Hyperphagia in cold-exposed rats is accompanied by decreased plasma leptin but unchanged hypothalamic NPY

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Bing, Chen, Helen M. Frankish, Lucy Pickavance, Qiong Wang, David F. C. Hopkins, Michael J. Stock, and Gareth Williams. Hyperphagia in cold-exposed rats is accompanied by decreased plasma leptin but unchanged hypothalamic NPY. Am. J. Physiol. 274 (Regulatory Integrative Comp. Physiol. 43): R62–R68, 1998.—Chronic cold exposure stimulates sympathetically driven thermogenesis in brown adipose tissue (BAT), resulting in fat mobilization, weight loss, and compensatory hyperphagia. Hypothalamic neuropeptide Y (NPY) neurons are implicated in stimulating food intake in starvation, but may also suppress sympathetic outflow to BAT. This study investigated whether the NPY neurons drive hyperphagia in rats that have lost weight through cold exposure. Rats exposed to 4°C for 21 days weighed 14% less than controls maintained at 22°C (P < 0.001). Food intake increased after 3 days and remained 10% higher thereafter (P < 0.001). Increased BAT activity was confirmed by 64, 96, and 335% increases in uncoupling protein-1 mRNA at 2, 8, and 21 days. Plasma leptin decreased during prolonged cold exposure. Cold-exposed rats showed no significant changes in NPY concentrations in any hypothalamic regions or in hypothalamic NPY mRNA at any time. We conclude that the NPY neurons are not activated during cold exposure. This is in contrast with starvation-induced hyperphagia, but is biologically appropriate since enhanced NPY release would inhibit thermogenesis causing potentially lethal hypothermia. Other neuronal pathways must therefore mediate hyperphagia in chronic cold exposure.

food intake; energy balance; thermogenesis; hypothalamus; neuropeptide Y

Cold exposure activates a series of physiological events to ensure survival, notably increased heat generation that is driven by increased sympathetic activity. In rodents, brown adipose tissue (BAT) is an important site of cold-induced thermogenesis. Catecholamines released from sympathetic endings in BAT act via β3-adrenoceptors to stimulate lipolysis and the expression of uncoupling protein-1 (UCP-1), which uncouples oxidative phosphorylation to produce heat from the oxidation of fatty acids. BAT is activated soon after acute cold exposure, and UCP-1 mRNA levels rise with increasing duration of cold exposure (17, 27). With chronic cold exposure, continuing mobilization of body fat stores results in weight loss and food intake increases by 15–50% after 7–10 days (1, 14, 21).

The neuronal pathways that mediate increased sympathetic activity and compensatory hyperphagia in cold exposure are not known. Neuropeptide Y (NPY)-ergic neurons of the hypothalamic arcuate nucleus (ARC) are thought to regulate autonomic function and energy balance. These neurons project to the paraventricular nucleus (PVN), dorsomedial nucleus (DMN), and medial preoptic area (MPO), all sites that are implicated in feeding and body temperature regulation (2). NPY injected into these sites powerfully stimulates food intake and also inhibits the sympathetic nerves supplying BAT, thereby reducing energy expenditure (8). The ARC NPY neurons become overactive when weight is lost through starvation, insulin-deficient diabetes, or lactation, and are hypothesized to mediate hyperphagia and reduced BAT activity in these conditions (7, 15, 33).

The role of the NPY-ergic ARC neurons in the homeostatic changes of long-term cold exposure has not been studied. We have previously observed that acute cold exposure (2.5 and 18 h) induces significant increases in hypothalamic NPY levels in its sites of release (the PVN and DMN), but with no change in hypothalamic NPY mRNA (6, 17). We have suggested that acute cold exposure blocks NPY release in the PVN and DMN, thus causing the peptide to accumulate in these nuclei, and that this disinhibits the sympathetic activation of BAT. With increased cold exposure, BAT activity would point to reduced activity of the NPY neurons. However, this would be inconsistent with the hyperphagia, as the same NPY neurons are overactive and are thought to stimulate feeding in other conditions of energy deficit such as starvation or diabetes (7, 33).

