Calorie-Restricted Weight Loss Reverses High-Fat Diet-Induced Ghrelin Resistance, Which Contributes to Rebound Weight Gain in a Ghrelin-Dependent Manner

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Twelve weeks of high-fat diet feeding causes ghrelin resistance in arcuate neuropeptide Y (NPY)/agouti-related protein (AgRP) neurons. In the current study, we investigated whether diet-induced weight loss could restore NPY/AgRP neuronal responsiveness to ghrelin and whether ghrelin mediates rebound weight gain after calorie-restricted (CR) weight loss. Diet-induced obese (DIO) mice were allocated to one of two dietary interventions until they reached the weight of age-matched lean controls. DIO mice received chow diet ad libitum or chow diet with 40% CR. Chow-fed and high-fat–fed mice served as controls. Both dietary interventions normalized body weight, glucose tolerance, and plasma insulin. We show that diet-induced weight loss with CR increases total plasma ghrelin, restores ghrelin sensitivity, and increases hypothalamic NPY and AgRP mRNA expression. We propose that long-term DIO creates a higher body weight set-point and that weight loss induced by CR, as seen in the high-fat CR group, provokes the brain to protect the new higher set-point. This adaptation to weight loss likely contributes to rebound weight gain by increasing peripheral ghrelin concentrations and restoring the function of ghrelin-responsive neuronal populations in the hypothalamic arcuate nucleus. Indeed, we also show that DIO ghrelin-knockout mice exhibit reduced body weight regain after CR weight loss compared with ghrelin wild-type mice, suggesting ghrelin mediates rebound weight gain after CR weight loss. (Endocrinology 154: 709–717, 2013)

Obesity is a major risk factor for many noncommunicable diseases, and as such, obesity-associated disease risk is associated with a significant economic burden. Modeling shows that small reductions in body mass index across the entire population would result in significant reductions in comorbid diseases (1). In humans, increased food intake is a key factor that drives obesity, and numerous studies over the past decade have examined the neurobiological causes of hyperphagia in the attempt to delineate the mechanisms that promote, and maintain obesity. In contrast, however, fewer studies have examined the neuroendocrine mechanisms that control rebound weight gain after diet-induced weight loss. In this study, we examine the neuroendocrine mechanisms regulating rebound weight gain after diet-induced weight loss.

Normally, hormonal and metabolic signals communicate short- and long-term energy stores to the brain, including the hypothalamus and caudal brainstem. These regions integrate and initiate appropriate behavioral and physiological responses. One such peripheral hormone that relays metabolic information from the periphery to the brain is ghrelin. Ghrelin is the only systemic orexigenic hormone and acts via the GH secretagogue receptor 1a...
(GHSR1a) on arcuate (ARC) neuropeptide Y (NPY)/agouti-related peptide (AgRP) neurons in the hypothalamus to increase neuronal activation, action potential firing, mRNA expression, and food intake (2–8). Ghrelin is increased in the plasma upon fasting or longer-term calorie restriction (CR) (9–12) and rises preprandially in humans, suggesting a role in meal initiation (11). Obese humans have an impaired postprandial ghrelin response, particularly after consuming a high-fat meal, and this is thought to contribute to reduced satiety and overeating (13).

We recently reported that diet-induced obesity (DIO) (12 weeks) causes ghrelin resistance in ARC NPY/AgRP neurons (14). This is surprising given that obesity is associated with increased appetitive drive. However, we showed that DIO causes NPY/AgRP neuronal dysfunction, which renders NPY/AgRP neurons unresponsive to ghrelin and other fasting-associated orexigenic stimuli (10, 14). Because obesity is associated with hypothalamic restructuring (15–17) and hormone resistance (18–21), it is important to understand the associated changes with diet-induced weight loss. In the current study, we hypothesized that diet-induced weight loss restores NPY/AgRP neuronal function and reverses ghrelin resistance. To test this, we subjected DIO mice (12 weeks) to 1 of 2 dietary interventions until they reached the weight of age-matched lean controls. The first dietary intervention involved simply switching the mice from a high-fat diet (HFD) to regular chow. The second dietary intervention involved switching the mice from HFD to regular chow with 40% CR (compared with lean controls). We show that diet-induced weight loss with CR restores ghrelin sensitivity and up-regulates the neuroendocrine ghrelin axis. We suspected that this adaptation to weight loss likely contributes to rebound weight gain by increasing peripheral ghrelin and by restoring the ability of NPY/AgRP neurons to respond to ghrelin. As such, we examined rebound weight gain after CR weight loss in ghrelin wild-type (WT) and ghrelin-knockout (KO) mice. DIO ghrelin-KO mice exhibited significantly reduced rebound weight gain after CR weight loss compared with WT controls, showing that ghrelin mediates rebound weight gain diet-induced weight loss.

