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Circulating Ghrelin Levels and Central Ghrelin Receptor Expression are Elevated in Response to Food Deprivation in a Seasonal Mammal *(Phodopus sungorus)*

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Key words: energy balance, photoperiod, seasonal body weight regulation, leptin.

Abstract

Ghrelin is an endogenous ligand for the growth hormone secretagogue receptor (GHSR). However, the functional interaction of ligand and receptor is not very well understood. We demonstrate that GHSR mRNA is up-regulated after food deprivation (48 h) in the hypothalamic arcuate nucleus and ventromedial nucleus of the seasonal Siberian hamster, *Phodopus sungorus*. This increase is accompanied by a two-fold elevation of circulating ghrelin concentration. Chronic changes in feeding state imposed by food restriction over a period of 12 weeks during long day-length induced increased GHSR gene expression, whereas food restriction for 6 weeks had no effect. *Phodopus sungorus* reveals remarkable seasonal changes in body weight, fat mass and circulating leptin levels. Ghrelin is generally regarded as having opposing effects on appetite and body weight with respect to those exhibited by leptin. However, our study revealed that seasonal adaptations were not accompanied by changes in either GHSR gene expression or circulating ghrelin concentration. Therefore, we suggest that ghrelin only plays a minor role in modulating long-term seasonal body weight cycles. Our findings imply that ghrelin predominantly acts as a short-term regulator of feeding.

Ghrelin, a 28-amino-acid gut peptide, has been identified as an endogenous ligand of the growth hormone secretagogue receptor (GHSR) and shown to stimulate growth hormone (GH) secretion (1, 2). However, accumulating evidence suggests that its major physiological role may be related to the regulation of energy homeostasis (3–5). Ghrelin is produced by the stomach and circulating plasma ghrelin concentrations are dynamically related to feeding state (6, 7). Thus, in man, it has been demonstrated that circulating ghrelin levels are decreased in chronic (obesity) and acute (feeding) states of positive energy balance. By contrast, plasma ghrelin levels are increased by fasting and in patients with anorexia nervosa (7–10). Furthermore, peripheral and central (intracerebroventricular) ghrelin administration in mice and rats caused weight gain by either reducing fat utilization or by a dose-dependent increase in food intake (5). Ghrelin modifies energy homeostasis independent of its GH-releasing activity, as demonstrated by studies performed in GH-deficient rats (4). Ghrelin undergoes post-translational processing where the hydroxyl group of one of its serine residues is acylated by *n*-octanoic acid (1, 11). Acylation of this peptide is regarded to be essential for its endocrine actions because it facilitates transport across the blood–brain barrier and is essential for binding to GHSR (12–14).

GHSR was originally cloned in 1996 from the pituitary of several species, including humans and the rat (15, 16). The name GHSR derived from a class of synthetic molecules, the growth hormone secretagogues (GHSs) which represent the first identified ligands of this receptor. GHSR is a G-protein-coupled receptor (15) and is encoded by a single gene across different species (17). In the rat, central GHSR mRNA expression is confined to the hypothalamus and the pituitary gland (18).

Recently, a link between feeding status and GHSR mRNA expression was demonstrated. Total hypothalamic mRNA of GHSR was increased after food deprivation (48 h) in the rat (19). However, whether the anatomical localization of mRNA changes according to feeding status within the hypothalamus is unknown. It may be important to establish whether feeding-induced mRNA changes occur in distinct hypothalamic nuclei that are important centres in the modulation of body weight.

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In the present study, we localized GHSR mRNA within the hypothalamus via *in situ* hybridization and investigated changes in the expression profile of GHSR mRNA within distinct hypothalamic nuclei. Moreover, we determined serum levels of total circulating ghrelin. We performed our studies in the seasonal Siberian hamster (*Phodopus sungorus*, also known as Djungarian hamster), which represents a unique model for the investigation of energy homeostasis. *Phodopus sungorus* reveals a remarkable natural body weight cycle determined by the prevailing photoperiod and reflected in changes of circulating leptin levels (20–22). Short day exposure (SD), either as a gradual change (natural conditions) or as an abrupt change (laboratory conditions), leads to a progressive reduction in body weight. This animal model allowed us to investigate the functional role of circulating ghrelin and its centrally expressed receptor in relation to chronically changed body weight induced by photoperiod. Effects of food deprivation on the interplay of this feeding related gut hormone and its receptor were analysed and, beyond that, the impact of food restriction and of the anorexigenic cytokine leptin on GHSR gene expression was studied, both in animals with a naturally high body weight and in animals that are reaching their body weight nadir induced by SD exposure.

