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## Deficiency of PTP1B in POMC neurons leads to alterations in energy balance and homeostatic response to cold exposure

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**De Jonghe BC, Hayes MR, Banno R, Skibicka KP, Zimmer DJ, Bowen KA, Leichner TM, Alhadeff AL, Kanoski SE, Cyr NE, Nillni EA, Grill HJ, Bence KK.** Deficiency of PTP1B in POMC neurons leads to alterations in energy balance and homeostatic response to cold exposure. *Am J Physiol Endocrinol Metab* 300: E1002–E1011, 2011. First published March 15, 2011; doi:10.1152/ajpendo.00639.2010.—The adipose tissue-derived hormone leptin regulates energy balance through catabolic effects on central circuits, including proopiomelanocortin (POMC) neurons. Leptin activation of POMC neurons increases thermogenesis and locomotor activity. Protein tyrosine phosphatase 1B (PTP1B) is an important negative regulator of leptin signaling. POMC neuron-specific deletion of PTP1B in mice results in reduced high-fat diet-induced body weight and adiposity gain due to increased energy expenditure and greater leptin sensitivity. Mice lacking the leptin gene (*ob/ob* mice) are hypothermic and cold intolerant, whereas leptin delivery to *ob/ob* mice induces thermogenesis via increased sympathetic activity to brown adipose tissue (BAT). Here, we examined whether POMC PTP1B mediates the thermoregulatory response of CNS leptin signaling by evaluating food intake, body weight, core temperature ( $T_c$ ), and spontaneous physical activity (SPA) in response to either exogenous leptin or 4-day cold exposure (4°C) in male POMC-Ptp1b-deficient mice compared with wild-type controls. POMC-Ptp1b<sup>-/-</sup> mice were hypersensitive to leptin-induced food intake and body weight suppression compared with wild types, yet they displayed similar leptin-induced increases in  $T_c$ . Interestingly, POMC-Ptp1b<sup>-/-</sup> mice had increased BAT weight and elevated plasma triiodothyronine ( $T_3$ ) levels in response to a 4-day cold challenge, as well as reduced SPA 24 h after cold exposure, relative to controls. These data show that PTP1B in POMC neurons plays a role in short-term cold-induced reduction of SPA and may influence cold-induced thermogenesis via enhanced activation of the thyroid axis.

protein tyrosine phosphatase 1B; proopiomelanocortin; phosphatase; leptin; energy expenditure; thermogenesis; thyroid

THE ADIPOSE TISSUE-DERIVED HORMONE LEPTIN mediates effects on energy balance via suppression of food intake and stimulation of energy expenditure (for review, see Ref. 6). Within the arcuate nucleus (ARC) of the hypothalamus, two distinct populations of neurons synthesize either agouti-related protein (AgRP) or proopiomelanocortin (POMC) and mediate opposing effects on energy balance. Circulating leptin activates these and other central nervous system (CNS) neurons, leading to

decreased food intake and increased energy expenditure, in part through simultaneous suppression of AgRP signaling and stimulation of POMC signaling. Deletion of the leptin receptor (LepR) in POMC neurons (POMC-Cre: LepR<sup>loxP/loxP</sup>) results in increased body weight and adiposity, hyperleptinemia, and altered hypothalamic neuropeptide expression (1, 45), demonstrating the importance of POMC neurons to energy balance control.

In addition to leptin's effects on food intake and energy expenditure, the hormone also regulates thermogenesis and locomotor activity via central circuits. Mice lacking the leptin gene (*ob/ob* mice) have decreased body temperature and are cold intolerant (50), yet these mice can survive at low temperatures (4°C) when properly acclimated (9). Leptin delivery to *ob/ob* mice induces thermogenesis via increased sympathetic activity to brown adipose tissue (BAT) and induction of uncoupling protein 1 (UCP1) expression (10, 11). However, data from mice lacking both leptin and UCP1 (compound *ob/ob*; *UCP1*<sup>-/-</sup>) suggest a more complex picture, namely that UCP1-independent mechanisms, induced by either peripheral leptin or active thyroid hormone injections, can sufficiently rescue and maintain body temperature during cold exposure (52). The neurons that mediate the effects of leptin on body temperature are not clear, although they are likely to involve activation of downstream melanocortin 3 and/or 4 receptors (MC3R/MC4R) within the hindbrain and hypothalamus (46, 47, 65). Cold exposure induces a reversible enhancement of LepR mRNA expression in lean mice within the ARC (31). Interestingly, a recent report found that selective re-expression of LepR in POMC neurons of LepR-deficient *db/db* mice resulted in increased locomotor activity (19), suggesting that leptin signaling in POMC neurons may be an important regulator of physical activity.

The leptin signaling pathway is tightly controlled by tyrosine phosphorylation (for review, see Refs. 34 and 54). Protein tyrosine phosphatase 1B (PTP1B) is an important negative regulator of leptin signaling that acts via direct dephosphorylation of Janus kinase 2 (7, 35, 60). Consistent with a role for PTP1B in the inhibition of leptin signaling, whole body and brain-specific Ptp1b<sup>-/-</sup> mice are lean and resistant to diet-induced obesity due to increased energy expenditure (22, 40). Ptp1b<sup>-/-</sup> mice are leptin hypersensitive, as demonstrated by increased hypothalamic phosphorylated signal transducer and activator of transcription 3 (pSTAT3) activation and enhanced suppression of food intake and body weight in response to exogenous leptin (7, 60). These studies, along with notable

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associations between PTP1B polymorphisms and type 2 diabetes in humans, have identified PTP1B as an attractive therapeutic target for obesity, diabetes, and metabolic syndrome (5, 14, 15, 32, 33, 39, 62, 64).

Although PTP1B is ubiquitously expressed, PTP1B is highly enriched in the ARC, an important site of leptin action (60). We have reported recently that POMC neuron-specific deletion of PTP1B (POMC-Ptp1b<sup>-/-</sup>) in mice results in reduced high-fat diet (HFD)-induced body weight and adiposity, lower serum leptin levels, increased energy expenditure, and improved leptin sensitivity (2). Although body weights were similar between POMC-Ptp1b<sup>-/-</sup> and wild-type mice on a low-fat chow diet, leptin sensitivity is improved in POMC-Ptp1b<sup>-/-</sup> mice under these conditions. However, it is unknown what role POMC PTP1B signaling has in mediating leptin's effects on physical activity and core temperature.

Given the links between POMC neuron leptin signaling and thermogenesis and physical activity in mice, we sought to examine the thermoregulatory and locomotor responses of leptin-hypersensitive POMC-Ptp1b<sup>-/-</sup> mice to exogenous leptin or sustained cold exposure (4°C) compared with littermate controls. We also examined BAT markers of thermogenesis as well as plasma thyroid hormone, ghrelin levels, hypothalamic TRH, and growth hormone secretagogue receptor (GHS-R) mRNA in cold-exposed animals to assess potential genotypic differences in the homeostatic response to cold.