### Materials and Methods

**Animals**

Male C57BL6 (– 8 weeks old) were group housed (5 per cage) under controlled conditions (20°C with 12-hour light, 12-hour dark cycle) and maintained on either a chow diet (9% fat, 8720610; Barastoc Stockfeeds, Victoria, Australia) or an HFD (23.50% fat, SF04-001; Specialty Feeds, Perth, Western Australia, Australia) ad libitum with free access to tap water for 12 weeks (10). All experiments were conducted in accordance with the Monash University Animal Ethics Committee guidelines.

### Study design

**Study 1: diet-induced weight loss**

Our previous study showed that 12 weeks on an HFD induced ghrelin resistance in NPY/AgRP neurons (14), so we next sought to determine whether this was reversible. In the current study, we placed mice on an HFD for 12 weeks after which 2 groups were placed on a chow diet (HFD-chow [HF-C]). After 5 days for acclimatization, one of these groups was CR to 60% ad libitum (HF-CR). A third group remained on the HFD (high-fat group), and chow-fed mice (chow group) served as controls (Figure 1A). Once the HF-CR group reached the weight of controls, plasma samples were taken, a glucose tolerance test (GTT) was performed, and ghrelin sensitivity was assessed before the mice were killed and the hypothalami were harvested to assess gene expression.

**Study 2: ghrelin deletion prevents rebound weight gain**

Study 1 showed that diet-induced weight loss with CR increased plasma ghrelin and downstream orexigenic signals. In study 2, we placed ghrelin WT or KO mice on a chow or HFD for 12 weeks (n = 14–16), and then mice either continued on chow ad libitum or HFD ad libitum or were placed on a chow CR or HFD CR diet regime (60% CR; n = 6–8). The absolute amount of daily food provided for the 60% CR groups was taken from study 1. CR mice were maintained on the CR diet for 13 days until body weights were indistinguishable from ad libitum chow and HFD-fed mice. At this time, all mice were fed chow diet ad libitum until the end of the experiment, and body weight was monitored for another 9 weeks (64 days) to monitor body weight regain. The approach was designed to model dietary intervention and weight regain in humans, in which subjects try to maintain weight loss after diet restriction simply by eating low-fat food (for a mouse, chow).

**Dual-energy x-ray absorptiometry**

Body composition was measured using a dual-energy x-ray absorptiometry (DEXA) scanner (Lunar PIXImsus2; GE Healthcare, Sydney, New South Wales, Australia). All scans were analyzed using the PIXImsus2 software (version 2.10). The head region is excluded from the analysis.

### Table 1. Nutritional Content of Chow and HFD

<table>
<thead>
<tr>
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<th>Chow diet (Barastock 8720610)</th>
<th>HFD (Specialty Feeds SF04-001)</th>
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<tbody>
<tr>
<td>Protein, %</td>
<td>22</td>
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<tr>
<td>Total fat, %</td>
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<td>AD fiber, %</td>
<td>4.4</td>
<td>5.4</td>
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<tr>
<td>Digestible energy, MJ/kg</td>
<td>13.2</td>
<td>19</td>
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</table>
Glucose tolerance test
For ip GTT, a tail-tip blood sample was taken from fasted mice (5 hours). We used short-term fasting, rather than long-term fasting, because this studies insulin action in a physiological context (22). Blood glucose concentration was immediately measured with ACCU-CHEK Active (Roche Diagnostics GmbH, Tokyo, Japan), after which D-glucose (50% solution) was injected (2g/kg, ip). Additional samples for blood glucose were taken at 15, 30, 60, and 90 minutes after injection.

