Regulation of Growth Hormone Secretagogue Receptor Gene Expression in the Arcuate Nuclei of the Rat by Leptin and Ghrelin

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The anorexigenic and orexigenic hormones leptin and ghrelin act in opposition to one another. When leptin signaling is reduced, as in the Zucker fatty rat, or when circulating ghrelin is increased during fasting, the effect of ghrelin becomes more dominant, indicating an influence of both hormones on ghrelin action. This effect could be mediated via the level of expression of ghrelin receptor (growth hormone secretagogue receptor [GHS-R]). For testing this, GHS-R expression was measured using in situ hybridization in Zucker fatty versus lean rats; in fed versus fasted (48 h) rats, treated with either ghrelin or leptin; and in GH-deficient, dwarf versus control rats. In the arcuate nuclei of the Zucker fatty rat and in fasted rats, GHS-R expression is significantly increased. A single leptin intracerebroventricular injection attenuated the fasting-induced increase in GHS-R but had no effect in fed rats 2 h after injection, whereas leptin infusion for 24 h or longer significantly decreased GHS-R expression in fed rats. Ghrelin significantly increased GHS-R expression but not in dwarf rats. These results show that the level of GHS-R expression in the ARC is reduced by leptin and increased by ghrelin and that the effect of ghrelin may be GH dependent.
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In mammals, neurons, particularly in the arcuate nuclei (ARC) of the hypothalamus, are involved in the regulation of energy homeostasis (1). One regulatory pathway consists of neurons that coexpress neuropeptide Y (NPY) and agouti gene–related protein (AgRP), potent stimulators of food intake, whereas an adjacent set of ARC neurons coexpress proopiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART), which suppress food intake (2). These cells respond to signals on both the long- and short-term energy status of the animal. Leptin, secreted by and in proportion to white adipose tissue, signals the status of energy stores and activates POMC/CART neurons and inhibits NPY/AgRP neurons (3), resulting in inhibition of feeding and an increase in energy expenditure, whereas ghrelin, secreted mainly by the stomach but also present in the hypothalamus, activates NPY/AgRP neurons (4,5), stimulating feeding and decreasing energy expenditure. Ghrelin, from the stomach, acts as a short-term signal of nutrient depletion, and levels rise rapidly in response to fasting and drop rapidly after feeding (6), whereas ghrelin in the hypothalamus is thought to link circadian rhythms and feeding activity (5). Ghrelin also functions as a growth hormone (GH)-releasing agent in hypothalamic GHRH neurons in the ARC and ventromedial nuclei (VMN) (7) and also directly in the pituitary (8).

Ghrelin acts via the GH secretagogue receptor (GHS-R). GHS-Rs are localized within the hypothalamus mainly in the ARC and the VMN (9). Both nuclei are important in the regulation of feeding, and GHS, including ghrelin, stimulate feeding and weight gain in short-term (10–14) and long-term experiments in humans and rodents (15–17). Administration of GHSs induces expression of immunoreactive Fos in the ARC as well as in the paraventricular and dorsomedial nuclei and the lateral hypothalamus, although the immunolabeling in regions other than the ARC is weak (14).

The long signaling form of leptin receptor (Ob-Rb) is also present in the ARC and the VMN (18), and leptin administration induces SOCS3 and Fos expression in these nuclei (19). Ghrelin has been shown to depolarize the majority of ARC neurons that are inhibited by leptin (20). In addition, administration of a GHS-R agonist to leptin-resistant Zucker fatty rats results in the induction of double the number of Fos-immunopositive cells in the ARC compared with lean animals, whereas central infusion of leptin to normal, fasted rats suppresses the GHS-R agonist–induced Fos response (21). This suggests that the lack of a leptin-signaling pathway increases sensitivity to GHSs, whereas leptin administration diminishes this effect, supporting the hypothesis that leptin functions to inhibit the effects of ghrelin.