The main aim of this study was, therefore, to determine how the NPY-ergic ARC neurons respond to the conflicting demands of chronic cold exposure and, specifically, whether they might mediate hyperphagia. Rats were exposed to cold (4°C) for 2, 8, or 21 days, whereas controls were warm-maintained throughout. BAT activity was measured as UCP-1 mRNA levels, and NPY neuronal activity was assessed from NPY levels in the ARC and other key hypothalamic nuclei and from hypothalamic NPY mRNA levels, which show marked increases in food deprivation and diabetes.

We also measured plasma leptin levels to determine whether these might explain changes in food intake, BAT thermogenic capacity, and NPY neuronal activity. Leptin acts centrally to inhibit feeding and stimulate sympathetically driven BAT activity and thermogenesis. These actions may be mediated at least partly by leptin’s inhibition of the ARC NPY neurons, which express the OB-Rb isoform of the leptin receptor (18, 24, 31). However, the thermogenic response to short-term cold exposure is not mediated by leptin, as its expression and plasma concentrations decrease rapidly in cold-exposed rodents, apparently through increased sympathetic activity (29). The role of leptin in chronic cold acclimation has not been investigated.
METHODS

Animals. Adult male Wistar rats (initial body wt 168 ± 16 g) were obtained from the animal unit of St. George’s Hospital Medical School. Rats were housed singly in hanging wire-bottomed cages in temperature-controlled (22°C) and light-controlled (12:12-h light-dark cycle; lights on at 0700) rooms for 3 days before the experiments. Rats were fed standard laboratory chow (Rat Mouse 1 Special Diets Services, Essex, UK) and water ad libitum. Total food consumption and body weight were recorded daily for each animal.

Cold exposure procedure. For each of the three experimental periods, the rats were divided into two weight-matched groups. One group was maintained at 4°C in a cold room, whereas the others were kept at 22°C throughout and served as controls.

The first study used 14 rats maintained at 4°C for 21 days and 14 controls kept at 22°C. This study was also used to define the time course of changes in feeding and weight during prolonged cold exposure and indicated that the second and eighth days of exposure to cold represented the stages of profound hypophagia and fully developed hypophagia, respectively. Fifteen rats were exposed to 4°C for 2 days and 17°C for 8 days, respectively, with the same numbers of weight-matched controls kept at 22°C for the same periods.

In each of the studies, eight rats from each group (cold exposed and controls) were used for measurement of regional hypothalamic NPY levels, and the remainder were used to measure hypothalamic NPY mRNA. Rats were killed within 45–60 s with the use of carbon dioxide inhalation and were immediately exsanguinated by cardiac puncture. Plasma was separated and stored at −40°C for subsequent measurement of leptin, insulin, and corticosterone levels. Interscapular BAT was rapidly dissected out, snap-frozen in liquid nitrogen, and stored at −70°C until measurement of uncoupling protein mRNA levels.

Hypothalamic microdissection. As previously described (32), the brain was quickly removed immediately after the rat was killed and was placed on its dorsal surface for dissection under a binocular microscope. A coronal tissue block containing the hypothalamus was excised from fresh brain and cut into 330- to 500-μm frontal slices using a vibrating microtome. Eight selected hypothalamic areas microdissected were the MPO, lateral preoptic area, anterior hypothalamic area, PVN, ventromedial nucleus, DMN, lateral hypothalamic area, and the ARC together with the median eminence. The tissue from each area in each rat was pooled and boiled for 10 min in 400 μl of 0.1 M HCl and then sonicated for 30 s to extract NPY. The extracts were frozen at −40°C until assayed for NPY and protein concentrations.

For NPY mRNA measurements, a slice of brain tissue was cut between the optic chiasm and the mammillary bodies, and the hypothalamus was removed by a horizontal cut immediately below the anterior commissure and vertical cuts tangential to the edge of the septum and passing through the perihypothalamic sulcus (32). These hypothalamic blocks were snap-frozen and stored at −70°C until measurement of NPY mRNA.

Assays. Plasma leptin levels were determined using a radioimmunoassay (RIA) kit that used mouse leptin as standard (Linco Research). The coefficient of variation within-assay was 4%. Plasma insulin levels were measured using a RIA kit that used human insulin as standard (Pharmacia Diagnostic, Cambridge, UK). The within-assay coefficient of variation was 3.6%. Plasma corticosterone concentrations were measured using a commercial RIA kit (DPL, Gwynedd, UK) with an intra-assay coefficient of variation of 2.3%.