## METHODS

**Animals.** Five-month-old male Ptp1b<sup>loxP/loxP</sup> POMC-Cre mice (hereafter termed POMC-Ptp1b<sup>-/-</sup>) and Ptp1b<sup>loxP/loxP</sup> (hereafter termed Ptp1b<sup>+/+</sup>) "wild-type" littermate controls were used for the following experiments. Mice were generated and genotyped by PCR, as described previously (2). Mice were housed individually in small plastic bins in a temperature- and humidity-controlled room following a 12:12-h light-dark cycle (lights on at 0800). Animals were maintained on pelleted chow (Lab Diet 5010), and water was available ad libitum unless otherwise indicated. All protocols and procedures were approved by the University of Pennsylvania Institutional Care And Use Committee.

**Telemetric transponder surgery.** Mice were anesthetized with ketamine (90 mg/kg im) prior to surgery. Under anesthesia, miniature telemetric transponders (G2 VitalView; Mini Mitter/Respironics, Bend, OR) were implanted within the abdominal cavity according to a slightly modified procedure (as described in Ref. 46) to electronically record core temperature (T<sub>C</sub>) and spontaneous physical activity (SPA).

**Metabolic measurements during leptin treatment or 4-day cold exposure.** Following a 1-wk recovery period from surgery, baseline measurements of food intake (FI), body weight (BW), T<sub>C</sub>, and SPA were taken for 2 days, with ad libitum access to food and water, followed by 2 days of measurements with no access to food during the light cycle only. Next, mice (POMC-Ptp1b<sup>-/-</sup>, *n* = 8; Ptp1b<sup>+/+</sup>, *n* = 9) were treated with exogenous mouse recombinant leptin (1 μg/g BW, every 12 h, for a total of 2 μg·g BW<sup>-1</sup>·day<sup>-1</sup> ip) over 2 days, with food and water available ad libitum. This relatively low dose of leptin was used to prevent maximal effects in the control animals, which may have masked any enhanced effects in POMC-Ptp1b<sup>-/-</sup> mice (2). In a second experiment, a new cohort of mice (POMC-Ptp1b<sup>-/-</sup>, *n* = 6; Ptp1b<sup>+/+</sup>, *n* = 6) were individually housed and allowed to acclimate for 2 days inside a thermally controlled chamber set at 23°C, followed by 4 days set at 4°C. These mice had free access to food and water and were not leptin treated. Upon the completion of cold exposure, these mice were euthanized via CO<sub>2</sub> inhalation and euthanized for trunk blood collection, tissue extraction, and analysis.

The mouse cages were placed on recording platforms for the indicated periods of either leptin treatment or 4°C cold exposure. T<sub>C</sub> data were collected every 5 min, and SPA was recorded as cumulative activity counts every 30 s, as described previously (18, 46). For analysis, the dark and light cycle data were averaged over a 12-h period and analyzed separately. FI and BW were measured daily at lights on.

**Tissue collection, RNA extraction, and real-time PCR.** Cold-exposed mice were euthanized immediately following 4-day cold exposure. An additional set of 4-day cold-exposed mice not implanted with telemetric transponders (POMC-Ptp1b<sup>-/-</sup>, *n* = 7; Ptp1b<sup>+/+</sup>, *n* = 4) were subjected to the same experimental procedure and euthanized for tissue analysis. Data between nonimplanted and implanted mice were similar and thus pooled for subsequent analysis. A cohort of non-cold-exposed mice (i.e., maintained at room temperature) were age matched when euthanized (POMC-Ptp1b<sup>-/-</sup>, *n* = 10; Ptp1b<sup>+/+</sup>, *n* = 7) and used as a control group for tissue analysis. Epididymal white adipose tissue (WAT), intrascapular BAT, and whole hypothalamus were carefully dissected, weighed, and flash-frozen in liquid nitrogen at euthanization.

For gene expression studies, total RNA was extracted from BAT and hypothalamus using TRIzol (Invitrogen) and the RNeasy kit (Qiagen). cDNA was synthesized from 1 μg of total RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The relative mRNA levels of BAT UCP1, UCP2, UCP3, cyclooxygenase-2 (COX-2), and hypothalamic pro-TRH and GHS-R were quantified by quantitative real-time PCR. Mouse β-actin (VIC-MGB, no. 4352341E; Applied Biosystems) or Hprt1 (PPM03559E; SABiosciences) was used as an internal control. PCR reactions were carried out using TaqMan gene expression kits (UCP-1: Mm00494069\_m1; UCP-2: Mm00627599\_m1; UCP-3: Mm01163394\_m1; GHS-R: Mm00616415\_m1) or SYBR Green PCR reagents from Applied Biosystems. Hypothalamic pro-TRH mRNA was assessed via custom assay (38) using the following primers: upstream pro-TRH, 5'-GGAGA-GGGTGTCTTAATGCCT-3'; downstream pro-TRH, 5'-GGCCT-GTTTGACCACAAGTCC-3'. COX-2 mRNA was examined via a custom assay used previously by Ukropec et al. (51) with the following primers: forward, GCCAATAGAAGCTTCCAATCCGTATAT; reverse, TGGTCGGTTTGATGTTACTGTTG; 5'-FAM probe, CTGATGC-CATCCCAGGCCGACTAA (Applied Biosystems). Samples were analyzed using the Eppendorf Mastercycler ep realplex. Relative mRNA expression was calculated using the comparative threshold cycle method, as described previously (4).

**Hematoxylin and eosin staining.** In a random subset of cold-exposed mice (POMC-Ptp1b<sup>-/-</sup>, *n* = 6; Ptp1b<sup>+/+</sup>, *n* = 6), BAT postfixed in 10% formalin was embedded in paraffin and cut into 4-μm sections via microtome and mounted onto slides. Hematoxylin and eosin staining was performing using SelecTech reagents (Surigipath/Leica Biosystems). Slides were dipped into HE 560 for 4 min, followed by 1-min wash (dH<sub>2</sub>O), and then submerged in Define solution for 20 s. After a 1-min wash, slides were dipped in Blue Buffer 8 (diluted 1:10) for 40 s. A final 1-min wash was performed before slides were exposed to Alcohol Eosin Y515 for 25 s. Slides were then dehydrated by sequential submersion in 95% ethanol, 100% ethanol, and then xylene before coverslipping. Images of slides were captured using a Nikon 80i fluorescence microscope and charge-coupled device camera at ×20 magnification and viewed using NIS elements software. Color images were taken on a Nikon E600 with a Nikon Coolpix color camera. Cell areas, diameters, and perimeters were measured using the object measurement tool (NIS elements). Only cells within the plane of focus and containing a clearly defined outer perimeter were selected for measurement. On average, 50–60 cells/animal were selected.