In the present study, we investigated the possibility that leptin may inhibit the effects of ghrelin by altering the expression of GHS-R. We examined the effects of central leptin administration on the expression of GHS-R in the ARC and VMN and the effects of the absence of leptin signaling in Zucker fatty rats and low levels of leptin during fasting. As ghrelin levels are raised when leptin

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AgRP, agouti gene–related protein; ARC, arcuate nuclei; CART, cocaine- and amphetamine-regulated transcript; GH, growth hormone; GHS-R, GH secretagogue receptor; ICV, intracerebroventricular; NPY, neuropeptide Y; POMC, proopiomelanocortin; VMN, ventromedial nuclei.

levels are reduced (22), we also investigated the influence of central ghrelin administration on GHS-R expression. The influence of ghrelin on the expression of leptin receptors Ob-Rb was also measured. Finally, to assess the role of GH, we also tested the effect of ghrelin in the dwarf rat.

RESEARCH DESIGN AND METHODS

All rats used were male and between 8 and 10 weeks of age apart from the Zucker rats, which were ~12 weeks of age. Zucker diabetic fatty rats (*fa/fa*) and Zucker lean rats (+/?) were bred and housed at the Rowett Research Institute (Aberdeen, U.K.) and killed by stunning and cervical dislocation followed by decapitation. Sprague-Dawley and the GH-deficient dwarf rat (DR) and wild-type Lewis rats were obtained from Harlan Ibérica (Barcelona, Spain) and housed at the University of Santiago de Compostela, where all experimental procedures were performed. In Spain, animals were housed in air-conditioned rooms (22–24°C) under a controlled light/dark cycle (14 h light) and fed standard rat diet and water ad libitum unless otherwise indicated. Animals were killed by decapitation in a separate room. Brains were rapidly removed, frozen, and stored at −80°C until cryosectioned and processed for in situ hybridization. All animal experimental procedures were conducted according to the regulations of Santiago de Compostela Medical School Animal Care Research Committee; $n = 6$ in all experimental groups.

Implantation of intracerebroventricular cannulas. Animals were anesthetized by an intraperitoneal injection of ketamine/xylazine (ketamine 100 mg/kg body wt + xylazine 15 mg/kg body wt). Chronic intracerebroventricular (ICV) cannulas were implanted stereotaxically using the following coordinates: 1.3 mm posterior to bregma, 1.9 mm lateral to the midsagittal suture, and a depth of 3.5 mm as described previously (23). The location of the cannula in the lateral ventricle was confirmed by methylene blue staining. Animals were individually caged and allowed to recover for 1 week before experiment. During the postoperative recovery period, the rats were handled regularly under nonstressful conditions.

Short-term ghrelin and leptin challenge. One group of rats was fed ad libitum, and the other group was deprived of food for 48 h. Rats then received either a single ICV injection of ghrelin (Bachem, Bubendorf, Switzerland; 5 μg/rat dissolved in 5 μl of distilled water saturated with argon) or vehicle. In a second experiment, rats were given a single ICV injection of vehicle or ghrelin at a dose of 0.5, 2, or 5 μg/rat. In both experiments, rats were killed 2 h after injection. In a third experiment, rats were fed ad libitum and received a single ICV injection of either ghrelin or vehicle and were killed 1 or 2 h after injection. In a separate experiment, animals were fed ad libitum or fasted for 48 h as described above. A single ICV injection of recombinant human leptin (Sigma, St. Louis, MO; 10 μg/rat) or vehicle was given, and animals were killed 2 h later. All treatments started at 0900 and were carried out in the light phase.

Long-term ghrelin challenge. Brain infusion cannulas were stereotaxically placed into the lateral ventricle as described above. A catheter tube was connected from the brain infusion cannula to the osmotic minipump flow moderator (model 2001D or 2ML2; Alza, Palo Alto, CA). A subcutaneous pocket on the dorsal surface was created using blunt dissection, and the osmotic minipump was inserted. The incision was closed with sutures, and rats were kept warm until fully recovered. Rats then received an infusion of either vehicle or ghrelin (5 μg/day) for 24 h, 48 h, or 7 days.

Long-term leptin challenge. Rats received an ICV infusion, as described above, of either recombinant human leptin (15 μg/day) or vehicle for 7 days into the lateral ventricle. For the last 48 h of the infusion period, rats were either fed ad libitum or fasted. During this time rats were kept in grid-bottomed cages to facilitate food intake measurements and were weighed daily. In a separate experiment, rats received an ICV infusion of vehicle or recombinant human leptin at a dose of 1 or 5 μg/day for 7 days into the lateral ventricle.

