Kinzig KP, Hargrave SL, Tao EE. Central and peripheral effects of chronic food restriction and weight restoration in the rat. Am J Physiol Endocrinol Metab 296: E282–E290, 2009. First published November 18, 2008; doi:10.1152/ajpendo.90523.2008.—Previous studies have demonstrated that some endocrine consequences of long-term caloric restriction persist after weight restoration in human subjects. Here we evaluate effects of chronic food restriction in rats that were restricted to 70% of control kcal for 4 wk and subsequently weight restored. Measures were taken from rats at 80% (chronically restricted; CR), 90% (partially weight restored; PR), 100% (fully weight restored; FR), and after 4 wk at 100% body weight of controls (extended weight restored; ER). Plasma insulin and leptin were decreased, and ghrelin was increased in CR compared with controls. Leptin and ghrelin normalized with weight restoration at PR, FR, and ER; however, baseline insulin was not normalized until the ER state. Hypothalamic mRNA expression levels for proopiomelanocortin (POMC), agouti-related protein (AgRP), and neuropeptide Y (NPY) revealed significantly less POMC mRNA expression in CR and PR rats, and significantly less arcuate NPY mRNA in PR and FR. In the dorsomedial hypothalamus, CR, PR, and FR rats had significantly increased NPY expression that was not normalized until the ER state. In response to a test meal, insulin and ghrelin release patterns were altered through the FR stage, and ghrelin remained affected at ER. Collectively, these data demonstrate that mere weight restoration is not sufficient to normalize hypothalamic gene expression levels and endocrine responses to a meal, and that meal-related ghrelin responses persist despite weight restoration for up to 4 wk.

ghrelin; insulin; leptin; hypothalamus

HYPOTHALAMIC PEPTIDE SIGNALING AND peripheral hormones play important roles in the control of food intake and regulation of body weight. Ideally, these systems work in concert to promote food intake and reduce energy expenditure when energy stores are low, and to inhibit food intake and promote energy expenditure when excess energy is stored. Changes in energy balance, both in cases of deficit and surplus, result in a number of neuroendocrine effects that act to restore energy homeostasis. Whereas many central and peripheral factors have been characterized for animals maintained on high-calorie, high-fat diets, the effects of chronic caloric restriction are less clear. Many studies investigating the effects of caloric restriction with regard to neuroendocrine controls of food intake have been done, but evaluating a single time point after an acute fast or short-term (<2 wk) caloric restriction. Insulin, leptin, and ghrelin provide signals about adiposity to the brain and affect food intake (27–29, 45, 46). The basal hypothalamus integrates these signals and provides outputs critical to the control of ingestive behavior and energy expenditure. Within the arcuate nucleus of the hypothalamus, neuropeptide Y (NPY)/agouti-related protein (AgRP) and proopiomelanocortin (POMC) neurons express leptin receptors (for review, see Ref. 24). Leptin inhibits NPY/AgRP neurons and suppresses expression of these orexigenic neuropeptides (31). Conversely, leptin has been shown to activate POMC neurons, resulting in increased synthesis and release of anorexigenic α-melanocyte-stimulating hormone (α-MSH) (5, 41). Neurons that co-express NPY and AgRP in the arcuate are also influenced by ghrelin. They express the ghrelin receptor, ghrelin administration stimulates expression of NPY and AgRP mRNA, and pharmacological blockade of either NPY or AgRP prevents the ghrelin-induced food intake (6, 8, 17, 25, 32, 42, 48). Collectively, these data demonstrate the involvement of NPY/AgRP and POMC in responding to anorexigenic and orexigenic signals that arise from the periphery to regulate food intake and body weight.

Recent work has demonstrated that human subjects who have undergone long-term caloric restriction associated with anorexia nervosa (AN) display altered endocrine responses to ingestion of a test meal that persist despite weight restoration (18). In the present experiments, we sought to evaluate how chronic food restriction and subsequent weight restoration would affect hypothalamic and peripheral feeding-related systems. Furthermore, we examined the effects of chronic food restriction and partial and full weight restoration on meal-induced insulin and ghrelin secretion. Because we found that many of the effects of chronic caloric restriction were not corrected on weight restoration, we evaluated an additional group of rats that experienced caloric restriction and subsequent weight restoration that was maintained for 4 wk beyond reaching 100% of control body weights.