Materials and methods

Animals

Procedures involving animals were licensed under the Animals (Scientific Procedures) Act of 1986 and received approval from the Ethical Review Committee at the Rowett Research Institute. All experimental animals were drawn from the Rowett breeding colony of Siberian hamsters (23–25), and were gestated and suckled in long day (LD) 16 : 8 h light/dark cycle. All hamsters were weaned at 3 weeks of age, and were individually housed either at weaning or, in the case of adult animals, at least 2 weeks before food deprivation. Where specified, hamsters were maintained from weaning in a short day (SD) 8 : 16 h light/dark cycle, but with all other environmental conditions unaltered. Food (Labsure pelleted diet; Special Diet Services, Witham, Essex, UK) and water were available *ad libitum* unless specified, and rooms were maintained at 22 °C. All animals were killed by cervical dislocation in the middle of the light phase, trunk blood was collected and brains were rapidly removed and frozen on dry ice.

Experimental procedure

To investigate acute changes in GHSR mRNA expression induced by food deprivation (48 h), archived brain sections were used from juvenile female LD hamsters weaned in LD and then held in LD (n = 12) or SD (n = 12) photoperiods. Eight weeks after weaning, half of the animals (n = 6) in each photoperiod were deprived of food while the remainder continued to feed *ad libitum* (26).

Chronic changes in GHSR gene expression following food restriction for 6 and 12 weeks, respectively, were examined by analysing archived brain sections of three groups of juvenile female hamsters. One group was maintained in LD (LD-ADLIB), the second was transferred to SD (SD-ADLIB) and the third group was also maintained in LD (LD-REST) but was food restricted so that the body weight trajectory was matched with that of the SD group (26).

In another experiment, the effect of leptin on GHSR gene expression was investigated. Archived brain sections (26) of juvenile female hamsters which had received a single intraperitoneal leptin injection 15, 30, 60 or 120 min before cervical dislocation were analysed. Control groups were injected with vehicle (26).

Serum ghrelin concentration was investigated in a second food deprivation experiment, which was carried out exactly as above with a new group of hamsters (n = 24). This repeated study was performed due to insufficient blood sampled from the first set of animals. To substantiate the results obtained from juvenile female hamsters, we also determined serum ghrelin concentration in adult male hamsters. Twenty adult male LD hamsters, aged 5–6 months, were divided into two groups, one of which was deprived of food for 48 h whereas the other group continued to feed *ad libitum*.

Radioimmunoassay

Serum concentrations of total immunoreactive ghrelin were measured using the commercially available radioimmunoassay kit from Phoenix Pharmaceuticals, Inc. (catalogue no. RK 031-31, Belmont, CA, USA).

Hypothalamic gene expression

Messenger RNA levels were quantified by *in situ* hybridization in 20-μm coronal hypothalamic sections, using techniques previously described in detail (24). A riboprobe complementary to GHSR (type 1a and b) was generated from cloned cDNA from the hypothalamus of rat. cDNA synthesis was performed by reverse transcription (cDNA synthesis Kit, Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. The 449-bp (Genebank NM032075) fragment of rat GHSR was amplified by polymerase chain reaction (PCR) with 35 cycles of 94 °C for 30 s, 60 °C for 30 s and 68 °C for 1 min and finally one cycle at 72 °C for 10 min. For the amplification, the primers 5′-GCGCTCTTCGTGGTGGGCATCT-3′ and 5′-GTGGCGCGGCATTCGTTGGT-3′ were used. The DNA fragments were ligated into PCR-script Amp cloning vector (Stratagene, Basingstoke, UK) and transformed into JM 109 cells (Promega, Southampton, UK). Automated sequencing was performed to verify the sequence of interest.