In situ hybridization. A 449-bp fragment of the GHS-R was cloned and amplified from rat hypothalamic cDNA. Automated sequencing was performed to verify the sequence. Messenger RNA levels were quantified using this probe by in situ hybridization, on 20-μm-thick coronal hypothalamic sections, using techniques described in detail elsewhere (24). Briefly, slides were fixed in 4% (wt/vol) paraformaldehyde in 0.1 mol/l PBS for 20 min at room temperature, washed in PBS, incubated in 0.1 mmol/l triethanolamine for 2 min, and acetylated in 0.1 mmol/l triethanolamine and 0.25% (vol/vol) acetic anhydride for 10 min. Sections were dehydrated in ethanol and dried under vacuum before hybridization with riboprobes at 10^6 cpm/ml for 18 h at 58°C. After hybridization, sections were desalted through a series of washes in standard saline citrate to a final stringency of 0.1× SSC at 60°C for 30 min, treated with RNase A, and dehydrated in ethanol. Slides were apposed to Biomax MR (Sigma, Poole, Dorset, U.K.) together with (^{14}C) microscale standards (Amersham International, Amersham, U.K.) for 1 week at room temperature.

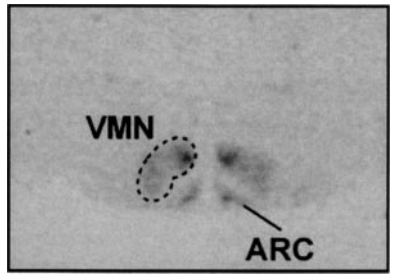

FIG. 1. In situ hybridization of the GHS-R probe in the mediobasal hypothalamus of the rat. Area of the VMN analyzed is outlined.

Quantification of in situ hybridization. Autoradiographs were scanned on a Umax Power Look II (UMAX Data Systems, Fremont, CA). Integrated optical densities of ARC and VMN were measured using the Image Pro-plus system (Media Cybernetics, Silver Spring, MD) and converted to nCi/g using the (^{14}C) microscale standard curve. Values were converted to percentages of control values (100%).

Radioimmunoassay for rat ghrelin. Plasma levels of ghrelin were assayed by means of a double-antibody radioimmunoassay using reagent kits (Phoenix Peptides, Belmont, CA) and methods as previously described (25). All samples were assayed in duplicate within one assay, and results were expressed in terms of the ghrelin standard. The limit of the assays sensitivity was 2.27 pg/ml; the intra- and interassay levels were, respectively, 5 and 13%.

Statistical analysis. Data are represented as mean ± SE and were analyzed by ordinary parametric ANOVA followed by a post hoc analysis. Differences between groups were evaluated using t tests. $P < 0.05$ was considered statistically significant.

RESULTS

GHS-R distribution. The highest levels of GHS-R expression in the hypothalamus are in the ARC and VMN. The distribution of GHS-R in the ARC nuclei seemed to be homogeneous, whereas in the VMN, relatively high levels of GHS-R expression were localized to a medial subdivision of the dorsomedial region of the VMN (Fig. 1). For the calculation of integrated optical density in the VMN, the whole nucleus was included (area within dotted lines).

GHS-R expression is higher in the ARC of the Zucker fatty rat. The expression of GHS-R in the ARC of the Zucker fatty (*fa/fa*) rats is significantly higher than in the Zucker lean rats (+/?) ($P < 0.05$), but the level of GHS-R expression in the VMN did not differ significantly between Zucker fat and lean animals (Fig. 2). Plasma ghrelin levels were unchanged in Zucker fatty rats in comparison with Zucker lean rats (68.8 ± 4.69 vs. 63.3 ± 6.99 pg/ml).

Fasting increases GHS-R levels in the ARC. The GHS-R mRNA content of the ARC in fasted rats was

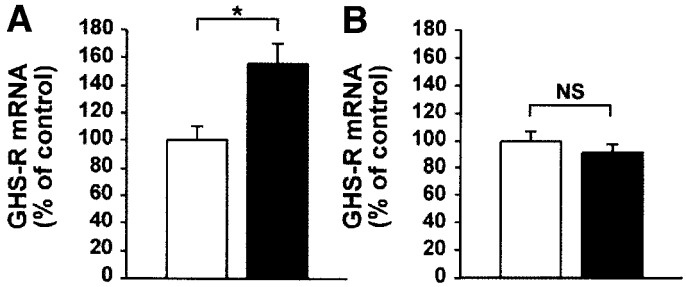

FIG. 2. GHS-R mRNA levels in the ARC (*A*) and VMN (*B*) of the Zucker fatty (*fa/fa*) (■) and the Zucker lean (+/?) (□) rats. *$P < 0.05$ fatty vs. lean in the ARC. No significant difference (NS) was detected in the VMN.