MATERIALS AND METHODS

Experiment 1

Animals and housing. Male Long-Evans rats (Harlan, Indianapolis, IN) weighing 270.48 ± 0.89 g were maintained at a constant temperature (25°C) on a 12:12-h light-dark cycle in hanging wire cages. All rats had 24-h access to tap water during all portions of the experiment. After 1 wk of acclimation to the laboratory, rats were weight matched and placed into one of four experimental groups: control (n = 6), chronically restricted (CR; n = 7), partially weight restored (PR; n = 7), or fully weight restored (FR; n = 7). Rats in the control group had ad libitum access to rodent chow (Harlan Teklad 2018 18% protein diet) at all times, except where noted. The CR, PR, and FR rats were restricted in the number of calories allowed each day such that they received 70% of the number of calories consumed by the control group in the previous 24 h. Food was presented to CR, PR, and FR rats

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within the hour before the onset of the dark cycle each day. Rats were handled and weighed daily. The Purdue University Animal Care and Use Committee approved all procedures.

CR, PR, and FR rats were restricted to 70% of control intake for 4 wk. At this point, the mean CR body weight was 80% of control body weights, and CR rats were killed. The remaining rats were then pair fed the number of calories consumed by the controls in the previous 24 h. PR rats were killed when the mean body weight was 90% of the mean control body weight, and FR rats were killed when the mean body weight was 100% of that of controls.

Blood and tissue collection. On the day of death, food was removed 12 h before the onset of the dark cycle. Two hours before the dark cycle, rats were deeply anesthetized by exposure to ether and rapidly decapitated. Brains were quickly removed, submerged into ice-sodium pentane for 25 s, and immediately stored in dry ice. Trunk blood was collected into K+EDTA vacutainer tubes, briefly placed in ice, and then centrifuged at 4°C for 15 min at 2,000 rpm. Plasma was aspirated into Eppendorf tubes. Blood and brains were stored at −80°C until processing. Epidydimal fat pads were also removed and weighed.

Radioimmunoassay. Radioimmunoassays (RIAs) (Linco Research) were used to determine baseline levels of plasma insulin, leptin, and ghrelin. The rat insulin RIA kit had a sensitivity of 0.1 ng/ml, the leptin RIA sensitivity was 0.5 ng/ml, and the ghrelin RIA sensitivity was 7.8 pg/ml. Volumes of 100 μl of plasma were used in duplicate samples for each assay, as directed by the manufacturer.

In situ hybridization. Brains were coronally sectioned at 14 μm, mounted onto electrostatically charged Superfrost Plus slides (Fisher Scientific), and stored at −80°C. Brain slices were fixed with 4% paraformaldehyde and dehydrated with an ascending series of alcohols. Sections from each rat containing the arcuate nucleus of the hypothalamus and sections containing the dorsomedial hypothalamus (DMH) were selected and stored at −80°C for future processing. Plasmids of NPY, AgRP, and POMC were linearized with the appropriate restriction enzymes. Antisense riboprobes were labeled with 35S-UTP (PerkinElmer), using in vitro transcription systems with appropriate polymerases (T3, SP6, and T7, respectively), according to protocols provided by the manufacturer (Promega). Probes were then purified using Quick Spin RNA columns (Roche Diagnostics).

For processing, slides were warmed and rinsed in triethylamylamine (TEA) buffer (pH 8.0) and TEA with acetic anhydride. Sections were incubated in hybridization buffer composed of 50% formamide, 0.3 M NaCl, 10 mM Tris-HCl (pH 8.0), 1 nM EDTA (pH 8.0), 1× Denhardt’s solution (Eppendorf), 10% dextran sulfate, 10 mM DTT, 500 μg/ml yeast tRNA, and 35S-UTP and incubated overnight in a 56°C humid chamber. After hybridization, sections were washed three times in 2× SSC followed by one wash in 2× SSC + DTT at 56°C. Slides were then treated with 20 μg/ml RNase A (Sigma) in buffer containing 5 M NaCl, 0.5 M EDTA, 1 M Tris, pH 7.5, and double-distilled H2O. Sections were washed twice in 2× SSC + DTT and then twice in 0.1× SSC + DTT and dehydrated in an ascending series of alcohols. Slides were exposed to Kodak Biomax film for 2 days. Autoradiographic images were then scanned and quantified with Scion Image software (National Institutes of Health), using autoradiographic 14C-labeled microscales (Amersham Pharmacia Biotech) as a standard. Data for each animal were means of the product of hybridization area × density, with the background density subtracted from the three sections, reflecting the region-specific levels of gene expression. Data for each animal were normalized to controls as 100% and are expressed as means ± SE.