**Thyroid hormone and ghrelin measurements.** Serum triiodothyronine (T<sub>3</sub>) and thyroxine (T<sub>4</sub>) levels in mice (cold-exposed POMC-Ptp1b<sup>-/-</sup>, *n* = 9; cold-exposed Ptp1b<sup>+/+</sup>, *n* = 10; nonexposed POMC-Ptp1b<sup>-/-</sup>, *n* = 6; nonexposed Ptp1b<sup>+/+</sup>, *n* = 6) were assessed

by radioimmunoassay and calculated according to the procedures and reagents provided by ICN Pharmaceuticals (Costa Mesa, CA) and Monobind (Costa Mesa, CA), respectively. The sensitivities of the T<sub>3</sub> and T<sub>4</sub> assays were 5.3 pg/ml and 0.16 ng/ml, respectively, and the intra- and interassay variabilities were ~5–7 and 10–11%, respectively. Deiodinase activity assays were performed according to Richard et al. (41) on cold-exposed mice. Briefly, extracted BAT tissue was homogenized in P100E2D10 [100 mM sodium phosphate (pH 7.2), 2 mM EDTA, 10 mM DTT]. Total protein concentrations were determined using a Bradford assay. Type 2 iodothyronine deiodinase (D2) activity in BAT samples was assayed using P100E2D25 and [<sup>3</sup>5'-<sup>125</sup>I]T<sub>4</sub> (3,000 cpm) with 100 mM 6-propyl-2-thiouracil [PTU (Sigma p3755) to block D1 activity] and 1 μM T<sub>3</sub> (to block D3 activity) in a total reaction volume of 0.5 ml. Samples were incubated at 37°C for 60 min, and reactions were stopped with 0.2 ml of ice-cold methanol. Samples were then centrifuged at 3,200 rpm for 30 min, and 0.4 ml of supernatant was removed and counted for radioactivity. For all samples, 0.7 mg of protein was used. All samples were run in the same assay on the same day.

Plasma ghrelin measurements were performed in an additional set of age-matched male mice (POMC-Ptp1b<sup>-/-</sup>, *n* = 6; Ptp1b<sup>+/+</sup>, *n* = 6) using a within-subjects design at room temperature and immediately following 24-h cold exposure. Blood was drawn via mandibular vein bleed and collected in separate EDTA tubes for measurements of unacylated and acylated ghrelin. For acylated ghrelin, p-hydroxymercuribenzoic acid (1 mM) was added to tubes to prevent degradation. These samples were then centrifuged at 3,500 rpm for 10 min at 4°C and supernatants transferred to separate tubes containing 100 μl of 1 N HCl/ml of collected plasma, and then samples were centrifuged again for 5 min and transferred to new tubes. Plasma unacylated and acylated ghrelin were analyzed via EIA kits (ghrelin: no. 10008953; acylated ghrelin: no. 10006307; Cayman Chemical).

**Statistics.** All data are expressed as means ± SE. For statistical analyses, leptin treatment days were compared with the second day of ad libitum food available, which served as baseline. In the cold exposure experiment, cold exposure days were compared with the 2nd day of room temperature acclimation as baseline. Percent suppression of BW from baseline due to leptin was calculated using the following formula: percent suppression = 1 - (experimental/baseline) × 100. Appropriate one- or two-way ANOVA, with repeated measurements where necessary, assessed the statistical difference between mean values (SAS 9.2; SAS Institute, Cary, NC). Tukey's HSD was used for post-hoc comparisons when applicable.

## RESULTS

**Differential leptin responses between POMC-Ptp1b<sup>-/-</sup> and Ptp1b<sup>+/+</sup> control mice.** Leptin significantly reduced food intake in POMC-Ptp1b<sup>-/-</sup> mice on the 2nd day of leptin injections, whereas Ptp1b<sup>+/+</sup> mice showed no change in intake (Fig. 1A). POMC-Ptp1b<sup>-/-</sup> mice also showed a significant reduction in BW following leptin treatment, whereas control mice did not (Fig. 1B). These data support previous findings in these animals of hypersensitivity to low doses of peripherally administered leptin (2). Leptin increased T<sub>C</sub> in the light phase on day 1 of treatment equally between genotypes (Fig. 1C). No effect on SPA in either genotype was noted following administration of this dose of leptin in the light or the dark cycle (Fig. 1D).

**POMC-Ptp1b<sup>-/-</sup> mice show similar changes in FI, BW, and T<sub>C</sub> following cold exposure but greater inhibition of SPA compared with Ptp1b<sup>+/+</sup> controls.** Exposure to cold (4°C) significantly increased food intake in both POMC-Ptp1b<sup>-/-</sup> and Ptp1b<sup>+/+</sup> mice to a similar magnitude across all 4 days (Fig. 2A). Similarly, all mice reduced core body temperature to

a significant extent over the 4-day duration of cold exposure and were able to maintain a reduced core temperature during cold exposure in both light and dark cycles (i.e., no individual mouse was unable to acclimate to the cold; Fig. 2B). Finally, SPA was decreased significantly by cold exposure in both POMC-Ptp1b<sup>-/-</sup> and Ptp1b<sup>+/+</sup> mice during the dark phase; however, alterations in SPA by cold exposure differed by genotype. POMC-Ptp1b<sup>-/-</sup> mice significantly inhibited SPA in the dark cycle beginning on the 1st day of cold exposure and subsequently on days 3 and 4, whereas in Ptp1b<sup>+/+</sup> mice, decreased SPA in the dark cycle was evident only on days 3 and 4 (Fig. 2C). No effects on BW were observed in response to cold on individual exposure days (data not shown).

**Cold exposure increases BAT weight in POMC-Ptp1b<sup>-/-</sup> mice but not in Ptp1b<sup>+/+</sup> controls.** Body weights between age-matched control cohorts of non-cold-exposed POMC-Ptp1b<sup>-/-</sup> and Ptp1b<sup>+/+</sup> mice at room temperature were no different than cold-exposed mice, regardless of genotype (Fig. 3A). Four-day cold exposure significantly reduced WAT weight of both POMC-Ptp1b<sup>-/-</sup> and Ptp1b<sup>+/+</sup> mice (Fig. 3B). In contrast, BAT weight in cold-exposed POMC-Ptp1b<sup>-/-</sup> mice was significantly greater than in cold-exposed Ptp1b<sup>+/+</sup> mice as well as nonexposed POMC-Ptp1b<sup>-/-</sup> mice. No differences in BAT weights were observed between cold-exposed or non-exposed Ptp1b<sup>+/+</sup> mice (Fig. 3C). Further examination of BAT cell parameters in cold-exposed mice showed significant increases in POMC-Ptp1b<sup>-/-</sup> BAT cell area, diameter, and perimeter relative to cold-exposed Ptp1b<sup>+/+</sup> mice (Fig. 3D, right). Results of hematoxylin and eosin staining of BAT illustrate the hypertrophy of BAT cells, as well as diminished presence of lipid droplets, following cold exposure in these mice (Fig. 3D, left).

**Increased cold-induced thyroid hormone levels in POMC-Ptp1b<sup>-/-</sup> vs. Ptp1b<sup>+/+</sup> mice.** mRNA expression of several markers of BAT thermogenesis was shown to be increased following cold exposure in both genotypes; UCP1, UCP2, UCP3, and COX-2 levels in BAT all exhibited enhanced expression compared with room temperature controls in both POMC-Ptp1b<sup>-/-</sup> and Ptp1b<sup>+/+</sup> mice (Fig. 4). UCP2 was significantly higher in POMC-Ptp1b<sup>-/-</sup> vs. Ptp1b<sup>+/+</sup> mice at room temperature. Although not reaching statistical significance, an interaction trend was observed for increased UCP2 levels in cold-exposed POMC-Ptp1b<sup>-/-</sup> mice compared with cold-exposed Ptp1b<sup>+/+</sup> controls (*P* = 0.06).