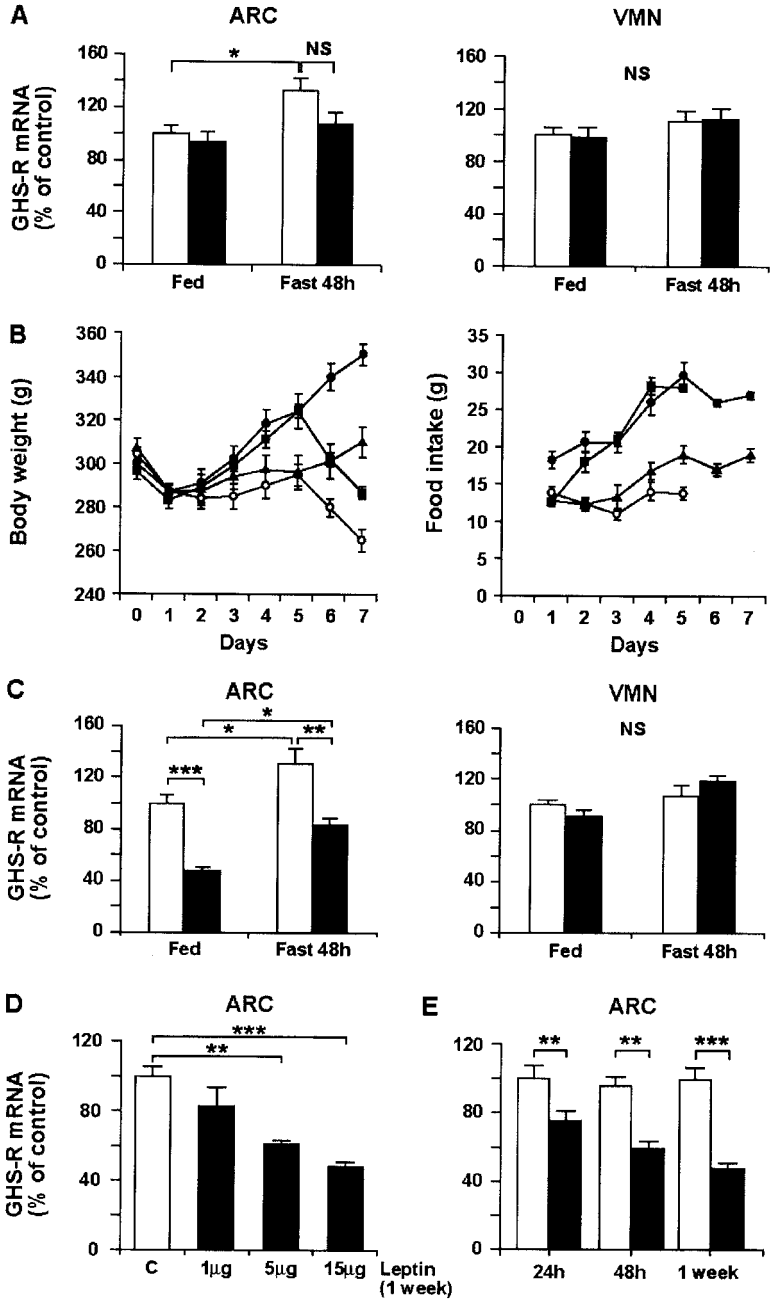

FIG. 3. *A*: **Effect of ICV leptin (10 μg) 2 h after injection in fed and 48-h–fasted rats on GHS-R expression in the ARC and VMN. *$P < 0.05$ fed vs. fasted. No significant difference (NS) between leptin (■) and vehicle (□).** *B*: **Body weights and food intake during long-term infusion of ICV leptin (15 μg/day). Food intake and body weight gain are inhibited by leptin. –●–, fed ad libitum; –■–, fasted 48 h; –▲–, fed + leptin (7 days); –○–, fasted 48 h + leptin (7 days).** *C*: **Effect of ICV leptin on GHS-R expression for 1 week in fed and 48-h–fasted rats. *$P < 0.05$ 48 h fasted vs. fed; **$P < 0.01$ leptin-treated 48-h fast group vs. vehicle; ***$P < 0.001$ leptin-treated fed vs. vehicle. □, vehicle; ■, leptin (1 week).** *D*: **Effect of leptin on GHS-R mRNA expression in the ARC after 1 week of infusion. **$P < 0.01$ leptin 5 μg/day vs. vehicle; ***$P < 0.001$ leptin 15 μg/day vs. vehicle.** *E*: **Effect of leptin (15 μg/day) on GHS-R mRNA expression in the ARC. **$P < 0.01$ 24- and 48-h leptin vs. vehicle. No significant difference (NS) was detected in the VMN between any treatment groups. □, vehicle; ■, leptin (1 week).**

significantly higher ($P < 0.05$) than in ad libitum–fed rats. No significant difference was seen in the level of GHS-R expression in the VMN between the fasted and the fed rats (Fig. 3*A* and *C*).