Food intake experiments
To assess the effect of peripheral ghrelin on feeding behavior, nonfasted animals received an ip injection of acyl-ghrelin (1 mg/kg, SC1357; NeoMPS, Strasbourg, France) or vehicle solution (sterile saline) just prior to the onset of the dark phase (6:00PM). Food intake was measured 5 hours later.

ELISA of acylated ghrelin, total ghrelin, and insulin
Tail blood or trunk blood was collected into EDTA tubes treated with Pefabloc SC (Roche Applied Science, Mannheim, Germany) to achieve a final concentration of 1 mg/ml. Blood was centrifuged, and collected plasma was acidified with HCl (final concentration 0.05N). Acylated ghrelin (EZRGRA-90K; Millipore, Billerica, MA), total ghrelin (EZRGRG-91K; Millipore), and insulin (ultrasensitive mouse insulin ELISA kit 90080; Crystal Chem, Inc, Downers Grove, Illinois) in mouse plasma were measured according to kit instructions.

Real-time PCR
Brains were quickly removed, the hypothalamus was dissected by making an anterior cut at the optic chiasm followed by a posterior cut at the mammillary nuclei, and the hypothalamus was blocked at the lateral margins. The entire hypothalamus was removed including the ARC, ventromedial, dorsomedial, and paraventricular nuclei and flash-frozen in liquid nitrogen. Tissue was stored at −80°C.

Purified RNA was extracted from frozen whole hypothalamic extracts using QIAGEN RNaseasy mini kit (74106; QIAGEN, Doncaster, Victoria, Australia). cDNA was synthesized using the iScript cDNA synthesis kit (170-8890; Bio-Rad Laboratories, Hercules, California). Real-time quantitative PCR was performed using TaqMan Gene Expression Master Mix (Applied Biosystems, Foster City, California) and TaqMan primers for NPY, AgRP, POMC, GHSR, LepR, InsR, and 18S. Amplifications were performed using a Real Plex4 Mastercycler (Eppendorf, Hamburg, Germany).

Statistical analyses
Data are presented as mean ± SEM. Statistical significance was determined by either a Student’s t test or a 2-factor ANOVA (with repeated measures where appropriate) followed by Bonferroni’s post hoc test using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, California). P < .05 was considered statistically significant.

Results
Diet-induced weight loss
Our previous study showed that 12 weeks on an HFD induced ghrelin resistance in NPY/AgRP neurons (14); we next sought to determine whether this was reversible. In the current study, we placed mice on an HFD for 12 weeks, after which HFD-fed mice were heavier than chow-fed mice (Figure 1B). Then, 2 groups were placed on a chow diet (HF-C). After 5 days for acclimatization, one of these
groups was CR to 60% ad libitum (HF-CR). A third group remained on the HFD (high-fat) and chow-fed mice served as controls (Figure 1A). After 15 days, the body weights of HF-C and HF-CR mice were not significantly different from the chow controls, although the HF-C mice were significantly heavier than the HF-CR mice (Figure 1C). The high-fat mice were heavier than all other treatment groups. This was reflected in an increased fat mass (Figure 1E) as measured by DEXA. There was no difference in lean mass between the groups (Figure 1D).

Diet-induced weight loss normalizes glucose tolerance

Once HF-CR mice reached the body weight of the chow group, we assessed glucose tolerance as above. The high-fat group had impaired glucose tolerance compared with the chow controls, as reflected in higher blood glucose levels at 60 and 90 minutes and area under the curve (AUC). The high-fat group had increased AUC compared with HF-CR mice, but not the HF-C mice (Figure 2A). Consistent with previous reports from human studies, diet-induced weight loss normalized glucose tolerance (Figure 2A) (23, 24). Thus, diet-induced weight loss (with and without enforced CR) normalized body weight, fat mass, and glucose tolerance to control levels.

Diet-induced weight loss with CR increases plasma ghrelin

To determine whether diet-induced weight loss normalized the endocrine profile of DIO mice, we measured plasma insulin and ghrelin. In accordance with our previous studies, DIO reduced acylated plasma ghrelin (Figure 2E) (14). HF-C and HF-CR mice had normalized acylated plasma ghrelin compared with high-fat mice. Total plasma ghrelin was higher in HF-CR than in all other treatment groups (Figure 2D). Blood glucose (Figure 2B) and plasma insulin (Figure 2C) was increased in the high-fat group and normalized by diet-induced weight loss.