As previously described (24), 20-μm forebrain sections were collected throughout the extent of the arcuate nucleus and the caudal part of the ventromedial nucleus (VMH), to which GHSR gene expression is confined, onto a set of eight slides with six or seven sections mounted on each slide. Accordingly, slides spanned the hypothalamic region approximating from −2.7 mm to −1.25 mm relative to Bregma according to the atlas of the mouse brain (27). One slide from each animal was hybridized. Briefly, slides were fixed, acetylated, and hybridized overnight at 58 °C using [^{35}S]-labelled cRNA probes ($1–2 \times 10^7$ c.p.m./ml). Slides were treated with RNase A, desalted, with a final high stringency wash (30 min) in 0.1 × SSC at 60 °C, dried and apposed to Kodak Biomax MR Film (Kodak, Rochester, NY, USA). Autoradiographic images were quantified using the Image-Pro Plus system. Equivalent sections of individual animals were matched according to the atlas of the mouse brain. Four sections from the arcuate nucleus and 3 sections from VMH of each animal spanning from −2.54 mm to 0.94 mm relative to Bregma were analysed. Integrated optical densities were calculated using a standard curve generated from ^{14}C autoradiographic microscales (Amersham Pharmacia Biotech, UK Ltd, Bucks, UK).

Statistical analysis

Data were analysed by one- or two-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls multiple comparison test, as appropriate, using SigmaStat statistical software (Jandel Corp., Erkrath, Germany). Where data failed normality tests, they were analysed by one-way ANOVA on ranks followed by Dunn's multiple comparison test. Data are presented as mean ± SEM. $P < 0.05$ was considered to be statistically significant.

Results

Localization of GHSR mRNA and protein in the hamster hypothalamus

The riboprobe complementary to rat GHSR mRNA hybridized within the hypothalamus of the Siberian hamster to the arcuate nucleus and the VMH (Fig. 1), as well as to the paraventricular (PVN) and suprachiasmatic nucleus (SCN) (data not shown). Differential gene expression was not observed in the PVN or SCN in any of the experiments reported below. A sense probe synthesized from the cloned