Short-term treatment with leptin does not change GHS-R mRNA levels in the ARC or the VMN. A single ICV injection of leptin did not affect the level of GHS-R expression in the ARC or VMN in the fed animals. The increase seen in GHS-R in the fasted animals seemed to be attenuated by leptin, with the fasting levels of GHS-R expression ~20% higher than in fasted rats that received the leptin injection. However, this attenuation was not statistically significant. There was no effect of leptin on GHS-R expression in the VMN (Fig. 3*A*).

Long-term treatment with leptin decreases GHS-R mRNA levels in the ARC but not the VMN. Long-term ICV leptin infusion significantly decreased food intake and body weight (Fig. 3*B*). GHS-R mRNA levels were significantly ($P < 0.001$) lower in the ARC but not in the VMN of animals that received ICV recombinant human leptin for 7 days compared with vehicle-treated animals in both ad libitum–fed ($P < 0.01$) and fasted animals (Fig. 3*C*). No effect of leptin was seen in the VMN (Fig. 3*C*). The effect of leptin on GHS-R gene expression was dependent on the dose of leptin infused daily. No significant effect of leptin was seen at 1 μg/day, but at 5 and 15 μg/day, GHS-R gene expression was significantly ($P < 0.01$ and $P < 0.001$, respectively) lower (Fig. 3*D*). The effect of leptin infused at 15 μg/dayay (Fig. 3*C*) has been included in this figure to illustrate the increasing effect of leptin with increasing quantity infused. GHS-R mRNA levels were significantly ($P < 0.01$) lower in the ARC of animals that received leptin infusions for 24 h, 48 h ($P < 0.001$), and 1 week (Fig. 3*E*). This difference