Statistical analyses. Differences were first analyzed by one-way ANOVA. Post hoc comparisons were made with Tukey’s honestly significant difference (HSD) tests. Differences were deemed significant when P value was <0.05.

Experiment 2

Animals and dietary maintenance. Sixteen male Long-Evans rats (338.71 ± 14.38 g; Harlan) were maintained on a 12:12-h light-dark cycle at a constant temperature (25°C) in Plexiglas tub cages. All rats had 24-h access to tap water during all portions of the experiment. During an acclimation period of 2 wk, all rats had ad libitum access to the standard laboratory chow described in Experiment 1. After acclimation, rats were weight matched and divided into two groups. The control group (n = 7) had ad libitum access to chow except where noted. The restricted group (n = 9) was given 70% of the number of calories consumed by the controls in the previous 24 h. Rats were maintained in the restricted (CR) state for 4 wk and then trained and tested in a meal testing paradigm as described below.

After the meal test, CR rats were allowed access to the same number of calories as were consumed by controls in the previous 24 h until body weights reached 90% of control body weight. At this time, rats were meal tested (PR). Access to 100% of the number of calories consumed by controls was continued until rats reached 100% of control body weights, at which time a final meal test was conducted (FR). Food intake and body weights were recorded daily.

Surgical procedures. Before experimentation, all rats had catheters implanted into the central branch of the jugular vein. The catheter, constructed of silastic tubing (inner diameter, 0.508 mm; outer diameter, 0.940 mm), was placed 42 mm into the vein, such that the tip minimally entered the heart. The catheter was exteriorized, attached to a bent and blunted 21G needle, and fixed to the top of the skull with dental cement. Catheters were flushed daily, and a lock solution composed of heparin and glycerol was used to maintain patency of the catheter.

Acute meal test. Rats were given access to vanilla-flavored Ensure (American Home Products) through the catheter on three separate training sessions before testing. For training, food and water were removed 3 h before the onset of the dark cycle, and a preweighed bottle of Ensure was placed on the cage when the lights were turned off. Access was allowed for 1 h, after which the bottle was removed, intake was recorded, and food and water were replaced on the cage. For testing, food and water were removed 3 h before the onset of the dark cycle. Blood was drawn via the jugular catheter immediately before rats gained access to Ensure (baseline) and then 15 min, 30 min, 1 h, and 2 h after the first lick. At each time point, 200 μl of blood were collected via a luer-lock syringe attached to the catheter while the rat was freely moving and feeding. The blood was then transferred into sterile vacutainer tubes containing K+EDTA and placed on ice for the remainder of the test. Once all samples were collected, blood was centrifuged at 4°C for 15 min at 2,000 rpm; plasma was transferred into sterile 1.5-ml Eppendorf tubes and stored at −80°C for further processing. The Ensure was weighed at baseline, 15 min after the first lick, and then at 30 min and 1 h, at which time the Ensure was removed. Food and water were replaced after the 2-h blood draw.

RIA. Insulin, ghrelin, and leptin were assayed using commercially available RIA kits (Linco Research) as described for Experiment 1. All samples were processed in duplicate and according to the manufacturer’s instructions.

Statistical analyses. Baseline plasma hormone levels were analyzed by one-way ANOVA. Post hoc comparisons of statistical differences were performed using Tukey’s HSD. Meal-related changes in plasma hormone levels were made by repeated measures ANOVA. Statistical significance was deemed at P < 0.05.