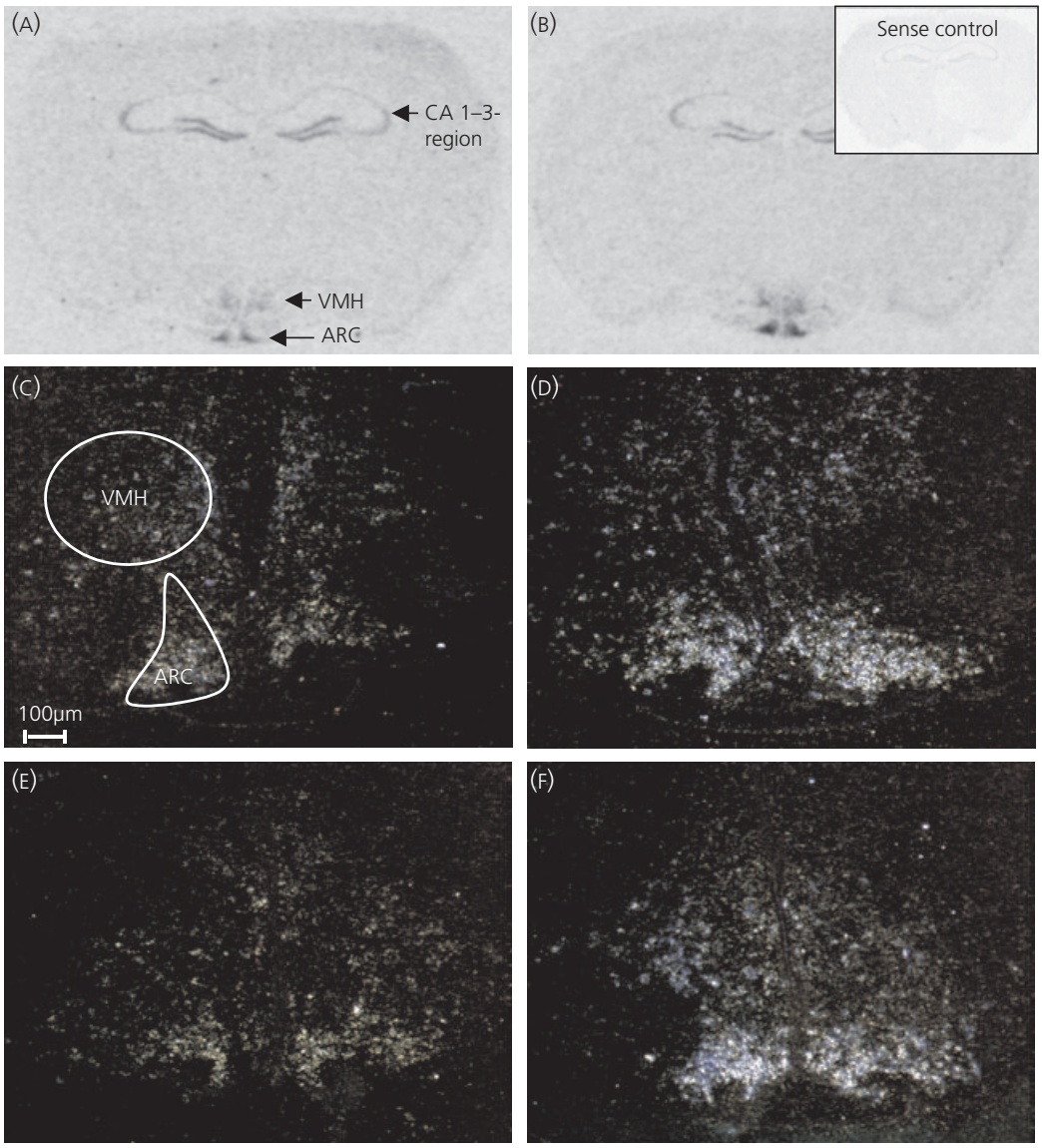

FIG. 1. Autoradiographs of LD female Siberian hamster brain sections (20 µm coronal sections; 8 weeks post weaning) either *ad libitum* fed (A) or 48-h food deprived (B) following *in situ* hybridization to an antisense ^{35}S-labelled riboprobe to growth hormone secretagogue receptor mRNA (inset depicting sense control). Also shown are representative sections of animals from each photoperiod. (C) to (F) Dark field photomicrographs showing high resolution images of the respective hypothalamic regions in LD (C,D) and SD (E,F) depicting induction of GHSR gene expression after food deprivation (C,E: *ad libitum*; D,F: food deprived). ARC, Arcuate nucleus; CA 1–3, CA 1–3 region; VMH, ventromedial hypothalamus.

rat cDNA generated a low intensity nonspecific signal (data not shown).

Effect of food deprivation(48 h) on GHSR gene expression in LD and SD hamsters

As described previously (26), SD hamsters gained 10.9 ± 1.0 g body weight, while hamsters in LD gained 16.8 ± 0.8 g during the 8 weeks following weaning. Food deprivation for 48 h led to a loss in body weight of 13.4 ± 2.3% in LD hamsters and 17.9 ± 2.3% in SD hamsters.

We found no difference in hypothalamic arcuate nucleus and VMH GHSR mRNA expression between LD and SD *ad libitum* fed hamsters, although there was a trend to increased gene expression in the arcuate nucleus and VMH in SD, which came close to, but did not achieve, statistical significance. However, food deprivation for 48 h led to a marked increase in GHSR gene expression in the arcuate nucleus (two-way ANOVA; $F = 18.17$; $P < 0.001$; Figs 1 and 2A) and VMH (two-way ANOVA; $F = 4.99$; $P < 0.05$; Figs 1 and 2B) irrespective of photoperiod.