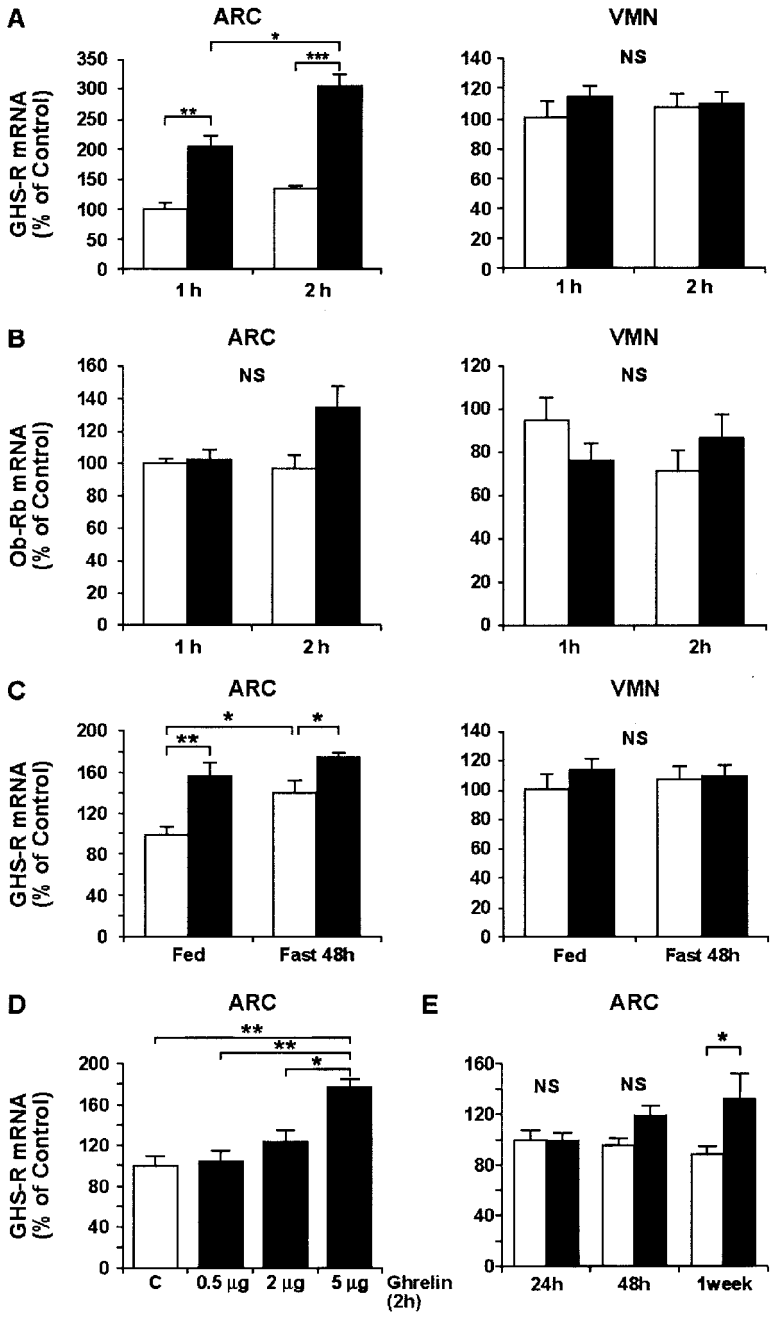

FIG. 4. ***A*****: Effect of ICV ghrelin (5 μg) 1 and 2 h after injection on GHS-R expression in the ARC and VMN. *****P*** **< 0.01 ghrelin vs. vehicle after 1 h; ******P*** **< 0.001 ghrelin vs. vehicle after 2 h; ****P*** **< 0.05 2 vs. 1 h. □, vehicle; ■, ghrelin.** ***B*****: Ob-Rb expression in the ARC and VMN 1 and 2 h after ICV ghrelin. No significant difference was found. □, vehicle; ■, ghrelin.** ***C*****: GHS-R expression in fed and 48-h–fasted rats, with ICV ghrelin or vehicle after 2 h. ****P*** **< 0.05 48-h fasted vs. fed; *****P*** **< 0.01 ghrelin vs. vehicle fed; ****P*** **< 0.05 ghrelin vs. vehicle fasted. □, vehicle; ■, ghrelin (2 h).** ***D*****: Effect of ghrelin on GHS-R expression in the ARC 2 h after ICV injection. *****P*** **< 0.01 5 μg ghrelin vs. vehicle; *****P*** **< 0.01 5 μg ghrelin vs. 0.5 μg ghrelin; ****P*** **< 0.05 5 vs. 2 μg ghrelin. □, vehicle; ■, ghrelin.** ***E*****: Effect of ghrelin infusion (5 μg/day) on GHS-R mRNA expression in the ARC. ****P*** **< 0.05 1 week ghrelin vs. vehicle. No significant difference (NS) was detected in the VMN between any treatment groups. □, vehicle; ■, ghrelin.**

is seen at 24 and 48 h before any significant change in body weight (Fig. 3*B*).

Short-term treatment with ghrelin increases GHS-R mRNA levels in the ARC but not the VMN in fed and fasted rats. A significant increase in GHS-R mRNA levels was observed in the ARC after treatment with ghrelin (Fig. 4*A*) at both 1 h ($P < 0.01$) and 2 h ($P < 0.001$) after injection compared with vehicle-treated animals. The increase at 2 h is significantly ($P < 0.05$) higher than that at 1 h. The administration of ghrelin caused a significant increase in food intake during the 1 h after injection (vehicle, 0.36 ± 0.15 g; ghrelin, 2.17 ± 0.37 g; $P < 0.001$). The increase in GHS-R gene expression was also seen in fasted animals (Fig. 4*C*). No change in GHS-R gene expression was seen in the VMN in any of the treatments. The effect of a single ICV injection of ghrelin 2 h after injection showed a steady increase in effect with dose with GHS-R gene expression being significantly ($P < 0.05$) higher after 5 μg compared with 2 μg ($P < 0.01$) and after 0.5 μg and vehicle injection (Fig. 4*D*).