Diet-induced weight loss with enforced CR increases AgRP and NPY mRNA

We then assessed the effect of diet-induced weight loss on hypothalamic neuropeptide gene expression. DIO suppresses hypothalamic NPY and AgRP mRNA expression (14, 25, 26), and fasting and enforced CR increases the expression of these genes (10, 27). Here, we show that HF-CR mice have increased NPY mRNA compared with chow-fed and high-fat-fed groups and increased AgRP mRNA compared with all groups (Figure 3, A and B). POMC is not different between groups, although there was a trend toward increased POMC expression in the high-fat group (Figure 3C). There were no differences in
the expression of GHSR, the insulin receptor, or the leptin receptor (Figure 3, D–F). Importantly, HF-CR mice have increased hypothalamic NPY and AgRP mRNA expression and increased plasma ghrelin despite having normal body weight compared with controls. This suggests that the state of food restriction (rather than body weight per se) is a key stimulus to increase ghrelin and NPY and AgRP mRNA to restore energy intake and cause rebound weight gain.

Diet-induced weight loss with enforced CR restores ghrelin sensitivity

To determine whether these changes restored ghrelin sensitivity, we assessed ip ghrelin-induced food intake. Interestingly, ghrelin sensitivity was completely restored in HF-CR mice (Figure 4). There appeared to be an improvement in ghrelin sensitivity in HF-C mice, but this was not significant. This is important because neither group differed from the control mice in terms of body weight or glucose tolerance. That enforced CR increased active plasma ghrelin and restored ghrelin sensitivity may have clinical implications for rebound weight gain after dieting.

Ghrelin promotes rebound weight gain after CR weight loss in HFD mice

Study 1 showed that diet-induced weight loss with enforced CR increased plasma ghrelin and downstream orexigenic signals. In study 2, we placed ghrelin WT or ghrelin KO mice on an HFD for 12 weeks, followed by CR to model diet-induced weight loss or ad libitum food intake as controls. After 12 weeks of HFD, both ghrelin WT and KO mice exhibited significant body weight gain relative to chow-fed WT and KO mice (Figure 5A); however, body weight gain was significantly attenuated in ghrelin-KO compared with WT mice, indicating the lack of ghrelin signaling restricts DIO weight gain, as previously described (28, 29). After ghrelin WT and KO Chow-fed and HFD-fed mice were CR for 13 days, mice were placed on Chow ad libitum to monitor body weight regain for 9 weeks after stopping CR. We observed a significant main effect of diet on rebound weight gain in Chow CR and HFD ad libitum-fed mice relative to Chow ad libitum-fed mice (Figure 5D). Moreover, there was greater rebound weight

Figure 3. Dietary interventions with enforced CR increases expression of hypothalamic NPY and AgRP mRNA. A–F, Hypothalamic expression of NPY (A), AgRP (B), POMC (C), GHSR (D), leptin receptor (E), and insulin receptor (F) mRNA (n = 6–9). All data are mean ± SEM. Data are analyzed by 1-way ANOVA with Bonferroni’s post hoc test: *P < .05 vs all other groups and P < .05 between the groups indicated by the line.

Figure 4. Dietary interventions with enforced CR restores ghrelin sensitivity. Food intake was measured in all groups in response to ip ghrelin. Exogenous ghrelin was administered ip (1 mg/kg) at the end (6:00 PM) of the light phase, and food intake was measured over 5 hours (n = 5–10). All data are presented as millijoules of energy consumed per animal. Data are mean ± SEM. Data were analyzed by 1-way ANOVA with Bonferroni’s post hoc test: *P < .05 vs all other groups and P < .05 between the groups indicated by the line.
gain in ghrelin WT mice on HFD then subjected to CR compared with ghrelin-KO mice on HFD then subjected to CR (Figure 5, C and D). These results show that dietary manipulation affects the rate of rebound weight gain and that ghrelin mediates rebound weight gain after CR weight loss in DIO mice.