Plasma levels of active thyroid hormone T<sub>3</sub> were elevated in 4-day cold-exposed POMC-Ptp1b<sup>-/-</sup> mice relative to room temperature POMC-Ptp1b<sup>-/-</sup> mice as well as to cold-exposed Ptp1b<sup>+/+</sup> controls (Fig. 5A). Plasma levels of T<sub>4</sub> were similar between all conditions and genotypes (Fig. 5B). Correspondingly, a trend toward increased BAT D2 activity was detected in cold-exposed POMC-Ptp1b<sup>-/-</sup> mice relative to Ptp1b<sup>+/+</sup> mice (*P* = 0.058; Fig. 5C). Hypothalamic mRNA expression of pro-TRH was upregulated in 4-day cold-exposed mice (Fig. 5D); however, no statistical differences were observed between genotypes. Additionally, GHS-R expression was increased similarly between POMC-Ptp1b<sup>-/-</sup> mice and Ptp1b<sup>+/+</sup> mice following 4-day cold-exposure (Fig. 5E). In 24-h cold-exposed mice, no differences were observed in unacylated plasma ghrelin (POMC-Ptp1b<sup>-/-</sup>: 1,531 ± 133 pg/ml vs. Ptp1b<sup>+/+</sup>: 1,294 ± 140 pg/ml) or acylated ghrelin (POMC-Ptp1b<sup>-/-</sup>: 836 ± 101 pg/ml vs. Ptp1b<sup>+/+</sup>: 887 ± 131 pg/ml). Similarly,

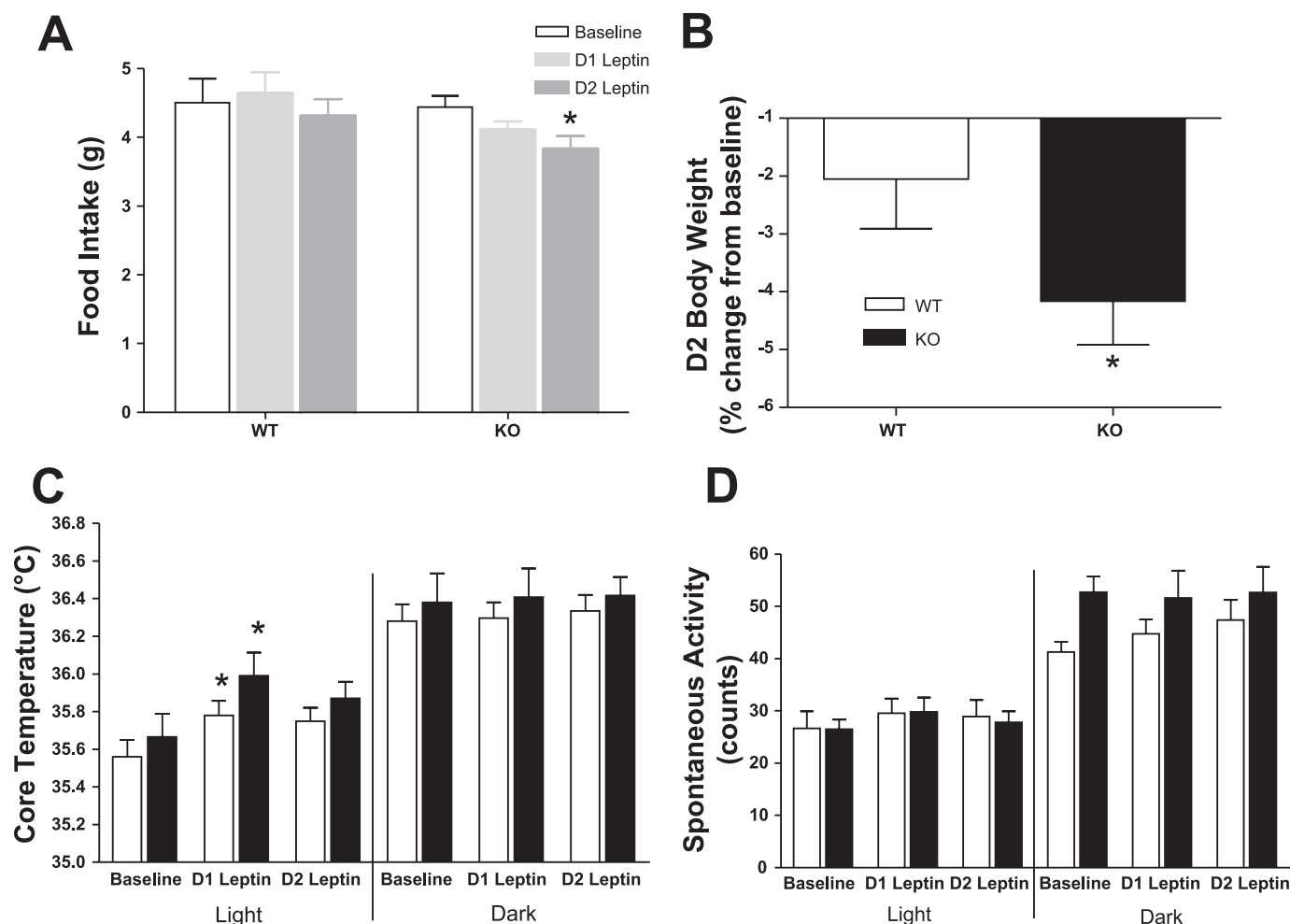


Fig. 1. *A*: leptin significantly reduced food intake in proopiomelanocortin (POMC)-protein tyrosine phosphatase (Ptp1b)<sup>-/-</sup> [knockout (KO)] mice on the 2nd day of leptin injections; however, Ptp1b<sup>+/+</sup> [wild-type (WT)] mice showed no change in intake. *B*: POMC-Ptp1b<sup>-/-</sup> mice showed significant body weight (BW) loss following leptin treatment, whereas control mice did not. *C*: leptin enhanced core temperature (T<sub>c</sub>) in the light phase on *day 1* of treatment equally between genotypes. *D*: no effect on spontaneous physical activity (SPA) was noted. \**P* < 0.05, baseline vs. leptin treatment within genotype.

POMC-Ptp1b<sup>-/-</sup> mice and Ptp1b<sup>+/+</sup> mice did not have differences in the levels of unacylated or acylated plasma ghrelin at room temperature (data not shown).

## DISCUSSION

Overall, our results show that deficiency of PTP1B in POMC neurons contributes to short-term cold-induced reduction of SPA and an upregulation of thyroid activity independent of UCPI-mediated thermoregulation in BAT. If POMC neurons mediate leptin-induced increases in core temperature, either solely or in part, we would expect leptin-hypersensitive PTP1B-deficient mice to show significantly elevated core temperature over controls in response to exogenous leptin. However, POMC-Ptp1b<sup>-/-</sup> mice showed similar leptin-induced increases in core temperature compared with controls despite enhanced leptin-induced suppression of food intake and body weight compared with Ptp1b<sup>+/+</sup> controls, suggesting that Ptp1b in POMC-expressing neurons may not be a critical mediator of leptin's effects on core temperature at low doses. Finally, PTP1B deficiency in POMC neurons resulted in mice with increased BAT weight and T<sub>3</sub> levels following cold exposure, which indicates that POMC-Ptp1b<sup>-/-</sup> mice may need to invoke a

more robust thermogenic response to cold to maintain their body temperature.