Short-term treatment with ghrelin has no significant effect on Ob-Rb mRNA levels in the ARC or the VMN. No statistically significant effect of ICV ghrelin on Ob-Rb could be found 1 or 2 h after injection in either the ARC or the VMN (Fig. 4*B*).

Long-term infusion with ghrelin increases GHS-R mRNA levels in the ARC. Long-term ICV infusion with 5 μg/day ghrelin did not produce a statistically significant increase in GHS-R mRNA after either 24 or 48 h. GHS-R mRNA was significantly ($P < 0.05$) increased after 1 week of ghrelin infusion (Fig. 4*E*)

Short-term treatment with ghrelin does not increase GHS-R mRNA levels in the ARC of GH-deficient rats. GHS-R gene expression was not significantly different in

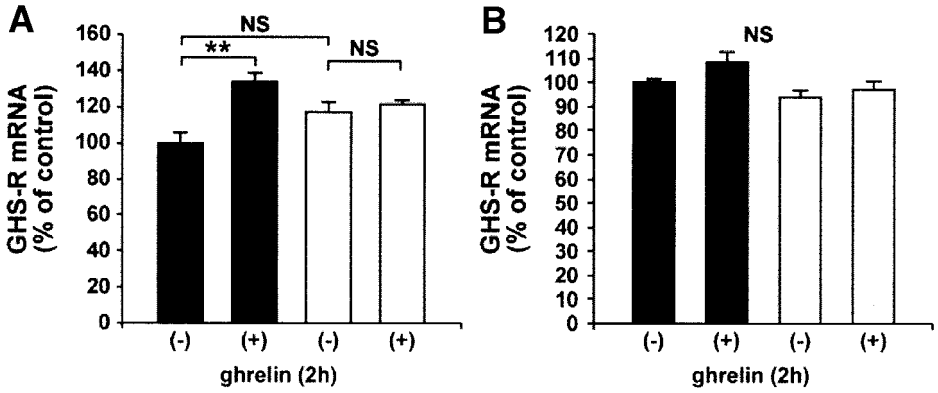

FIG. 5. GHS-R mRNA levels in the ARC and VMN in DR (□) and Lewis (■) rats 2 h after ICV ghrelin challenge. No significant difference was found in GHS-R gene expression between DR and Lewis rats in the ARC (*A*) or VMN (*B*). Ghrelin challenge significantly increased GHS-R expression in the ARC of the Lewis rats. $^{**}P < 0.01$ but had no effect in the DR.

either the ARC or the VMN between DR and Lewis rats (Fig. 5). However, whereas ICV ghrelin administration did not significantly change GHS-R gene expression in the ARC of the DRs, it caused a significant ($P < 0.01$) increase in the level of gene expression in the ARC of the Lewis rats. There was no difference in the level of GHS-R expression in the VMN between the DR or Lewis rats or in response to ghrelin challenge.

DISCUSSION

The hormone leptin, generated by and in proportion to body fat, acts as a negative feedback to the ARC, in part, by inhibiting the expression of AgRP and NPY, which in turn inhibit food intake and increase energy expenditure. Ghrelin, produced in the stomach and the hypothalamus (5,8), is the endogenous ligand for the GHS-R and acts to increase the expression of AgRP and NPY, stimulating appetite and decreasing energy expenditure (26–28). GHS-R expression has been localized to NPY-expressing cells with >90% of ARC neurons coexpressing NPY and GHS-R (29,30). The long signaling form of the leptin receptor (Ob-Rb) also colocalizes with NPY in the ARC (31,32). Electrophysiological studies have shown that leptin inhibits a subpopulation of GHS-responsive neurons (33) and that ghrelin acts directly on leptin-responsive cells in the ARC (20). Thus, ghrelin and leptin act on NPY- and AgRP-coexpressing cells in the ARC in opposition to one another (10,34). The relative sensitivity of the hypothalamus to these orexigenic and anorexigenic signals therefore is key in the delicate balance of body weight regulation.

