 h and entered a torpor-like state (4) (Fig. 5E and G). The incidence of torpor-like behavior was markedly higher in fasted D2.1KOs, reaching 55% (Fig. 5F and G). Apparent reductions in VO2 in fasted D2.1KOs versus L2.1KOs observed when all readings were averaged (Fig. 5B) were largely lost when mice that exhibited bouts of torpor were excluded from the analysis (Fig. 5C).

The initiation of a torpor-like state is characterized by a rapid suppression of metabolic rate, followed by a decrease in Tc (4); therefore, we assessed the effect of an overnight fast on Tc under group- and single-housing conditions in all experimental groups. Because we previously determined that L2.1KOs 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 Tc in response to fasting (Fig. 6A and C). When mice were group-housed, fasting induced a larger decrease in Tc in D2.1KOs compared with all other groups (controls, −1.07 ± 0.39°C; I2.1KO, −0.80 ± 0.74°C; L2.1KO, −1.70 ± 0.33°C; D2.1KO, −2.90 ± 0.20°C; P < 0.05; Fig. 6A).

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 Tc after an overnight fast in singly housed mice. Fasting-induced losses in Tc were the same in group-housed and singly housed controls (Fig. 6A). Although fasting-induced decreases in Tc were greater in singly housed compared with group-housed I2.1KOs and L2.1KOs, this difference did not reach significance (Fig. 6A). Preventing social thermoregulation in D2.1KOs increased the loss of Tc by ~69% (group, −2.9 ± 0.2°C vs. single, −4.9 ± 0.6°C; P < 0.05; Fig. 6A).

Under single-housing conditions, the average change in Tc in all fasted L2.1KOs was significantly higher than controls; this difference was further amplified in D2.1KOs (Fig. 6A). However, as we observed in the metabolic cages, there was a bimodal distribution of Tc phenotypes in fasted animals, and some defended a Tc over 32°C, whereas others did not (Fig. 6C). Although all singly housed controls maintained a Tc over 32°C, 6% of L2.1KOs (1 of 18), 22% of L2.1KOs (4 of 18), and 68% of D2.1KOs (22 of 32) had a Tc lower than 32°C after the overnight fast, consistent with the initiation of a torpor-like state (Fig. 6B). When mice that exhibited a fasting-induced Tc below 32°C were excluded from the analysis, the data were nearly identical to the fed data (Fig. 6C and D). These observations are consistent with the idea that leptin and insulin signals disrupted by Nkx2.1-Cre–mediated

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**Figure 5**—Increased torpor-like behavior after an overnight fast in D2.1KOs. Daily VO2, as measured in metabolic cages, of adult singly housed weight-matched fed (A) and fasted (B) L2.1KO and D2.1KO males (including all mice) and in fasted L2.1KO and D2.1KO males (C) after excluding mice that exhibited torpor-like behavior (n = 5 per group). Data are presented for light cycle, dark cycle, or 24 h total. VO2 in individual mice over a 24-h period in adult singly housed male controls (D), L2.1KOs (there was no difference in body weight of L2.1KOs that entered into torpor [38.25 ± 1.55 g] vs. those that did not [40.75 ± 0.66 g]) (E), and D2.1KOs (there was no difference in body weight of D2.1KOs that entered into torpor [51.17 ± 1.32 g] vs. those that did not [43.33 ± 6.12 g]) (F) (n = 10 per group). G: Percentage of mice exhibiting torpor-like behavior when fasted in the metabolic cages. A–C: All data are mean ± SEM. P values were calculated between age- and sex-matched groups.
recombination provide critical signals to maintain thermogenesis and prevent torpor under conditions of negative energy balance.

**D2.1KOs 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 L2.1KOs and D2.1KOs to maintain Tc during an acute 4°C challenge. Although Tc was significantly lower in singly housed fasted L2.1KOs and D2.1KOs at an ambient temperature of 20–22°C, within 15 min of exposure to 4°C, mice in both groups increased their Tc by 2.5°C and 4.5°C, respectively (Fig. 7). At 15 min postexposure, the Tc of singly housed fasted control, I2.1KO, L2.1KO, and D2.1KO adult males, after excluding mice that exhibited torpor-like behavior (i.e., those with Tc <32°C; n ≥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.

**DISCUSSION**

Merits and Limitations of the D2.1KO 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 I2.1KO males, a strength of the D2.1KO model is that reductions in VO2 and Tc compared with L2.1KOs can be attributed to interactions between

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**Figure 6**—Disruption of hypothalamic InsR signals promotes torpor-like behavior in fasted L2.1KOs. A: Change in Tc of group-housed and singly housed control, I2.1KO, L2.1KO, and D2.1KO adult males in response to an overnight fast (n ≥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 (Tc <32°C) when fasted overnight. C: Fed and fasted Tc of singly housed control, I2.1KO, L2.1KO, and D2.1KO adult males (n ≥10 per group). D: Tc of singly housed fasted control, I2.1KO, L2.1KO, and D2.1KO adult males, after excluding mice that exhibited torpor-like behavior (i.e., those with Tc >32°C; n ≥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.

**Figure 7**—L2.1KO and D2.1KO males can mount a thermogenic response to an acute cold challenge. Tc during a 75-min cold challenge in overnight-fasted control, L2.1KO, and D2.1KO adult males at 8–10 weeks (n ≥4 per group). All data are mean ± SEM. *P < 0.05 L2.1KO and D2.1KO vs. control; #P < 0.05 D2.1KO vs. control.
leptin- and insulin-signaling networks and not to secondary consequences of metabolic dysregulation in L2.1KOs.

Our observations support the hypothesis that interactions between leptin and insulin signals exert a mild but significant influence on body temperature set points (and hence 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 D2.1KOs relative to L2.1KOs (Fig. 5). However, the observation that fasted D2.1KOs 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 D2.1KOs 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 D2.1KO mice. Although the D2.1KOs 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 D2.1KOs 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 D2.1KOs subjected to an investigator-initiated fast exited from torpor (Fig. 5F). Moreover, when fasted singly housed D2.1KOs 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 D2.1KOs. Because all singly housed D2.1KOs subjected to an investigator-initiated fast exited from torpor (Fig. 5F), psychological factors responsible for the aphagic behavior also likely contributed to the failure to exit torpor. In addition, the observation that ~45% of fasted D2.1KOs did not initiate torpor (Fig. 5G) 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 D2.1KOs 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 Tc (20,45).

Dramatic differences in the nature of the response to fasting versus cold in L2.1KOs and D2.1KOs strongly support the idea that leptin- and insulin-sensing populations that influence Tc 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 Tc in L2.1KOs is ~0.5°C lower than in controls and that Tc in D2.1KOs 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 Tc 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 (7,12,20,47,48).

Neuronal LepRb is sufficient to support a thermogenic response to acute cold stress (8,49). Even though fasting Tc was 5°C lower in L2.1KOs and 12°C lower in D2.1KOs 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 stress (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 L2.1KOs. Leptin-sensing neurons in the midbrain and brainstem that form polysynaptic connections with BAT are strong candidates (7).

Future Directions
Suppression of Tc 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.

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