**Discussion**

In the current study, we show that diet-induced weight loss restores ghrelin sensitivity. We used two paradigms of diet-induced weight loss to separate the effects of diet composition and enforced CR: 1 group of DIO mice was switched to a chow diet (HF-C) and another to a chow diet with 40% CR (60% of chow ad libitum; HF-CR) (Figure 1A). Furthermore, we used ghrelin WT and KO mice to illustrate that ghrelin promotes rebound weight gain after diet-induced weight loss from a DIO state.

Diet-induced weight loss normalized body weights back to control weights in both groups, although HF-CR mice weighed less than HF-C mice (Figure 2C). Interestingly, weight loss with enforced CR had additional effects on the ghrelin system compared with weight loss without enforced CR. First, total ghrelin was increased in HF-CR mice compared with all other groups (Figure 2D). Second, acyl-ghrelin was increased in HF-CR compared with both chow and high-fat mice (Figure 2E). Acyl-ghrelin was decreased in the high-fat group compared with chow controls, consistent with our previous findings (14). In our study, switching a DIO mouse to a chow diet normalized body weight and acyl-ghrelin, although in a similar study by Chandarana et al (30), low levels of acyl-ghrelin per-
sisted after normalization of body weight in humans. In addition, acyl-ghrelin did not increase in formerly DIO mice with enforced CR. These differences may be related to the differences in the experimental design, particularly the use of step-down CR and the lower fat content of the chow diet (30). This supports previous studies that negative energy balance up-regulates the neuroendocrine ghrelin system (9, 12, 31, 32). HF-CR mice also had increased hypothalamic AgRP mRNA expression compared with all other groups as well as increased hypothalamic NPY mRNA expression compared with the chow and high-fat groups (Figure 3, A and B). This is consistent with increased NPY mRNA and AgRP mRNA levels responding to hormonal metabolic cues signaling negative energy (9), because either fasting or CR increases NPY and AgRP gene expression (10, 33–36), whereas DIO suppresses NPY and AgRP gene expression (14, 37, 38) and impairs hypothalamic AgRP circuits innervating the paraventricular nucleus (16).

Taken together, our data suggest that DIO mice subjected to diet-induced weight loss with enforced CR exhibit signals of acute negative energy balance such as increased plasma ghrelin and increased NPY and AgRP gene expression. These markers of negative energy balance in HF-CR mice exist even though body weight does not differ from chow controls, although we acknowledge there is a significant difference in body weight between HF-C and HF-CR groups. This indicates that the acute rate of change in body weight is a stronger predictor of the perception of negative energy rather than long-term absolute body weight. This supports the set-point theory of energy homeostasis, whereby body weight is regulated at a level by a feedback control mechanism (reviewed in Ref. 39). However, the body weight set-point appears malleable and adjustable over the long term and does not represent the immediate metabolic needs of the animal. For example, our studies suggest that 12 weeks of HFD feeding significantly increases the body weight set-point, as diet-induced weight loss in the HF-CR drives mechanisms that signal and sense negative energy balance (ghrelin, NPY, and AgRP), despite sufficient metabolic stores. The body strongly defends the set-point against weight loss by promoting weight regain, but the defense against overfeeding is less robust because it does not produce a short-to-medium-term threat to survival. We postulate that the rate of diet-induced weight loss predicts a state of negative energy balance, because HF-CR mice are significantly lower in body weight compared with HF-C. Thus, HF-CR provokes a compensatory increase in orexigenic signals at the level of the ARC.

Total and acyl-ghrelin are increased by diet-induced weight loss with enforced CR, and this is associated with a restoration of ghrelin sensitivity, and consequent increases in NPY and AgRP mRNA. It is interesting to note that although there was an improvement in ghrelin sensitivity in the HF-C group, ghrelin did not significantly increase food intake in these mice (Figure 4). Others have also shown that ghrelin sensitivity is fully restored by switching DIO mice to a chow diet (58). This difference may be methodological; as in the Perreault study, ip ghrelin sensitivity was assessed during the light phase, when we have shown DIO mice are only partially resistant to peripheral ghrelin (14). Regardless, this further supports our hypothesis that energy homeostasis is affected by signals of negative energy balance rather than body weight per se.