Experiment 3

Animals and analyses. Given persistent differences in hypothalamic and endocrine responses following chronic restriction and weight restoration, an additional set of rats were calorie restricted to 80% body weight of controls, maintained at 80% body weight for 2 wk, and then pair fed to control food intake until weight was restored to 100% for a period of 4 wk (extended weight restored; ER). Sixteen
male Long-Evans rats (Harlan) weighing 302 ± 2.6 g were main-
tained as described in Experiment 1. Before the start of restriction, rats
were weight matched and divided into two groups. Controls (n = 8)
were allowed ad libitum access to food and water, except where
described, and ER rats (n = 8) received 70% of the calories consumed
by controls over the previous 24 h. After ER body weights reached
80% of control body weights, rats were given the same number of
calories per day as were consumed by controls to restore body weight
in a controlled manner. Matched calorie access was continued for 4
wk beyond the point when the ER rats reached 100% body weight of
controls. At this time, all rats were killed, and blood and brains were
collected and processed precisely as described in Experiment 1, such
that plasma insulin, leptin, and ghrelin were analyzed by RIA, and
hypothalamic gene expression levels were quantified for arcuate NPY,
AgRP, POMC, and DMH NPY.

Statistical analyses. Differences were first analyzed by one-way ANOVA. Post hoc comparisons of statistical differences
were performed using Tukey’s HSD. Meal-related changes in plasma
hormone levels were made by repeated measures ANOVA. Statistical
significance was deemed at P < 0.05.

Experiment 4

To evaluate whether the meal-related effects of caloric restriction
persisted beyond attainment of 100% body weight of controls, rats
that were previously restricted, weight restored, and maintained at
100% body weight of controls for 4 wk were meal tested, as described
in Experiment 2. Eight control and eight ER rats with indwelling
jugular catheters were given three 1-h training sessions with Ensure.
For training, food and water were removed 3 h before the onset of the
dark cycle, and a preweighted bottle of Ensure was placed on the cage
when the lights were turned off. Access was allowed for 1 h, after
which the bottle was removed, intake was recorded, and food and
water were replaced on the cage. For testing, food and water were
removed 3 h before the onset of the dark cycle. Blood was drawn via
the jugular catheter immediately before rats gained access to Ensure
(baseline) and then 15 min, 30 min, 1 h, and 2 h after the first lick.
Blood was treated and processed for plasma levels of insulin and
grelin, as described in Experiment 2. Food and water were replaced
after the 2-h blood draw.

Statistical analyses. Baseline plasma hormone levels were analyzed
by one-way ANOVA. Post hoc comparisons of statistical differences
were performed using Tukey’s HSD. Meal-related changes in plasma
hormone levels were made by repeated measures ANOVA. Statistical
significance was deemed at P < 0.05.

Fig. 1. Body weight and fat pad weight during and after chronic caloric
restriction. Rats were allowed access to 70% of the daily kcal consumed by
controls for 4 wk (chronically restricted; CR), which resulted in significantly
lower body weight. A: after 4 wk, rats were pair fed to the amount of kcal
consumed by controls [partially weight restored (PR) and fully weight restored
(FR)] before death. At PR, body weights remained significantly lower than for
controls (P < 0.05). B: epidydimal fat pad weights were significantly lower in
CR and PR compared with controls. At the FR stage of weight restoration,
epidydimal fat weight was restored to that of controls. *P < 0.05.

Fig. 2. Basal insulin, leptin, and ghrelin during and after chronic caloric
restriction. A: baseline plasma insulin levels were significantly lower in all
groups regardless of weight status compared with controls (P < 0.05 in all
cases). B: leptin levels were only significantly lower in CR rats compared with
controls. C: baseline plasma ghrelin levels were significantly elevated in CR
rats compared with controls (P < 0.05 for CR). At the PR and FR stages of
weight restoration, ghrelin levels were not different from controls. *P < 0.05.
RESULTS

Experiment 1: peripheral and central effects of chronic caloric restriction and partial and full weight restoration. As depicted in Fig. 1A, chronic food restriction resulted in reduced body weight compared with controls, and access to food following the restriction period resulted in weight gain to the level of controls. After 4 wk of restriction to 70% of the calories consumed by controls, CR body weights were 81.0% of control body weights ($P < 0.001$). One-third of the CR rats were then killed for the previously described analyses (see MATERIALS AND METHODS). Body weights were restored to baseline plasma insulin levels (Fig. 1A). As depicted in Fig. 2A, baseline plasma insulin levels were significantly lower than in controls in all groups, regardless of weight status ($P < 0.05$ for CR; Fig. 2A). At the PR and FR weight stages, leptin was not different than in control animals. Baseline plasma ghrelin levels were significantly elevated in CR compared with controls (Fig. 2B). At the PR and FR stages of weight restoration, ghrelin levels were not different from controls.