Effect of chronic food restriction on GHSR gene expression in LD and SD hamsters

This experiment investigated changes in GHSR gene expression related to chronic manipulation of feeding state. As described previously (26), the body weight trajectory of LD-REST hamsters was matched to the body weight traject-

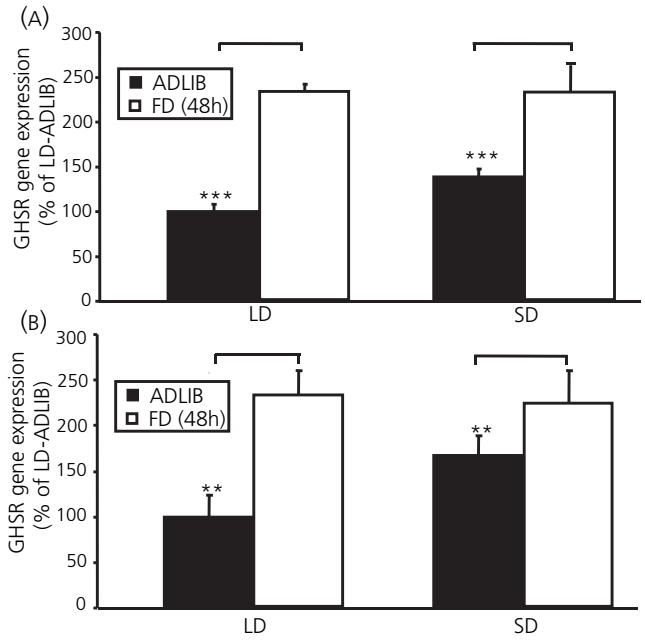

FIG. 2. Growth hormone secretagogue receptor (GHSR) gene expression in the hypothalamic arcuate nucleus (A) and in the ventromedial hypothalamus (B) of juvenile female Siberian hamsters. Hamsters were either *ad libitum* fed (ADLIB) or food deprived for 48 h (FD) (n = 6) in long (LD) or short day-length (SD). Values are expressed as percentages of values in LD hamsters fed *ad libitum*. Data are mean ± SEM. **P < 0.01, ***P < 0.001.

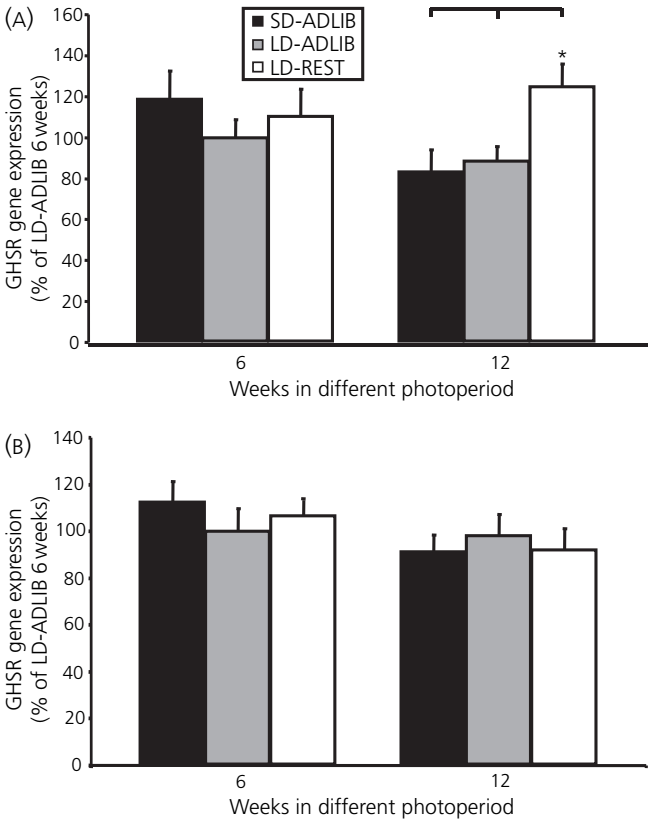

FIG. 3. Growth hormone secretagogue receptor (GHSR) gene expression in the hypothalamic arcuate nucleus (A) and in the ventromedial hypothalamus (B) of juvenile female Siberian hamsters (n = 9–12 in each group), fed *ad libitum* in long (LD-ADLIB) or short day-length (SD-ADLIB) for either 6 or 12 weeks, or held in long day-length with restricted food from day 0 post weaning onwards to mimic short-day-length body weight trajectory (LD-REST). Values are expressed as percentages of values in LD hamsters fed *ad libitum* (6 weeks). Data are mean ± SEM, *P < 0.05