Regional hypothalamic NPY concentrations were measured in 35-μl samples of tissue extract, using an in-house RIA that employed 125I-labeled porcine NPY (pNPY; Amersham International, Amersham, UK) and pNPY as standard (Bachem, Saffron Walden, UK). NPY antiserum (APT 140; Affiniti Research Products, Ilkeston, UK), raised in our laboratory in a rabbit against porcine NPY, was used in a final dilution of 1:36,000. The assay sensitivity of the assay was 1.6 fmol/tube, with an intra-assay coefficient of variation of 3.1%. Samples from each experiment were measured in duplicate in a single assay.

Protein concentrations in hypothalamic extracts were determined by a modified Lowry method, and NPY levels in each region were expressed as femtomoles per microgram protein.

Northern blotting for hypothalamic NPY mRNA. Total hypothalamic mRNA was isolated from frozen hypothalamic tissue blocks using the guanidinium thiocyanate phenol-chloroform method (12). Briefly, each hypothalamic sample was homogenized, using a Polytron tissue homogenizer (Kinematica, Switzerland), in 500 μl guanidinium isothiocyanate extraction buffer including 0.75 M sodium acetate (pH 7.0), 10% sarcosyl, and 0.1 M 2-mercaptoethanol, to which were added sequentially 50 μl 2 M sodium acetate (pH 4.0), 500 μl phenol, and 100 μl chloroform-isooamylalcohol (49:1). The mixture was vortexed and kept on ice for 15 min, followed by centrifugation at 13,000 revolutions per minute (rpm) for 20 min at 4°C. The aqueous phase was removed and precipitated with 500 μl isopropanol overnight at −20°C. Following centrifugation at 13,000 rpm for 20 min at 4°C, the pellet was dissolved in 500 μl extraction buffer and precipitated with 500 μl isopropanol for 1 h at −20°C. This mixture was recovered by centrifugation and rinsed with 75% ethanol, and finally the pellet was dissolved in 0.5% sodium dodecyl sulfate (SDS). The RNA concentration was determined from the absorbance at 260 nm; 20–25 μg RNA was obtained from each hypothalamus.

Twenty micrograms of total RNA from each sample was applied to a 1% agarose-formaldehyde gel and separated by electrophoresis. The RNA was transferred overnight to a nylon membrane (Hybond N; Amersham) by capillary blotting. The RNA was cross-linked under ultraviolet (UV) light for 10 min. Prehybridization was performed at 68°C for 20 min with 4 ml Quikhyb (Strategene, Cambridge, UK) followed by hybridization at 68°C for 1 h with a NPY cDNA probe (0.51 kb, kindly provided by Dr. Steven L. Sabol, Laboratory of Biochemical Genetics, National Institutes of Health) labeled with 32P and 200 μl denatured salmon sperm DNA. Finally, the membrane was washed in a solution of 2× standard sodium citrate (SSC); 1× SSC is 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0, 0.1% SDS at 22°C for 15 min, followed by two further washes at 60°C for 15 min each. Blots were autoradiographed by exposure to X-ray film, and the images were quantified with a scanning densitometer. To exclude nonspecific effects on protein synthesis and to normalize the amounts of RNA loaded, tubulin mRNA was probed at the same time as a reference, using a 32P-labeled cDNA probe provided by Dr. Charles Barker (Molecular Biology Laboratory, Maharishi International University, Fairfield, IA). NPY mRNA levels were expressed as the ratio of NPY/tubulin mRNA signals.

Northern blotting for UCP-1 mRNA. UCP-1 mRNA was measured by a Northern blotting method described previously (28). Briefly, 15 μg of total RNA extracted from interscapular BAT with guanidinium isothiocyanate-phenol was subjected to electrophoresis on a 1% agarose gel. The RNA was then transferred to a charged nylon membrane (Boeh-
Table 1. Metabolic data at 2, 8, and 21 days of cold exposure

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<th>22°C</th>
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<td>17</td>
<td>14</td>
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<td>327±5*</td>
<td>662±11</td>
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<td>257±3</td>
<td>248±2</td>
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<td>200±5</td>
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<td>Final body wt</td>
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<td>14±1</td>
<td>8±1†</td>
<td>66±2</td>
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<td>weight gain/total</td>
<td>19.4±0.5</td>
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<td>21.0±0.6</td>
<td>14.7±0.4†</td>
<td>27.5±0.5</td>
<td>18.1±0.6†</td>
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<tr>
<td>food intake ×100%</td>
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<tr>
<td>Plasma leptin</td>
<td>1.6±0.6</td>
<td>1.3±0.5</td>
<td>2.6±0.8</td>
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<td>Plasma insulin</td>
<td>22±1</td>
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<td>130±20</td>
<td>100±17</td>
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Data are means ± SE. *P < 0.01, †P < 0.001, ‡P < 0.05, vs. respective 22°C group.