Analysis of hypothalamic gene expression levels revealed unexpected differences in NPY mRNA in the arcuate between control and CR, PR, or FR rats. As depicted in Fig. 3A, arcuate NPY mRNA expression levels were not different from those of controls in CR but were significantly lower in PR and FR (69.4 and 72.3%, respectively; $P < 0.05$ for both cases). Figure 3B demonstrates no differences in AgRP mRNA expression in any groups compared with controls. POMC mRNA expression was significantly lower in CR (67.8%) and PR (47.6%) rats compared with controls ($P < 0.05$ for all comparisons; Fig. 3C).

Chronic caloric restriction also affected NPY mRNA expression levels in the DMH. CR rats had significantly elevated NPY mRNA in the DMH compared with controls (451.6% increase in CR; $P < 0.05$), and this difference persisted in PR and FR rats such that PR rat DMH NPY mRNA expression levels were 399.07% and FR levels were 240.83% of control levels ($P < 0.05$ in both cases; Fig. 3D).

Experiment 2: meal-related plasma insulin and ghrelin after chronic caloric restriction and subsequent weight restoration. Following a period of chronic caloric restriction, rats were allowed to consume Ensure at the start of the dark cycle for 60 min on three separate occasions to accustom them to the procedure. During a fourth access period, intake was measured and blood was sampled. As depicted in Fig. 4, Ensure intake was similar among groups at all time points.

Chronic caloric restriction resulted in altered patterns of plasma insulin in rats that had undergone chronic restriction and weight restoration. In control rats, plasma insulin was significantly increased 30 min after the onset of the test meal ($P < 0.05$). It remained significantly elevated through the 60-min time point and returned to a value that was not different from baseline at the 120-min measurement. As depicted in Fig. 5A, plasma insulin was significantly lower in CR rats compared with controls at baseline, and the difference between CR insulin at baseline and CR insulin at 15 min was significantly different from those in control rats in the CR stage; however, they were significantly reduced in PR (69.4%) and FR (72.3%) compared with control values ($P < 0.05$ in both cases). B: analysis of agouti-related protein (AgRP) mRNA revealed no effect of body weight status. C: there were reductions in proopiomelanocortin (POMC) mRNA levels in CR (67.8%), PR (47.6%), and FR (74.4%). All differences were significantly different from controls ($P < 0.05$ in all cases). D: CR rats had significantly elevated NPY mRNA in the dorsomedial hypothalamus (DMH) compared with controls (451.6% increase in CR; $P < 0.05$). This difference persisted in PR and FR rats, such that PR rat DMH NPY mRNA expression levels were 399.07% and those of FR rats were 240.83% of control levels, $P < 0.05$ compared with controls.
significantly greater \((P < 0.05)\). It remained elevated throughout the testing period such that at the 120-min time point, CR plasma insulin was greater than CR baseline plasma insulin \((P < 0.01)\). Comparison of the area under the curve (AUC) for control compared with CR insulin revealed a significant difference such that the AUC for CR insulin was less than that of control rats \((P < 0.05; \text{Fig. } 5\text{D})\).

The pattern of insulin release in PR rats was similar to that of controls. At baseline, PR insulin was lower than control baseline insulin \((P < 0.05)\), and PR insulin was significantly elevated compared with baseline PR insulin at the 30-min time point. It remained elevated at 60 min and returned to baseline levels at the 120-min measurement (Fig. 5B). Interestingly, the FR insulin values rose more quickly than those of controls or PR rats such that at 15 min, FR insulin was increased compared with FR baseline insulin \((P < 0.05)\). It remained elevated with respect to the baseline FR level for the duration of the test session such that it was significantly elevated at 120 min. FR insulin at 120 min was also significantly higher than control insulin at this time point (Fig. 5C). Despite the prolonged elevation, the AUC for the FR group was not different from that of controls.