Recent data from our laboratory examining PTP1B in POMC cells support a critical role for these neurons in mediating HFD-induced increases in body weight, body temperature, and energy expenditure (2). On a chow diet, POMC-Ptp1b<sup>-/-</sup> mice do not show differences between wild-type controls in adiposity, food intake, or BW. Despite this similarity, chow-maintained POMC-Ptp1b<sup>-/-</sup> mice exhibit hypersensitivity to exogenous leptin, as demonstrated by enhanced suppression of food intake and BW, as well as increased POMC pStat3 activation compared with wild-type mice. When comparing POMC-Ptp1b<sup>-/-</sup> mice to controls in the current data, we again show that exogenous leptin induces greater decreases in food intake and BW; however, we do not see differential effects on leptin-induced core temperature between genotypes. In addition, SPA is not altered by leptin in either genotype. This apparent disconnect between sensitivities of energy intake vs. output parameters in response to leptin injection could indicate that either PTP1B does not regulate leptin's effects on core temperature or POMC neurons are not the primary mediators of leptin's effects on core temperature.

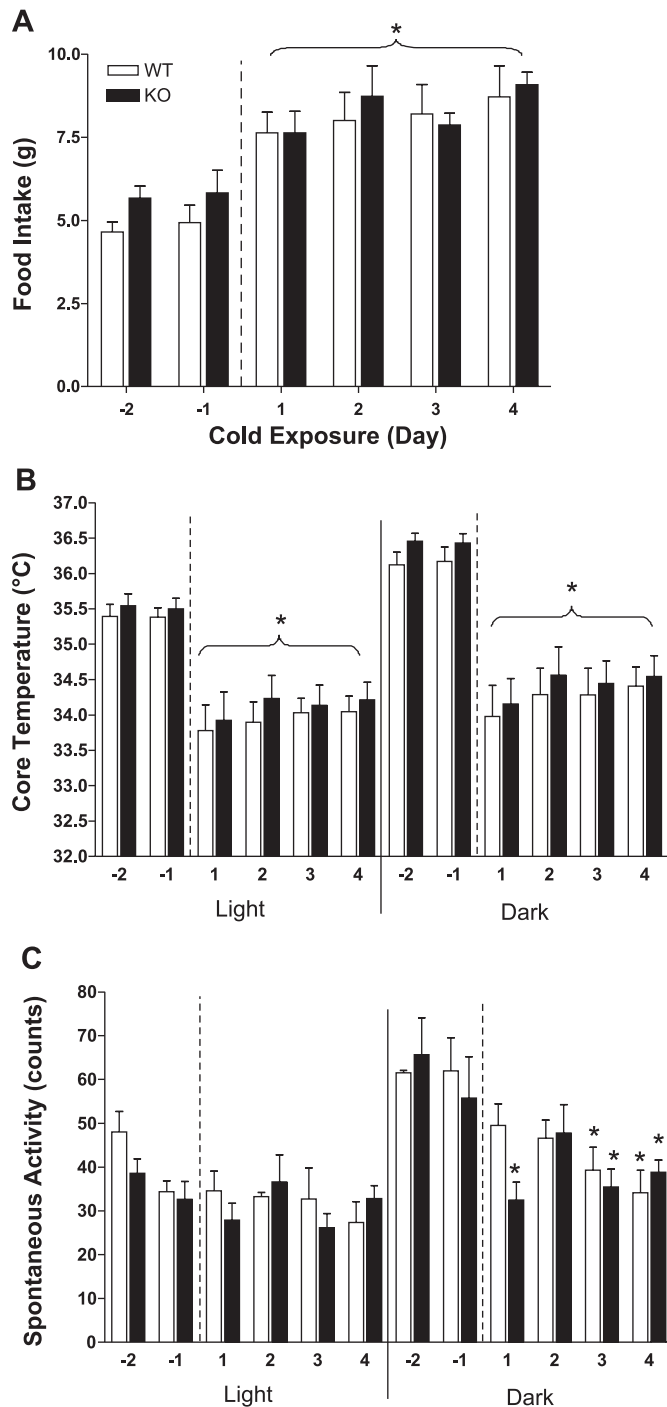


Fig. 2. A: exposure to cold (4°C) significantly increased food intake in both POMC-Ptp1b<sup>-/-</sup> (KO) and Ptp1b<sup>+/+</sup> (WT) mice to a similar magnitude across all 4 days. B: all mice reduced T<sub>C</sub> to a significant degree over the 4-day duration of cold exposure. SPA was decreased significantly by cold exposure in both POMC-Ptp1b<sup>-/-</sup> and Ptp1b<sup>+/+</sup> mice during the dark phase. C: POMC-Ptp1b<sup>-/-</sup> mice exhibited significant inhibition of activity beginning on the 1st day of exposure and also subsequently on days 3 and 4, whereas in Ptp1b<sup>+/+</sup> mice, decreased activity was evident only on days 3 and 4. \*P < 0.05, nonexposed vs. cold exposed within genotype.

For the SPA measurements, it is possible that the dose of leptin used in our study is not sufficient to affect activity. For example, in the *ob/ob* mouse, leptin administered at low doses normalizes glucose homeostasis without changing food intake

or body weight. In contrast, large doses of leptin are required to normalize lower body temperatures and V<sub>O</sub><sub>2</sub> seen in these mice (37).

Recent data suggest a role for POMC neurons in the regulation of body temperature and activity. For example, animals lacking LepR in AgRP and POMC neurons (AGRP + POMC; *Lepr*<sup>-/-</sup> mice) show reduced body temperature as well as locomotor activity (53). Unfortunately, the thermal or activity effects of LepR knockdown only in POMC neurons remain unknown. Additionally, POMC neurons may regulate thermogenic responses of leptin through central glucose sensing (53). We have also reported recently that POMC-Ptp1b<sup>-/-</sup> mice exhibit higher body temperatures when maintained on a HFD (2). Given our current results, we speculate that at low doses of leptin in chow-fed mice, PTP1B in POMC neurons does not affect leptin-induced increases in core temperature; however, in cases of chronically high circulating leptin levels (such as those achieved on a HFD), inactivation of Ptp1b in POMC neurons could result in elevated body temperature via enhanced leptinergic stimulation of POMC neurons.