...ringer-Mannheim, Mannheim, Germany) by capillary blotting overnight and was fixed with UV light.

Prehybridization was performed at 42°C for 1.5 h in a buffer containing 50% formamide, 5× SSC, 2% blocking reagent (Boehringer Mannheim), 0.1% 3’-lauroylsarcosine, 50 mM sodium phosphate (pH 7.0), and 7% SDS. Hybridization was at 42°C overnight in the prehybridization solution, together with a 32-mer oligonucleotide (25 ng/ml) that was end-labeled (3’ and 5’) with digoxigenin. Posthybridization washes were performed with 2× SSC/0.1% SDS for 15 min at room temperature (twice) and 0.1× SSC/0.1% SDS for 15 min at 48°C (twice). The membrane was then rinsed (3 min) with washing buffer (pH 7.5), containing 0.1 M maleic acid, 0.15 M NaCl, and 0.3% Tween 20. The membrane was blocked by incubation with 1% blocking reagent, 0.1 M maleic acid, and 0.15 M NaCl (pH 7.5) for 30 min at room temperature and then incubated in the blocking buffer with an antibody against digoxigenin (Fab fragment; Boehringer), which was conjugated to alkaline phosphatase, for 30 min at room temperature. After being washed twice for 15 min each with washing buffer, the membrane was immersed in 0.3 M diethanolamine, 0.02% sodium azide, 1.0 mM MgCl₂ (pH 9.5) for 3 min, and sprayed with 0.25 mM chemiluminescence substrate CDP-star (Tropix) for 10 min. Signals were obtained by exposure of the membrane to X-ray film for 1–5 min at room temperature, and the 1.5-kb band corresponding to UCP-1 mRNA was quantified using densitometry. UCP-1 mRNA band densities were normalized by 18S rRNA band densities probed separately with a digoxigenin-labeled oligonucleotide, and the amount of UCP-1 mRNA was expressed as the UCP-1 mRNA/18S rRNA ratio.

Statistical analyses. Unless otherwise indicated, all data are expressed as means ± SE. For NPY concentrations in individual hypothalamic regions, two-way analysis of variance (ANOVA) was performed initially to determine whether there were significant differences between groups, with the intention of proceeding to examine differences in individual nuclei between groups using the unpaired Student's t-test. For food intake, body weight, plasma hormone concentrations, NPY mRNA, and UCP-1 mRNA levels in each experiment, one-way ANOVA was used to compare the differences between groups. A P value of 0.05 or less was considered to be significant. The statistical software package used was ARCUS (Medical Computing, Aughton, UK).

RESULTS

Metabolic data. Chronic cold exposure had marked effects on food intake and body weight during all the study periods (Table 1). Compared with controls, rats acclimated to cold for 3 wk significantly increased their total food intake by 8–10% (P < 0.01). This increase became statistically significant at day 3 and remained consistent thereafter (Fig. 1A). Cold-exposed rats gained weight more slowly than controls, the difference reaching statistical significance from day 7 onward (Fig. 1B). During cold acclimation for 21 days, body weight gain was 71% of that in controls (P < 0.001) and final body weight was 14% lower (P < 0.001). Feed efficiency
(weight gain/total food intake × 100%) was reduced by 34% (P < 0.001) in rats acclimated to cold for 3 wk.

Food intake and body weight gain patterns from rats exposed to cold for 2 days and 8 days were similar to those during the same intervals in the study of 3 wk cold exposure (data not shown).