Plasma ghrelin levels in response to the test meal were also affected by chronic caloric restriction. Whereas CR rats had significantly elevated ghrelin levels in the baseline condition of Experiment 1 (Fig. 2C), the baseline measure in the meal testing paradigm was significantly lower than that of controls \((P < 0.01)\). Figure 6A depicts the meal-induced changes in plasma ghrelin levels in controls compared with CR rats. Controls displayed the expected meal-related decrease in plasma ghrelin such that by 15 min after the start of the meal, ghrelin levels were significantly reduced compared with baseline. This pattern was not observed in CR rats. There was no preprandial elevation in this group, nor did consuming the meal affect ghrelin at any time point measured. In addition to lower preprandial ghrelin in CR rats, ghrelin levels were significantly lower than those of controls at 15, 30, and 120 min of testing \((P < 0.05 \text{ in all cases})\).

Similarly, PR baseline ghrelin was significantly lower than control ghrelin (Fig. 6B; \(P < 0.01\)). Ingestion of Ensure did not result in any measurable changes from baseline PR ghrelin levels; however, at 60 and 120 min, PR ghrelin levels were significantly greater than those of control rats \((P < 0.05 \text{ in both cases})\). In the FR rats, baseline plasma ghrelin was less than that of controls \((P < 0.05)\), but unlike CR and PR rats, ghrelin levels decreased in FR rats 15 min after the onset of the test meal \((P < 0.05 \text{ compared with FR baseline; Fig. } 6\text{C})\). At this time point, FR ghrelin levels were lower than control levels. The lower FR ghrelin level persisted at the 30-min time point; however, as was the case for the PR rats, FR rats had significantly higher ghrelin levels than controls at 60 and 120 min \((P < 0.05)\).
Experiment 3: peripheral and central effects of prior caloric restriction after extended weight restoration. After 4 wk at the weight-restored state, body weights and epididymal fat pad weights were indistinguishable between controls and ER rats (Table 1). Additionally, baseline levels of plasma insulin, leptin, and ghrelin were not different between groups. Analysis of hypothalamic gene expression levels revealed that, while levels of NPY mRNA in the arcuate remained altered in FR, after 4 wk at the weight-restored state, there were no longer differences between groups (Fig. 7). Similarly, AgRP, POMC, and DMH NPY levels in ER rats were found to be similar to those of controls.

Experiment 4: meal-related plasma insulin and ghrelin after 4 wk at the weight-restored state. As depicted in Fig. 8A, insulin responses to a test meal in ER rats resembled those of controls. Insulin levels rose significantly in both groups compared with baseline levels and remained similar to one another for the duration of the test session.

Figure 8B depicts the ghrelin response to a meal in ER rats. As was the case with FR rats in Experiment 2, plasma ghrelin levels resembled those of controls but were significantly lower at baseline and at 15 min after the start of the meal and higher than control levels at 60 and 120 min.

DISCUSSION

The present data demonstrate that chronic food restriction affects peripheral and central systems involved in the control of food intake and regulation of energy balance, and that weight restoration alone does not fully reverse these effects. Restricted access to food resulted in reduced body weight gain, epididymal fat mass, and leptin and insulin, with significantly greater baseline ghrelin levels than those measured in control animals. Meal-related insulin and ghrelin responses were also affected by chronic caloric restriction, and patterns of insulin and ghrelin release did not fully normalize to those of control animals despite weight restoration.
Levels are also evidenced in the meal feeding paradigm. A finding that after 4 wk at the weight-restored state, baseline levels, plasma insulin remained lower than control insulin despite no difference in body weight, epidydimal fat, or plasma leptin levels. We have previously demonstrated that meal-related insulin secretion (18). It may be that insulin levels are more sensitive to food restriction than leptin and require weight restoration continued to display decreased basal insulin. This effect remained through the FR stage such that, despite no differences in body weight, epidydimal fat, or plasma leptin levels, plasma insulin remained lower than control insulin levels. We have previously demonstrated that meal-related insulin secretion is altered in human subjects with AN, and weight restoration does not fully reverse the effects of AN on meal-related insulin secretion (18). It may be that insulin levels are more sensitive to food restriction than leptin and require more time at a weight-restored state to return to levels comparable to controls. This conclusion is supported by our present finding that after 4 wk at the weight-restored state, baseline insulin levels were no longer suppressed compared with controls.