Leptin-induced increases in locomotor activity are likely regulated in large part through POMC neuron activation. Restoration of LepR in the ARC of LepR-null mice robustly increases voluntary physical activity (12). Furthermore, when LepR signaling in *db/db* mice is restored specifically within POMC neurons, a similar increase in activity is observed (19). However, neither of these studies reported potential alterations in core body temperature as a result of LepR signaling reinstatement, thus providing few clues as to the role of LepR signaling in POMC-induced thermogenesis. The dose of leptin examined here did not affect SPA even in wild-type control animals. It must be noted that, in the studies mentioned above, increases in SPA after restored POMC neuron leptin signaling were observed as the rescue of an activity deficit relative to controls (i.e., baseline activity in *db/db* or LepR-null mice was significantly lower than in normal mice). Since baseline SPA in POMC-Ptp1b<sup>-/-</sup> mice is similar to controls, a failure to increase SPA due to leptin may not be unexpected since there is no inherent deficit in POMC-Ptp1b<sup>-/-</sup> mice requiring “rescue.” (see Fig. 1 or 2).

POMC-Ptp1b<sup>-/-</sup> and Ptp1b<sup>+/+</sup> mice showed similar increases in food intake and decreases in body temperature following cold exposure, suggesting that the POMC-Ptp1b<sup>-/-</sup> mice are not different relative to controls in defending body temperature when cold-stressed for 4 days. In contrast, POMC-Ptp1b<sup>-/-</sup> mice reduce dark-phase physical activity in response to cold significantly earlier than control mice. This observed difference suggests altered behavior to initial cold exposure to acclimate to lower temperatures. Indeed, *ob/ob* mice will die when maintained at 4°C, unless acclimated by successive decreases in environmental temperature (9). It is possible that, in cold-exposed POMC-Ptp1b<sup>-/-</sup> mice, overall core temperature is maintained at a level similar to controls through a relatively heightened increase in thermoregulation response at the expense of spontaneous activity. That is, it may be that the animals show less initial movement because they are more efficiently engaging homeostatic thermogenic mechanisms to maintain core temperature. Consistent with this notion, examination of SPA using average activity in 5-min bins during the first 24 h of cold showed that POMC-Ptp1b<sup>-/-</sup> mice tend to

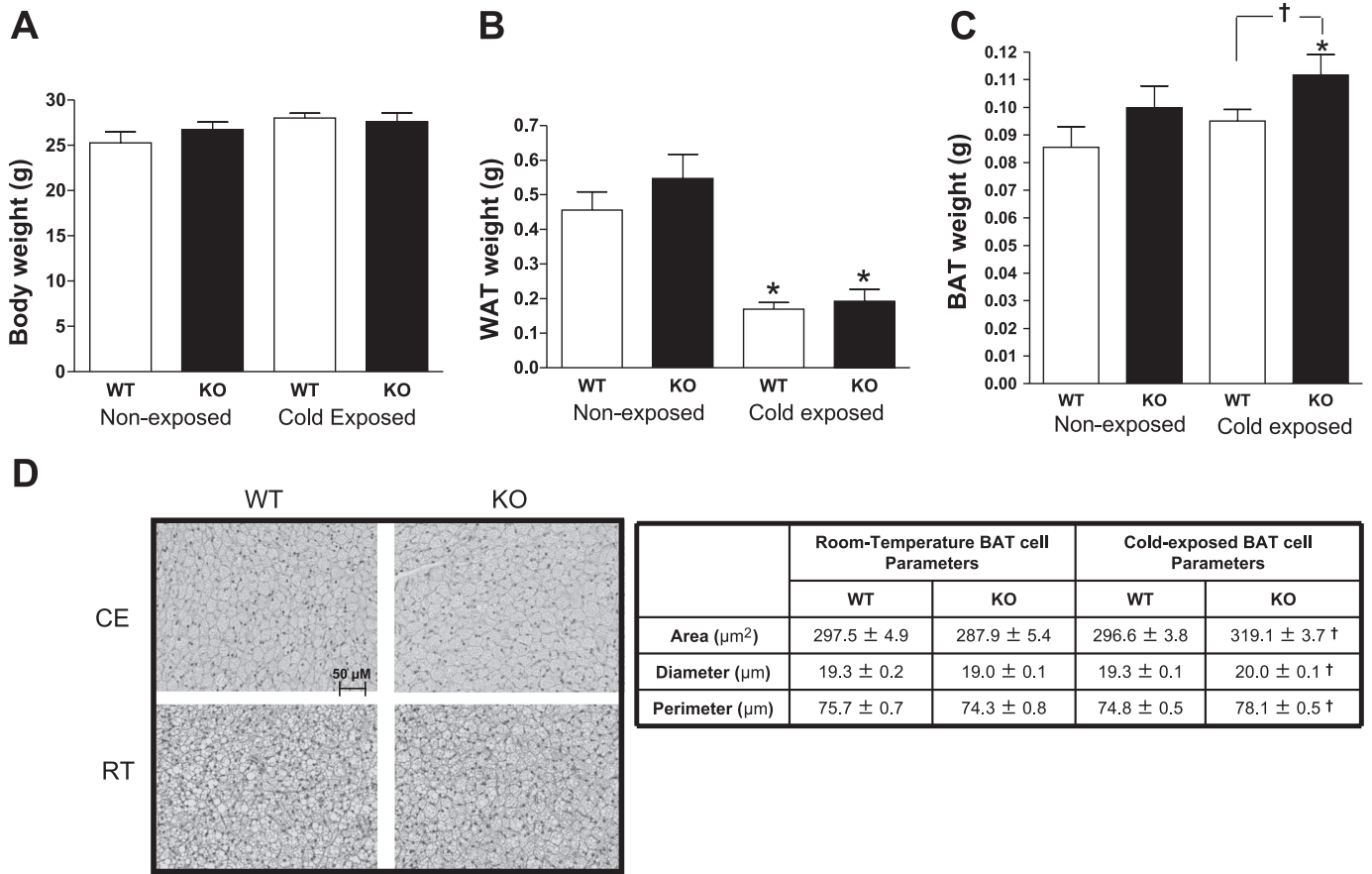


Fig. 3. A: BW between age-matched controls of non-cold-exposed POMC-Ptp1b<sup>-/-</sup> (KO) and Ptp1b<sup>+/+</sup> (WT) mice at room temperature was no different than in cold-exposed mice, regardless of genotype. B: 4-day cold exposure significantly reduced white adipose tissue (WAT) weight of both POMC-Ptp1b<sup>-/-</sup> and Ptp1b<sup>+/+</sup> mice. C: brown adipose tissue (BAT) weight in cold-exposed POMC-Ptp1b<sup>-/-</sup> mice was significantly greater than in nonexposed POMC-Ptp1b<sup>-/-</sup> mice, whereas no such difference was observed in Ptp1b<sup>+/+</sup> mice. D: hemotoxylin and eosin staining of BAT and cold-exposed BAT cell parameters show hypertrophy of BAT cells in POMC-Ptp1b<sup>-/-</sup> mice. \*P < 0.05, nonexposed vs. cold exposed within genotype; †P < 0.05, POMC-Ptp1b<sup>-/-</sup> vs. Ptp1b<sup>+/+</sup> mice.

decrease activity more quickly than in controls within the first few hours of dark (see Fig. 6).