During fasting, the hypothalamus shows an increased responsiveness to GHS, with induction of Fos immunoreactivity by GHS in the ARC being threefold higher in fasted rats (35). An increase in GHS-R mRNA in the hypothalamus of fasted rats has been reported using quantitative PCR techniques (36). However, this study did not distinguish between hypothalamic nuclei. The results of the present study confirm this effect and also show that the increase in GHS-R expression is specific to the ARC of fasted rats. Fasted rats have both suppressed circulating leptin (37) and increased ghrelin levels (38), and to ascertain whether these changes in ghrelin and/or leptin modulate GHS-R expression, we compared GHS-R expression in leptin- and ghrelin-treated animals that were fasted or fed ad libitum. Levels of GHS-R were also measured in the Zucker fatty rat, which lacks fully functional Ob-Rbs.

The Zucker fatty rat shows a variety of metabolic and endocrine abnormalities, including hyperphagia and excessive lipogenesis (39). The *fa* allele codes for an altered Ob-Rb protein (40) with decreased binding affinity and reduced signal transduction (41). The Zucker fatty rat is more sensitive to the effect of GHS than the Zucker lean rat (21). In the present study, significantly higher levels of GHS-R were found in the ARC of Zucker fatty rat than in lean animals, indicating that in the absence of full leptin signaling, GHS-R expression is increased in the ARC.

The influence of leptin on GHS-R expression was confirmed in rats that were challenged with ICV leptin for 24 h or more. Although leptin failed to influence hypothalamic GHS-R mRNA expression 2 h after a single ICV injection in fed rats, leptin infusion for 24 h and longer had a significant effect of GHS-R gene expression. The half-life of GHS-R mRNA in the pituitary gland is 8 h (42), and it is not implausible that GHS-R mRNA has a similar half-life in the hypothalamus, indicating that the effect of leptin on GHS-R expression would take longer than 2 h to become apparent.

To investigate whether the high levels of ghrelin often found in conjunction with low circulating levels of leptin could influence the sensitivity of the hypothalamus to ghrelin, we tested the effect of ghrelin challenge on GHS-R expression. Ghrelin, given as a single ICV injection, increased GHS-R expression in the ARC in both fed and fasted rats in a dose-dependent manner. However, infusion of ghrelin took longer to show a significant effect. Considering that the same dose (5 μg) was given either acutely, as a single ICV challenge, or over 24 h, this is not a surprising result.

GH-deficient rats were also challenged with ghrelin. The GH-deficient rat model provides us with the ability to study the regulation of hypothalamic GHS-R expression in the absence of endogenous GH. Previous studies have shown that GH-deficient rats have higher levels of GHS-R in both ARC and VMN compared with wild type (43). However, other studies have found differences in GHS-R expression only between female dwarf and wild-type rats with no difference between males (44). In the present study, GHS-R expression in the ARC seems to be only moderately increased (17%) in GH-deficient rats, and this is not statistically significant. Treatment of wild-type rats with ghrelin for 2 h increased GHS-R mRNA expression in the ARC but did not change GHS-R mRNA expression in GH-deficient rats, indicating that GHS-R expression is not sensitive to GH status but that the influence of ghrelin on GHS-R expression in the ARC may be GH dependent as

ghrelin fails to stimulate GHS-R expression in the absence of GH.

In conclusion, we have shown that both ghrelin and leptin are involved in the regulation of GHS-R in the ARC but not in the VMN, with ICV ghrelin increasing GHS-R expression and ICV leptin decreasing ARC GHS-R mRNA. Moreover, during fasting (high levels of ghrelin and low levels of leptin) and in obese Zucker rats (insensitive to leptin), there is increased GHS-R mRNA expression in the ARC. Finally, GHS-R mRNA seems to be regulated by ghrelin via a GH-dependent mechanism. These results seem particularly relevant as GHS-Rs are reported to be constitutively active and the presence of agonist stimulation may be relatively minor compared with the basal activity of the receptor (45). This indicates that factors that influence receptor expression could be more important than agonist stimulation.

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