Studies from KO mice have suggested a role for ghrelin in glucose homeostasis. Under severe, chronic CR, mice lacking components of the ghrelin system are unable to maintain euglycemia (32, 40). Thus, ghrelin increases blood glucose. This had led to speculation that ghrelin plays a pathogenic role in the development of diabetes. However, HF-CR significantly improves glucose tolerance relative to HF mice, despite an increase in plasma ghrelin, indicating that the precise role of ghrelin in glucose metabolism is strongly affected by metabolic status (41) and requires further research.

In the current study, diet-induced weight loss in both groups normalized glucose tolerance, blood glucose, and plasma insulin (Figure 2). Thus, normalization of plasma insulin and glucose homeostasis to chow control levels alone is not sufficient to completely restore ghrelin sensitivity. It is worth noting that although not significant, insulin is lower in the HF-CR group than in the HF-C group, because this likely contributes to restored ghrelin sensitivity in this group (Figure 2C). Insulin infusion suppresses ghrelin secretion (42, 43), and obesity-associated hyperinsulinemia may contribute to the impaired postprandial ghrelin response in obese individuals (13).

The restoration of ghrelin sensitivity in HF-CR mice likely contributes to rebound feeding and weight regain commonly seen in human dieters. Most studies show that diet-induced weight loss in humans increases plasma ghrelin, and the amount of weight lost positively correlates with plasma ghrelin (44–47). Indeed, we show for the first time that ghrelin plays an important and direct role in rebound weight gain, because ghrelin-KO mice show significantly reduced rebound weight gain compared with ghrelin WT mice. Our results strongly support the human studies, described above, that suggest a role for plasma ghrelin in rebound weight gain. Furthermore, our findings that ghrelin-KO mice show attenuated weight gain on an HFD or after CR from a DIO state (Figure 5) are supported by previous studies showing the deletion of ghrelin or its receptor restricts weight gain on an HFD (28, 29), al-
though not all studies show this effect (40). This function of ghrelin is likely an adaptive response to limit weight loss in an attempt to defend the set-point, because weight loss is also associated with decreased energy expenditure and increased hunger (47). Again, animal studies show that the ghrelin system suppresses locomotor activity and energy expenditure (48) as well as preserving fat stores by promoting carbohydrate metabolism (49) to increase body weight and energy reserves. Intriguingly, ghrelin-KO mice fed a chow diet showed equivalent body weight gain to chow-fed WT mice after CR (Figure 5D), indicating that ghrelin mediates rebound weight gain only after HFD exposure. It is likely that HFD exposure affected hypothalamic circuits regulating appetite, and this impacted rebound weight gain after CR weight loss. Indeed, HFD is well described to suppress NPY and AgRP gene expression (10, 37, 38) compared with chow-fed mice. Therefore, after CR-induced weight loss, we speculate that NPY/AgRP neurons, previously exposed to HFD, remain in a low-activity state in the absence of ghrelin to signal increased activity. Although this is a potential explanation as to why ghrelin mediates rebound weight gain only after DIO, this requires experimental examination.

Based on the results of the current study, we propose that antagonism of the ghrelin system immediately after diet-induced weight loss may provide protection from rebound weight gain. Indeed, inhibition of the ghrelin system by pharmacological antagonism of ghrelin O-acyltransferase (50), an acyl-ghrelin–specific neutralizing antibody (51), GHTR antagonism (52), or ghrelin vaccination (53) reduces acute weight gain in normal mice. However, this approach may have negative side effects on numerous ghrelin-regulated behaviors, such as stress, anxiety, neuroprotection, learning, memory, and motivation (31, 54–57).

In conclusion, we show that CR weight loss after DIO restores the ability of ghrelin to induce food intake, indicating a reversal of DIO ghrelin resistance with diet-induced weight loss. Furthermore, CR weight loss induces markers of negative energy balance, such as increased plasma ghrelin and NPY and AgRP gene expression, despite no differences in body weight relative chow ad libitum-fed control mice. Importantly, ghrelin-KO mice exhibit attenuated rebound weight gain after CR weight loss, illustrating that ghrelin is a key driver of rebound weight. We suggest long-term DIO changes the body weight set-point, and as the body interprets CR weight loss as negative energy balance, ghrelin fights to defend this higher body weight. This represents a novel target to restrict rebound weight gain in humans.

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