It has been shown previously in the rat that ghrelin administration can cause decreased physical activity (49). However, mouse models of ghrelin/GHS-R deficiency provide conflicting data on the role of ghrelin in locomotor activity, and thus its role remains controversial. For example, ghrelin-null mice are reported to have higher spontaneous activity on HFD (58), whereas another report shows the opposite in GHS-R mice (66). Recent evidence from Komori et al. (24) suggests a potential link between leptin stimulation and the downregulation of hypothalamic GHS-R expression, raising the possibility that GHS-R expression may be lower in leptin hypersensitive

POMC-Ptp1b<sup>-/-</sup> mice compared with Ptp1b<sup>+/+</sup> mice. Here we have shown that hypothalamic GHS-R expression is increased in cold-exposed mice, but there was no effect of genotype. Similarly, no differences in plasma ghrelin were noted between genotypes. Thus, our current data do not suggest a role of ghrelin/GHS-R pathways in the differential reduction of spontaneous activity during cold exposure between POMC-Ptp1b<sup>-/-</sup> and Ptp1b<sup>+/+</sup> mice.

Our findings of increased BAT weight and cell hypertrophy in cold-exposed POMC-Ptp1b<sup>-/-</sup> compared with Ptp1b<sup>+/+</sup> mice suggest altered thermogenic capacity of BAT in POMC-Ptp1b<sup>-/-</sup> mice. It is clear the BAT undergoes hypertrophy in response to cold (16, 61), which signifies increased thermo-

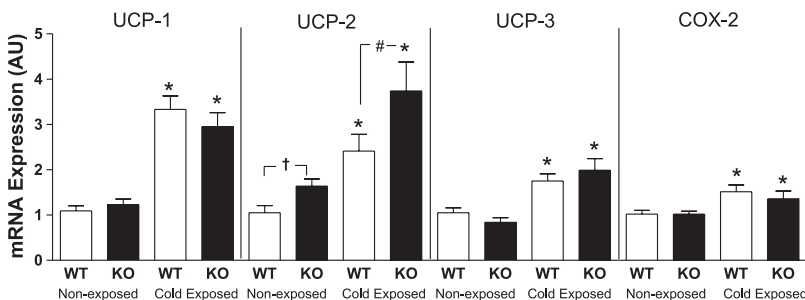
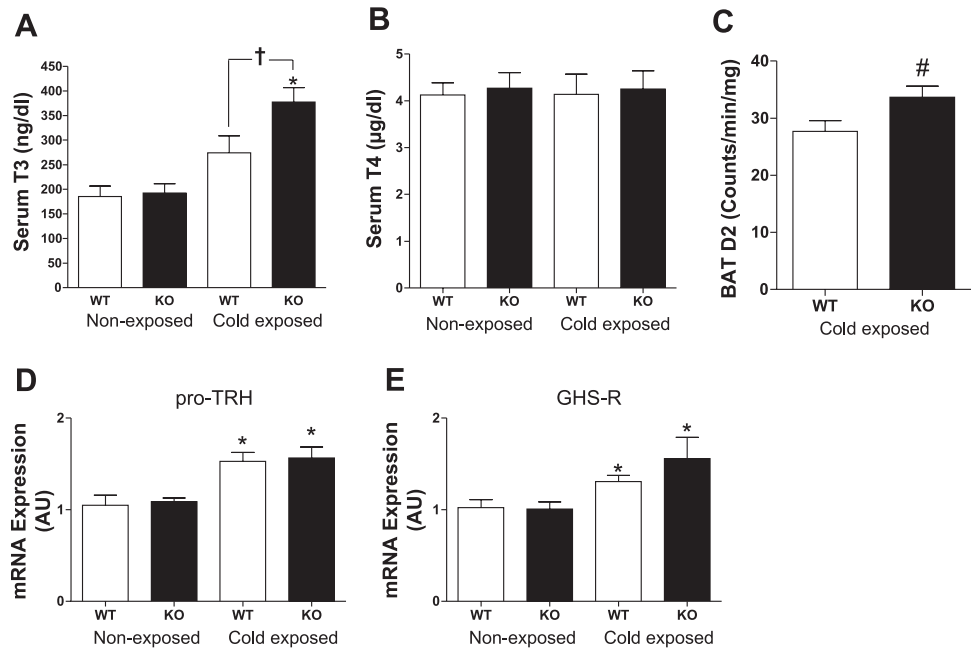


Fig. 4. Uncoupling protein (UCP)1, UCP2, UCP3, and cyclooxygenase (COX-2) mRNA in BAT from cold-exposed tissue exhibited enhanced expression compared with nonexposed BAT in both POMC-Ptp1b<sup>-/-</sup> (KO) and Ptp1b<sup>+/+</sup> (WT) mice. UCP2 levels are elevated in POMC-Ptp1b<sup>-/-</sup> mice compared with Ptp1b<sup>+/+</sup> controls. \*P < 0.05, nonexposed vs. cold exposed within genotype; †P < 0.05; #P = 0.06, POMC-Ptp1b<sup>-/-</sup> vs. Ptp1b<sup>+/+</sup> mice.

Fig. 5. *A*: plasma levels of triiodothyronine ( $T_3$ ) were elevated in cold-exposed POMC-Ptp1b<sup>-/-</sup> (KO) mice relative to POMC-Ptp1b<sup>+/-</sup> mice at room temperature as well as to cold-exposed Ptp1b<sup>+/-</sup> (WT) controls. *B*: plasma levels of thyroxine ( $T_4$ ) were similar between all conditions and genotypes. *C*: correspondingly, a trend toward increased BAT D2 activity was detected in cold-exposed POMC-Ptp1b<sup>-/-</sup> mice relative to Ptp1b<sup>+/-</sup> mice ( $P = 0.06$ ). *D* and *E*: hypothalamic pro-TRH (*D*) and growth hormone secretagogue receptor (GHS-R; *E*) mRNA expression was upregulated following cold exposure similarly between genotypes. \* $P < 0.05$ , nonexposed vs. cold exposed within genotype; † $P < 0.05$ ; # $P = 0.06$ , POMC-Ptp1b<sup>-/-</sup> vs. Ptp1b<sup>+/-</sup> mice.



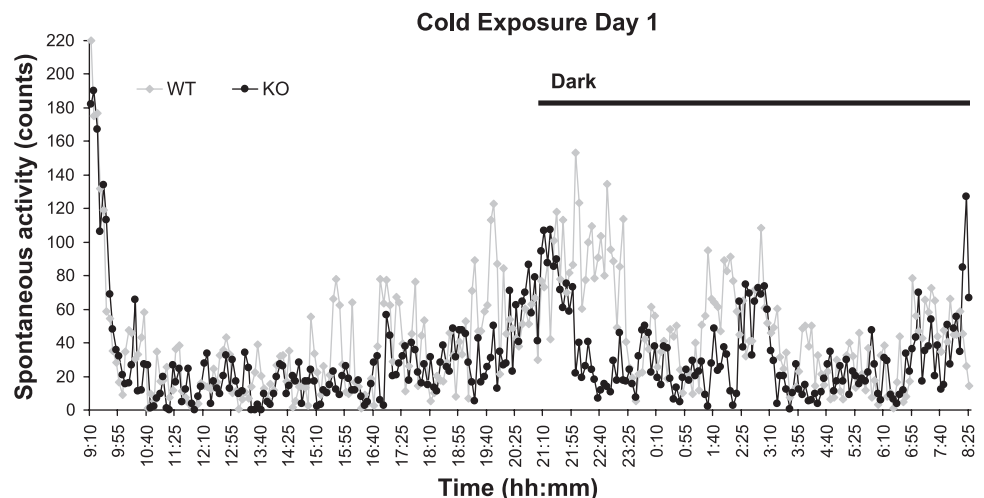
genesis. It is also likely that intact hypothalamic LepR signaling is required for maintaining cold tolerance (30). Both leptin (10, 44) and cold exposure (17, 21) are potent inducers of BAT mitochondrial UCP1, which can largely mediate thermoregulation of body temperature. Our data show no differential induction of BAT UCP1 mRNA expression between cold-exposed POMC-Ptp1b<sup>-/-</sup> compared with Ptp1b<sup>+/-</sup> mice, suggesting that UCP1-independent pathways may play a role in the thermogenic response to cold in POMC-Ptp1b<sup>-/-</sup> mice.