Plasma leptin was lower in the cold-exposed rats at all three time points than in the respective control groups and showed significant 50–60% reductions (P < 0.01) after 8 and 21 days (Table 1). The relationships between plasma leptin concentrations and body weight in the warm- and cold-exposed groups are shown in Fig. 2. In the warm-maintained rats, plasma leptin concentrations were significantly positively correlated with body weight (P < 0.001). A similar significant relationship was seen in the cold-exposed rats, with no significant divergence from that in the warm-maintained group (P > 0.05).

Plasma insulin levels were significantly lower in all groups of cold-exposed rats than in controls (Table 1). There were no significant changes in plasma corticosterone levels in any of the cold-exposed groups (Table 1).

UCP-1 mRNA. The autoradiograms of Northern blots showed bands corresponding to 1.5 kb for UCP-1 mRNA in BAT. UCP-1 mRNA levels were higher in cold-exposed rats than in controls at all three time points (Fig. 3A). The increase in uncoupling protein mRNA levels rose with increasing duration of cold exposure, being 164% over warm-maintained controls at 2 days (P < 0.01), 196% at 8 days (P < 0.01), and 335% at 21 days (P < 0.01; Fig. 3B).

Hypothalamic NPY and NPY mRNA. NPY concentrations in the eight hypothalamic regions in rats acclimated to cold for 2 days and in their warm-maintained controls are shown in Fig. 4A. Cold exposure did not cause significant effects on NPY levels in any of these hypothalamic regions at any time point.

No significant differences in hypothalamic NPY mRNA levels were found between the control and cold-exposed rats at days 2, 8, or 21 (Fig. 4B).

**DISCUSSION**

Prolonged cold exposure, by increasing BAT thermogenesis and energy expenditure, creates an energy deficit that progressively depletes the body's energy stores; adaptive hyperphagia later supervenes, presumably when fat stores have been reduced to a critical level. This study shows that chronic cold exposure induced large and progressive increases in UCP-1 mRNA expression in interscapular BAT, which parallels other indexes of activity in this tissue (27). We have also confirmed that chronic cold exposure significantly reduces the rate of weight gain and induces a persistent 10% increase in food intake (14, 19, 21). Cold-exposed rats weighed progressively less than controls with increasing duration of cold exposure, finally weighing 14% less after 21 days. Hyperphagia became significant after day 3 of cold exposure, slightly earlier than the onset at 1 wk reported by Leung and Horwitz (14).

Weight loss induced by chronic cold exposure differs in important respects from that occurring in other states of energy deficit, notably starvation and insulin-deficient diabetes or lactation. Hyperphagia in cold-exposed rats is less than the marked increases (100% or more) seen when comparable weight (15–20%) is lost through starvation, diabetes, or lactation (7, 15, 33).

Crucially, BAT activity is inhibited in these other
conditions. These divergences suggest that the hypothalamic regulation of energy homeostasis differs markedly between these various states of energy deficit. In food deprivation, diabetes, and lactation, the hypothalamic NPY neurons are apparently overactive, in that NPY synthesis is increased in the ARC; NPY concentrations are elevated in the ARC, PVN, DMN, and MPO (12, 15, 16, 32); hypothalamic NPY receptors are down-regulated (9), and NPY release within the PVN is increased (13). Given the orexigenic effects of NPY injected into the PVN and DMN, we and others have postulated that overactivity of these neurons mediates the hyperphagia and reduced BAT activity characteristic of these conditions.

In contrast, cold-exposed rats in this study showed no changes in hypothalamic NPY or NPY mRNA, either before or during hyperphagia. Hyperphagia per se may tend to counteract any increases in NPY neuronal activity, as the raised NPY and NPY mRNA levels in food-deprived rats return to normal 1–2 days after refeeding. This possibility could have been tested directly by including an additional cold-exposed group of rats that were not allowed to overeat; for reasons of economy, we were unable to do this. However, the normalization of hypothalamic NPY during refeeding occurs while body weight is rising toward control values and while food intake is declining toward normal; this acute situation is therefore quite different from chronic cold exposure when body weight is maintained at 10–15% below controls and hyperphagia persists. We contend that increased NPY neuronal activity would have been revealed by raised NPY and NPY mRNA levels, especially as NPY mRNA levels rose severalfold after 2 wk food restriction that caused 20% weight loss (7). Cold acclimation-induced hyperphagia therefore seems unlikely to depend on increased hypothalamic NPY neuronal activity. However, this needs to be substantiated by the direct demonstration, e.g., using push-pull sampling (13), that NPY release in the PVN is not increased in hyperphagic, cold-exposed animals. If confirmed, this implies that the increase in ARC NPY neuronal activity, normally triggered by weight loss of this degree, is overridden by cold exposure.