ory of SD hamsters, whereas LD hamsters which continued to feed *ad libitum* gained significantly more weight than the remaining two groups (approximately 30% after 12 weeks).

Neither SD acclimation nor food restriction for 6 weeks had significant effects on arcuate nucleus (Fig. 3A) GHSR gene expression. By contrast, after 12 weeks, the LD-REST group showed significantly elevated arcuate nucleus GHSR gene expression compared to the LD-ADLIB and SD-ADLIB animals (one-way ANOVA on ranks; $H = 5.39$; $P < 0.01$). GHSR gene expression in the VMH (Fig. 3B) after either food restriction period was not different from the respective *ad libitum* fed groups.

Effect of leptin injection on GHSR gene expression

There was no effect of leptin injection on GHSR gene expression in the arcuate nucleus in either LD or SD hamsters over the 15–120 min time course postinjection compared to the vehicle-injected controls. Furthermore, GHSR gene expression of LD and SD vehicle injected hamsters was not different (Fig. 4).

Serum ghrelin concentration

In this repetition of the first experiment, over the 8-week postweaning period, SD hamsters gained 10.7 ± 1.0 g, while hamsters in LD gained 15.4 ± 1.1 g. Following food deprivation, LD hamsters lost 18.4 ± 2.4%, and SD hamsters 20.4 ± 3.2%, of their initial body weight before food deprivation. These values are similar to those observed previously (26).

Serum ghrelin levels recorded in the Siberian hamster are within the range measured in different mammalian species (28–30). No significant effect of photoperiod on serum ghrelin concentration was observed, although there was a trend for higher levels in SD. In food-deprived juvenile female hamsters, serum ghrelin concentration was significantly elevated in comparison to *ad libitum* fed hamsters (Fig. 5). This increase was observed in both LD and SD hamsters with a slightly greater elevation in SD (Fig. 5). The overall effect of feeding status was highly significant (two-way ANOVA; $F = 9.20$; $P < 0.001$). A similar increase in serum ghrelin concentration was also apparent in adult male LD hamsters after 48 h of food deprivation (LD-ADLIB: 795.2 ± 83.5 pg/ml, LD-FD: 1510.6 ± 189.0 pg/ml; n = 10 in each group, *t*-test; −3.31; $P < 0.001$).

Discussion

In this report, we demonstrate, for the first time, a marked elevation of GHSR gene expression in the arcuate nucleus and VMH in response to food deprivation for 48 h. By contrast chronic food restriction imposed to match SD body weight trajectory in LD hamsters (LD-REST) for 6 weeks