UCP1-independent pathways, induced by either leptin or thyroid hormone injections, can sufficiently rescue body temperature in the absence of BAT UCP1 activation in cold-exposed animals (52). Furthermore, *ob/ob* and double-mutant *ob/ob;UCP1<sup>-/-</sup>* mice show no differences in adiposity, energy expenditure, or obesity phenotypes, suggesting that UCP1-induced thermogenesis in BAT does not play a role in the mediation of leptin effects on energy balance. Presumably, POMC-induced stimulation of BAT thermogenesis occurs following downstream melanocortin activation of sympathetic

projections via MC4R-expressing neurons originating within the forebrain and hindbrain (48, 56, 57). Although we have reported previously that  $\alpha$ -MSH within the ARC and  $\alpha$ -MSH containing projections to the paraventricular nucleus of the hypothalamus (PVH) in POMC-Ptp1b<sup>-/-</sup> mice are similar to controls (2), it is possible that downstream effects such as MC4R activation in the PVH and/or hindbrain may be altered, leading to increased cold-induced activation of sympathetic outflow to BAT in POMC-Ptp1b<sup>-/-</sup> animals. It is also possible that the hypothalamic-pituitary-thyroid axis is upregulated in our mice. Indeed, plasma levels of  $T_3$  were increased in POMC-Ptp1b<sup>-/-</sup> mice compared with Ptp1b<sup>+/-</sup> mice. Furthermore, D2 activation, required for thermogenesis through local BAT  $T_3$  production (13), showed a trend toward increased activity in POMC-Ptp1b<sup>-/-</sup> mice, suggesting a possible upregulation of thyroid hormone action at the level of BAT in these animals.

TRH is released from PVH neurons in response to leptin both directly (via LepR activation) and indirectly (via MC4R

Fig. 6. SPA during the first 24 h of cold exposure. POMC-Ptp1b<sup>-/-</sup> mice ( $n = 6$ ) reduce activity during initial periods of the dark phase relative to Ptp1b<sup>+/-</sup> mice ( $n = 6$ ). Error bars are omitted for clarity.





activation by leptin-induced  $\alpha$ -MSH signaling) as well as through cold-induced adrenergic stimulation via norepinephrine (NE)-containing neurons of the hindbrain (see Ref. 36 for review). The downstream effects of TRH release include increased TSH release from the anterior pituitary and increased circulating thyroid hormone levels. It is possible that enhanced  $T_3$  levels in POMC-Ptp1b<sup>-/-</sup> mice and perhaps local  $T_4$  to  $T_3$  conversion in BAT are a result of increased cold-induced stimulation of the hypothalamic-pituitary-thyroid axis, via increased sensitivity to TRH, through either actions of greater NE stimulation of TRH neurons and/or sensitivity to downstream effects of endogenous leptin. Recent findings have shown that dorsomedial hypothalamic LepR-containing neurons project to BAT via connections through hindbrain nuclei, where a population of thermoregulatory circuits are located, suggesting that hypothalamic LepR activation contributes to thermoregulation in part via the hindbrain (63). Similarly, leptin may interact with TRH in the activation of hindbrain thermogenesis (3, 42, 43) by “priming” hindbrain neuron responses to TRH in the nucleus of the solitary tract. Although the current data show similar increases in hypothalamic mRNA expression of TRH in POMC-Ptp1b<sup>-/-</sup> and -Ptp1b<sup>+/+</sup> mice following cold exposure, it may be possible that comparable cold-induced release of endogenous TRH could produce enhanced thermogenic stimulation in leptin-hypersensitive POMC-Ptp1b<sup>-/-</sup> mice relative to controls. This would support the notion of increased sensitivity of thyroid signals in thermogenesis, the increased conversion of plasma  $T_4$  to active  $T_3$ , and an increase in BAT D2 activity relative to controls. We did not directly assess the absolute requirement of elevated  $T_3$  on the induction of BAT thermogenesis in our model; however,  $T_3$  action within the CNS has been shown to increase BAT thermogenesis via sympathetic  $\beta$ -adrenergic receptors and is essential for regulating many markers of thermogenesis in BAT (28). Therefore, although the net thermoregulatory phenotypes between POMC-Ptp1b<sup>-/-</sup> and Ptp1b<sup>+/+</sup> mice are largely similar in response to cold exposure, POMC-Ptp1b<sup>-/-</sup> mice appear to show an enhanced ability to upregulate sympathetic controls of thermogenesis in BAT via downstream effects of higher  $T_4$ -to- $T_3$  conversion.

Our results also show an increase in UCP2, UCP3, and COX-2 in BAT of all cold-exposed animals, although no significant difference in cold-induced increases was observed between POMC-Ptp1b<sup>-/-</sup> and Ptp1b<sup>+/+</sup> mice. UCP2 mRNA levels were elevated in POMC-Ptp1b<sup>-/-</sup> compared with Ptp1b<sup>+/+</sup> mice, the significance of which is unclear. Whole body deficiency of PTP1B in mice results in increased mRNA expression of BAT UCP1, UCP2, and UCP3 as well as UCP1 protein expression, AMP-activated protein kinase activity, and mitochondrial density (59). Coupled with the current data, it may be that increased UCP2 in whole body PTP1B knockouts could be related to POMC-PTP1B deficiency. Centrally, UCP2 has recently been shown to regulate peripheral glucose homeostasis potentially via modulation of hypothalamic MCH neuron activity (25). The role of UCP2 in BAT per se is less clear; however, acute  $T_3$  injections have been shown to increase UCP2 levels in isolated liver cells and heart tissue (26, 27, 55).

We conclude that whole body and POMC neuron-specific PTP1B-knockout mice exhibit a negative energy balance phenotype in large part through enhanced energy expenditure (2, 23). The current results show that deficiency of PTP1B in

POMC neurons plays a role in short-term cold-induced reduction of SPA and may influence core temperature regulation via enhanced priming of thyroid activity rather than alterations in BAT UCP-induced thermoregulation, which likely contributes to the increased energy expenditure phenotype of POMC-Ptp1b<sup>-/-</sup> mice.

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#### DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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