These findings may cast further light on the physiological function of the ARC NPY neurons, which have been suggested to act as a “last-ditch” defense of body fat stores. As well as stimulating feeding, these neurons are apparently involved in regulating the sympathetically mediated stimulation of BAT activity. Administration of NPY intracerebroventricularly or into the PVN suppresses sympathetic outflow to BAT and reduces UCP-1 mRNA expression (5, 8), whereas a negative association has been observed between NPY mRNA and UCP-1 gene expression (10). The NPY-ergic ARC-PVN neurons may therefore act tonically to inhibit BAT thermogenesis. Activation of the ARC-PVN projection would both stimulate feeding and inhibit heat production; these changes would minimize energy losses in starvation and diabetes, but could lead to potentially lethal hypothermia during cold exposure. The failure to activate the ARC NPY neurons in the cold is therefore appropriate and would help to ensure survival.

Several neural pathways could drive hyperphagia in cold exposure and prevent the usual compensatory increase in NPY neuronal activity in response to weight loss. Serotonin injected into the PVN or MPO inhibits feeding and stimulates thermogenesis, and serotonergic neurons may be influenced by NPY (20, 23). Serotonin’s actions may be mediated by corticotropin-releasing factor (CRF), which is expressed by PVN neurons and has similar effects on energy balance (23). CRF has been shown to decrease NPY mRNA levels and to antagonize NPY-induced feeding (3). In cold exposure, increased release of serotonin and/or CRF could therefore stimulate thermogenesis and suppress NPY neurons, and their hypophagic action could explain why the hyperphagia of cold exposure is less marked than that of starvation (200–400%). Other appetite-modifying peptides include galanin and glucagon-like peptide-1 (GLP-1). Like NPY, galanin injected centrally stimulates feeding but also inhibits BAT thermogenesis.
(4) and so would appear unlikely to mediate hyperphagia during chronic cold exposure. GLP-1 decreases feeding, possibly by inhibiting NPY neurons (30), but its central effects on BAT activity are unknown.

We investigated three circulating hormones (insulin, leptin, and corticosterone) that can all enter the hypothalamus and influence the ARC NPY neurons. Insulin and leptin both inhibit feeding and stimulate BAT thermogenesis and may exert these actions partly by inhibiting the ARC NPY neurons that express the OB-Rb leptin receptor mRNA (11, 18, 22, 24, 25). Insulin levels fall in cold-exposed animals, and that leptin expression by fat is also rapidly suppressed following acute cold exposure (29). Leptin and insulin secretion are both inhibited by sympathomimetic agents, and the increased sympathetic activity to adipose tissue and the pancreatic β cells may explain the falls in both hormones in cold exposure. We have shown here that plasma leptin levels remain suppressed during prolonged cold acclimation, but that this was proportional to the lower body weight (and presumably fat mass) compared with warm-maintained controls (see Fig. 2). Theoretically, the decrease in leptin and/or insulin concentrations could contribute to hyperphagia in cold exposure, but in view of their central thermogenic effects, BAT activity would then be predicted to decrease.

Glucocorticoids stimulate feeding and may act to reduce thermogenesis and BAT activity, possibly by inhibiting the hypothalamic CRF neurons or directly stimulating the ARC NPY neurons (26). Corticosterone levels are increased by acute cold exposure (17), but in our study, did not rise during prolonged cold acclimation.

In summary, sustained thermogenesis in chronically cold-exposed rats leads to weight loss and moderate compensatory hyperphagia. In contrast to other catabolic states, there were no increases in hypothalamic NPY or NPY mRNA, suggesting that the ARC NPY neurons are not activated and are therefore unlikely to drive the hyperphagia. This suggests that cold exposure may prevent the NPY-ergic system from being activated by weight loss. This is an appropriate response, as activation of the ARC NPY neurons is in fact an appropriate response, as another hypothalamic action of NPY is to inhibit heat production, which in severe cold exposure could cause fatal hypothermia. This is a striking example of the versatility and interplay between the numerous neural pathways that regulate energy balance precisely in response to the animal’s overall needs.

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