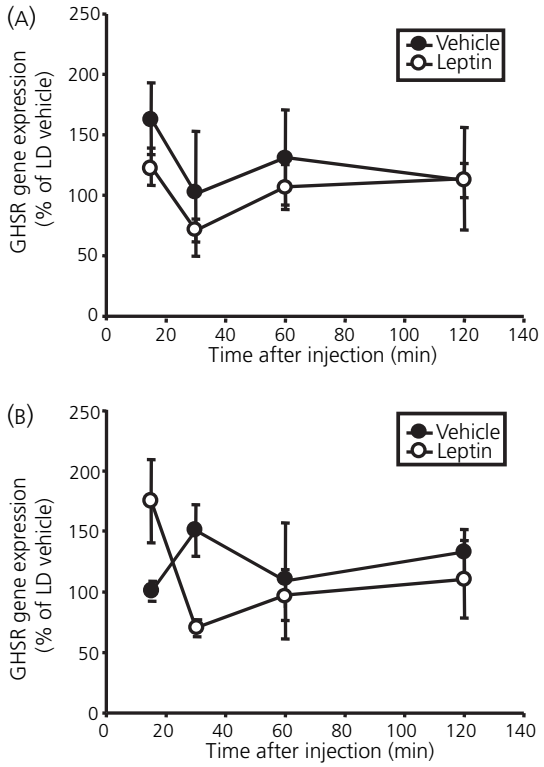

FIG. 4. Time-dependent effect of leptin injection on Growth hormone secretagogue receptor (GHSR) gene expression in the hypothalamic arcuate nucleus of juvenile female hamsters held in short (A) or long (B) day-length for 8 weeks (n = 3). Leptin was injected intraperitoneally at different time-points (15, 30, 60 and 120 min) before preparation of the brains; values are expressed as percentages of values in LD 15 min vehicle. Data are mean ± SEM.

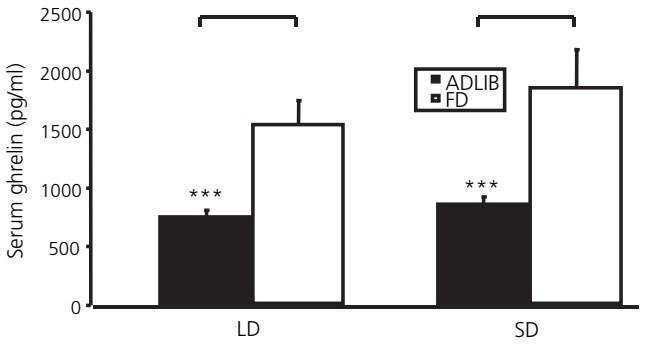

FIG. 5. Serum ghrelin levels in juvenile female Siberian hamsters 8 weeks post weaning. Hamsters were either *ad libitum* fed (ADLIB) or food deprived for 48 h (FD) (n = 4–6) in long (LD) or short day-length (SD). Data are mean ± SEM. ***P < 0.001.

had no effect and after 12 weeks led to only a slight increase in GHSR gene expression. Remarkably, the distinct photoperiod induced changes in body weight of *P. sungorus* did not affect GHSR gene expression within the examined hypothalamic regions. The arcuate nucleus and the VMH are both regarded to be key centres for the central integration of peripheral signals that convey energy homeostasis (31). Across different mammalian species, including the seasonal hamster *P. sungorus*, it is well established that body weight regulatory hormones such as leptin are processed in these hypothalamic nuclei to transduce their bioenergetic information into a central response. Recently, GHSR was demonstrated to be the primary ghrelin receptor that is able to modulate appetite in mice (32). We demonstrate that an increase of GHSR gene expression following food deprivation (48 h) is associated with a two-fold elevation of serum ghrelin levels. In addition to the fact that hypothalamic differential GHSR gene expression is confined to the arcuate nucleus and VMH, this implies that the orexigenic function of ghrelin would appear to depend on signal processing in these hypothalamic nuclei.