The effects of chronic caloric restriction on plasma insulin levels are also evidenced in the meal feeding paradigm employed in our present studies. The CR, PR, and FR rats displayed decreased plasma insulin levels at baseline compared with controls. Plasma insulin levels in control rats peaked 30 min after the start of the test meal and returned to baseline by the end of the meal testing session (120 min). In CR rats, plasma insulin increased more rapidly, such that it was elevated compared with CR baseline 15 min after the start of the test meal. Unlike control insulin, CR insulin remained significantly elevated with respect to CR baseline insulin for the duration of the test session. With partial weight restoration, the pattern of insulin release in response to the test meal more closely resembled that of controls; however, while the initial (first 60 min) insulin responses in PR and FR rats were similar, insulin remained elevated in FR rats to 120 min. This difference is attenuated at the ER stage, suggesting that the extended elevation of insulin in response to the test meal at FR was related to the active process of weight restoration that is corrected when weight is maintained for a period of time.

In our present experiments, plasma ghrelin measured 2 h before the onset of the dark cycle was significantly increased in CR rats compared with controls, but not in PR or FR rats. This finding concurs with other demonstrations of a relationship between low body weight and increased plasma ghrelin, such as in cases of underfeeding associated with dietary restriction, AN, and cancer anorexia, among others (see Ref. 43 for review). Given the baseline measures in our study, the meal-related ghrelin responses were unexpected. Control plasma ghrelin was at its highest at the onset of the dark cycle and was significantly decreased 15 min after the start of the liquid test meal. For the rats that had undergone caloric restriction, regardless of weight status at testing, ghrelin levels were significantly lower than for controls at the start of the dark cycle. In CR and PR, ingestion of the test meal did not result in changes in plasma ghrelin levels compared with baseline at any time point. The FR and ER groups demonstrated decreased plasma ghrelin levels 15 min after the start of the meal, compared with baseline, in response to the test meal. Whereas control ghrelin levels remained significantly decreased 120 min after the start of the test meal, they were not different from baseline in any of the experimental groups at this time point. It is possible that the discrepancies in ghrelin measured 2 h before the onset of the dark cycle (Experiments 1 and 3) and those measured at baseline for the meal test (at the onset of the dark cycle; Experiments 2 and 4) are related to the patterns of intake that developed after a history of experiencing chronic caloric restriction. Differences between the anticipation and the presentation of meals have been demonstrated to affect plasma ghrelin levels. Many studies have shown preprandial ghrelin release in rats that are fed ad libitum, as ghrelin peaks just before the end of the light period when they normally eat their largest meal of the day (15, 20, 34, 35, 37, 39, 47). While it is clear that ghrelin secretion is involved in stimulating food intake, ghrelin secretion may be entrained to the timing of habitual meal feeding. In rodents, Drazen et al. (9) demonstrated that both pre- and postprandial ghrelin can be entrained to meal feeding that is independent of whether the rats were fed or fasted. Scheduled access to food resulted in increased ghrelin just before food presentation. Similarly, plasma ghrelin levels peaked in ad libitum rats just before the onset of the dark cycle, when rats generally consume the majority of their food. The preprandial peak in ghrelin was absent in rats that were