Ad libitum fed LD acclimated hamsters adjust their body weight corresponding to a postulated 'set-point' encoded by unknown neuronal mechanisms. In the present study, LD-REST hamsters were manipulated to a much lower body weight than imposed by this 'set point'. By contrast, SD hamsters defend a body weight that is appropriate to the lowered 'set-point' induced by SD acclimation. In terms of appetite, SD-ADLIB and LD-REST hamsters, despite having the same body weight, are in different satiety states due to their photoperiodic history. Therefore, LD-REST hamsters are expected to exhibit a permanently increased appetite, reflecting the drive to regain their individual body weight to the desired LD 'set point'. Indeed after 12 weeks of food restriction, GHSR gene expression in the arcuate nucleus of LD-REST hamsters was significantly elevated by approximately 50% compared to LD-ADLIB hamsters, but food restriction for 6 weeks had no effect. This discrepancy may be explained by the respective body weight differentials established between LD-ADLIB and LD-REST hamsters in these studies. In juvenile female hamsters, 6 weeks of food restriction led to a body weight differential of 6.3 g between LD-ADLIB (27.7 ± 2.6 g) and LD-REST (21.4 ± 1.4 g), whereas 12 weeks of food restriction caused a body weight differential of 8.6 g between LD-ADLIB (30.6 ± 2.6 g) and LD-REST (22.0 ± 1.0 g) (26). The larger body weight differential established after 12 weeks of food restriction may be indicative for stronger appetite in these hamsters. Interestingly, imposed food restriction in LD-REST hamsters led to a more dramatic decrease of circulating leptin levels after 12 weeks compared to 6 weeks [6 weeks: LD-ADLIB: 26.3 ± 0.8, LD-REST: 15.4 ± 3.2 ng/ml, SD-ADLIB: 10.8 ± 1.5; 12 weeks: LD-ADLIB: 26.7 ± 8.1; LD-REST: 3.8 ± 0.6 and SD-ADLIB: 9.1 ± 2.4 ng/ml (26)]. Only after 12 weeks were serum leptin levels in LD-REST hamsters clearly decreased even below the level measured in SD-ADLIB hamsters. Thus, leptin may exert an inhibitory effect on GHSR gene expression that is released only in catabolic states associated with extremely low serum leptin levels. The inhibitory potential of leptin on ghrelin sensitivity may be fully exploited in *ad libitum* fed hamsters. This may be one reason why leptin injections had no effect on GHSR gene expression in *ad libitum* fed hamsters with normal leptin levels. Hewson *et al.* (33) demonstrated that leptin alters ghrelin sensitivity only in food deprived rats, in which ghrelin and ghrelin mimetics were able to increase the number of cells expressing Fos protein in the arcuate nucleus. Our observed increase in GHSR gene expression and the accompanied elevation in circulating ghrelin concentration,

after 48 h of food deprivation, although being in a different species, may be a general mechanism through which ghrelin sensitivity could be altered in this catabolic state. This may be mediated by the abrupt decline of circulating leptin levels induced by food deprivation (26). Clearly, the increase in gene expression after 12 weeks of food restriction was less profound than that exhibited following food deprivation for 48 h. Together with the finding that GHSR gene expression in the VMH was not at all affected by chronic food restriction but clearly induced by acute food deprivation, our data support the hypothesis that ghrelin is primarily involved in the short-term regulation of appetite and body weight.

Across different species, including man, a negative correlation between circulating ghrelin and leptin has been described (10, 28, 34); obesity is associated with high leptin and low ghrelin levels. However, in *P. sungorus*, ghrelin levels were not negatively correlated with body weight. Although LD and SD hamsters exhibited a body weight differential of 15% after 8 weeks acclimation to the opposite photoperiod, no changes in serum ghrelin concentration could be detected. As published previously (26), serum leptin levels in LD hamsters are elevated by two- to three-fold in LD compared to SD. Thus, the lack of SD photoperiod-induced changes of both GHSR gene expression as well as circulating ghrelin levels implies that in seasonal body weight regulation, leptin may not counteract ghrelin. Barazzoni *et al*. (34) demonstrated that, in lean rats, subcutaneous leptin infusion prevented the rise in serum ghrelin levels in response to moderate caloric restriction. However, our results in *P. sungorus* suggest that leptin has no effect on serum ghrelin levels in the ad libitum fed state.

Long-term changes in serum leptin concentrations induced by photoperiod in the female juvenile hamster do not affect central expression of GHSR or ghrelin serum concentration, suggesting that ghrelin does not play a major role in seasonal body weight regulation. To generalize this conclusion, further photoperiod experiments in adult male and female hamsters are required. As such, ghrelin does not appear to be a functional antagonist to leptin (at least for the regulation of long-term body weight changes) but perhaps a signal regulating responses to short-term changes in energy homeostasis, such as food deprivation. The data obtained from the seasonal species *P. sungorus* contribute additional information to the poorly understood interaction of leptin and ghrelin. This discrepancy implies that the interaction of the important feeding related hormones leptin and ghrelin, an interesting enigma in body weight regulation, certainly requires further investigation.

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