![Fig. 8. Plasma insulin and ghrelin in response to a test meal after extended weight restoration. Four weeks at the weight-restored stage normalized insulin, but not ghrelin, responses to a test meal in ER rats. A: baseline plasma insulin levels were similar in controls and ER rats. There were no differences between the 2 groups throughout the testing session. B: ER ghrelin was significantly lower at baseline than in controls. Fifteen minutes after the start of the meal, ghrelin levels in both groups were decreased significantly, and at 60 and 120 min, ER ghrelin was elevated compared with controls (P < 0.05). *P < 0.05 compared with controls.](image-url)
fasted and not on a scheduled meal feeding regimen, suggesting that the ghrelin surge can be learned and precede an expected meal. In our present studies, food was replenished each day 1–2 h before the onset of the dark cycle during the CR, PR, FR, and ER stages. In contrast to rats fed ad libitum, rats that are calorie restricted eat when the food is available regardless of the light cycle. We did not measure patterns of intake in this study, although, based on observation, the majority of food was consumed within the first 2 h of access. Characterization of meal patterns in this paradigm would allow further examination of the relationships between caloric restriction, timing of food intake, and plasma ghrelin levels. Given that differences in ghrelin responses to the test meal persisted in the ER group, future work could address issues pertaining to changes in patterns of intake following experience with chronic caloric restriction.

In the hypothalamus, we measured reduced POMC in CR and PR rats. It is known that elevated POMC is associated with decreased food intake and that leptin activates POMC neurons (4). Therefore, it follows that in CR, reduced levels of circulating leptin in conjuction with low body weight and adiposity would be associated with decreased activity of POMC neurons. Continued suppression of POMC during weight restoration, as seen in the PR stage, may lend to promoting this process. Differences in POMC were attenuated by the time of weight restoration (FR).

Previous studies have demonstrated that acute food deprivation results in increased NPY mRNA expression levels in the arcuate (3, 12). This is also the case in studies in which rats have undergone food restriction for up to 14 days (2). Our results in chronically food-restricted rats are inconsistent with these findings. In the hypothalamus, chronic caloric restriction did not affect arcuate NPY gene expression in CR rats. This was an unanticipated finding, because not only was body weight reduced compared with controls, so were food intake and leptin levels. Given that leptin receptor mRNA is co-expressed with NPY in the arcuate (23), and leptin administration reduces arcuate NPY expression levels (30), we had predicted that NPY mRNA would be increased in the arcuate in CR rats. Compared with previous studies reported by others, one potentially important difference in our study is that rats were food restricted for 28 days. A potential explanation is that perhaps, with prolonged caloric restriction, adaptations occur such that NPY is normalized in CR. More surprising was the finding that arcuate NPY was significantly lower in PR and FR rats compared with controls. It is possible that this is related to the increases in plasma leptin levels during the weight restoration process. While there was no difference in plasma leptin levels between controls and PR/FR rats, there was a significant increase in PR/FR compared with CR. One possibility for the effects on NPY mRNA expression is that, during weight restoration, as plasma leptin levels rise, NPY neurons are responsive to the change in leptin, and that responsiveness requires more than weight restoration (i.e., time) for normalization. Evaluation of sensitivity to leptin, in terms of effects on food intake and NPY mRNA expression, during the weight restoration process may provide insight into whether chronic restriction results in such effects.

A role for the DMH energy balance has long been suggested by studies demonstrating that DMH-lesioned animals develop hypophagia and show reduced body weight (1). Interestingly, paradigms of both an energy deficit (lactation, intense exercise, and chronic food restriction; Refs. 2, 16, 22, and 33) and excess [animal models of obesity, including the lethal yellow Ay, MC-4 receptor knockout, and diet-induced obesity (13, 14, 17a)] demonstrate increases in DMH NPY expression. The multiplicity of challenges to energy homeostasis that influence DMH NPY suggests an important role, the precise nature of which remains unknown. In our present experiments, we demonstrated that NPY in the DMH was elevated in rats at the CR, PR, and FR stages. It was not until rats had been weight restored for 4 wk that levels of NPY resembled those of controls. This finding is consistent with the study of Bi et al. (2), in which rats were restricted to 70% of the calories consumed by control rats for 14 days, and provides further support for a role for DMH NPY in overall regulation of energy homeostasis (2, 40).

In conclusion, the present data indicate that chronic caloric restriction affects multiple factors involved in the control of food intake and regulation of energy balance. While some of these effects are abolished with weight restoration, the persistence of decreased basal plasma insulin and arcuate NPY, increased DMH NPY, and altered insulin and ghrelin responses to a test meal demonstrates that weight restoration alone is insufficient to fully reverse the effects of previous chronic caloric restriction. Indeed, the majority of parameters measured within these experiments were corrected after weight restoration, but only after weight had been restored for an extended period of time.

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