

# The Rat Arcuate Nucleus Integrates Peripheral Signals Provided by Leptin, Insulin, and a Ghrelin Mimetic

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**The hypothalamic circuits controlling food intake and body weight receive and integrate information from circulating satiety signals such as leptin and insulin and also from ghrelin, the only known circulating hormone that stimulates appetite following systemic injection. Activation of arcuate neurons by ghrelin and ghrelin mimetics (the growth hormone secretagogues) is augmented in 48-h-fasted rats compared with fed rats, as reflected by a greater number of cells expressing Fos protein in response to administration of the same maximally effective dose. Here we sought to determine whether this increased responsiveness in fasting might reflect or be influenced by low levels of circulating satiety factors such as leptin or insulin. Chronic central infusion of insulin or leptin during a 48-h fast suppressed the threefold increase in the Fos response to intravenous injection of a maximally effective dose of growth hormone-releasing peptide (GHRP)-6, a synthetic growth hormone secretagogue. This appears to be a direct central action of insulin and leptin because the marked decrease in plasma levels of insulin, leptin, and glucose during fasting were unaffected by central administration of either hormone. Furthermore, the GHRP-6-induced Fos response was twofold greater in obese leptin- and insulin-resistant Zucker rats compared with lean controls. These data provide evidence that the ghrelin-sensitive circuits in the hypothalamus are dynamically regulated by central insulin and leptin action. *Diabetes* 51:3412–3419, 2002**

**F**ollowing the discovery of ghrelin (1), an endogenous ligand for the growth hormone secretagogue (GHS) receptor (GHS-R) (2), accumulating data has suggested that this gut-derived peptide participates in the central regulation of food intake and body composition. Thus, administration of either ghrelin or synthetic ghrelin mimetics (e.g., the GHS and growth hormone-releasing protein [GHRP]-6) acutely stimulates food intake in satiated rats and following chronic administration induces adiposity in rodents (3–7). More recently, a marked preprandial increase in plasma ghrelin levels in

humans has been reported, suggesting a physiological role in meal initiation (8). The stimulatory action of ghrelin on food intake appears to be mediated via the orexigenic neuropeptide Y (NPY)/agouti-related peptide (AgRP) pathway, since blockade of NPY receptors or immunoneutralization of AgRP attenuates ghrelin-induced feeding (9–11). Consistent with this, the majority of NPY neurons in the arcuate nucleus express the GHS-R (12), and ~50% of cells activated (that express *c-fos* mRNA) following GHS administration contain NPY mRNA (13). In addition, both NPY and AgRP mRNA expression in the arcuate nucleus are increased following ghrelin administration to fed rats (9–11,14).

Previously we showed that a dose of GHRP-6/ghrelin that was maximally effective for the induction of Fos protein in fed rats induced a two- to threefold greater response in the arcuate nucleus of 48 h-fasted rats (15,16). One possible explanation for this is that ghrelin's hypothalamic actions are unopposed in the absence of circulating satiety signals such as leptin and insulin. Both hormones have an established role as "adiposity" signals that regulate long-term body weight homeostasis through actions on the central nervous system, particularly the hypothalamic arcuate nucleus (17). Messenger RNA for both insulin and leptin receptors is highly expressed in the arcuate nucleus (18,19), and central administration of either insulin or leptin reduces both food intake and body weight (20,21). Critical roles for leptin and insulin in maintaining body weight homeostasis are clearly demonstrated in genetic models of hyperphagia and obesity, resulting from leptin deficiency (*ob/ob* mice; 22), leptin receptor mutations (*db/db* mice and *fa/fa* Zucker rats; 23,24) and neuronal insulin receptor knockouts (NIRKO mice; 25). Furthermore, the increased expression of arcuate nucleus NPY mRNA in conditions of hypoinsulinemia/hypoleptinemia (e.g., fasting, insulin-deficient diabetes, and *ob/ob* mice) can be reversed by appropriate replacement of insulin or leptin but not in insulin/leptin-resistant states (e.g., obese *fa/fa* Zucker rats and *db/db* mice), suggesting an inhibitory action of insulin and leptin on arcuate neuron NPY gene expression (26–30). Moreover, electrophysiological recordings demonstrated a direct inhibitory action of both insulin and leptin on a subpopulation of arcuate nucleus neurons in lean but not obese Zucker rats (31,32). In our own electrophysiological studies we have shown that GHS-responsive cells in the arcuate nucleus are predominantly inhibited by leptin, consistent with an inhibitory role of leptin on GHS-responsive circuits (33). Therefore, we examined the possibility that the increased responsiveness of arcuate nucleus neurons to GHS administration in fasted rats may

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Received for publication 9 April 2002 and accepted in revised form 6 September 2002.

AgRP, agouti-related peptide; CNS, central nervous system; CSF, cerebrospinal fluid; GHRH, growth hormone-releasing hormone; GHRP, growth hormone-releasing protein; GHS, growth hormone secretagogue; GHS-R, growth hormone secretagogue receptor; ICV, intracerebroventricular; NPY, neuropeptide Y.

be due to reduced central activity of insulin and/or leptin, as both are decreased by fasting. In addition, we compared the central response to GHRP-6 in fed and fasted lean (+/?) and obese (*fa/fa*) Zucker rats to determine whether reduced leptin/insulin signaling alters the hypothalamic response to GHS.

## RESEARCH DESIGN AND METHODS

**Animals.** Male Wistar rats were obtained from Charles River (Kent, U.K.) and used at age 10–12 weeks (250–350 g). Male obese (*fa/fa*; 482 ± 9 g) and lean (+/?; 373 ± 8 g) Zucker rats were obtained from Charles River (Chatillon Sur Chalaronne, St. Germaine Sur L'Arbresle, France) and used at 18 weeks of age. The genotype of lean littermates can be either +/+ or +/*fa*; in this study lean control Zucker rats are therefore referred to as +/?. All animals were allowed to acclimatize for at least 1 week after arrival at the animal unit in a temperature-controlled environment (21–22°C) on a 12-h light/dark cycle (lights on 0700–1900) before undergoing any procedures. Unless otherwise stated, all animals had free access to pelleted rat diet (RM-1; Special Diet Services, Essex, U.K.) and water throughout the study. All experiments were performed in accordance with the 1986 U.K. Animals (Scientific Procedures) Act.

**Central insulin or leptin infusion during fasting.** The effect of central infusion of leptin and insulin on GHS responsiveness during fasting was carried out as two separate studies each with its own control groups; here we describe these experiments together, however, as the procedure was identical for both. Wistar rats were anesthetized with tribromoethanol/amyl hydrate (Avertin, 1 ml/100 g i.p.) for placement of a jugular vein catheter as previously described (15). Rats were then placed in a stereotaxic frame for insertion of an intracerebroventricular (ICV) cannula (Alzet Brain Infusion Kit II, Charles River). The coordinates used for lateral ventricle cannulation were as follows: 0.6 mm posterior, 1.6 mm lateral to bregma, and 4.5 mm below the skull surface with the skull level between bregma and lambda (34). An osmotic minipump (Alzet Model 2001, flow rate 1  $\mu$ l/h for 7 days; Charles River) was implanted subcutaneously in the flank and connected to the ICV cannula with a short length of polypropylene tubing. The ICV cannula was secured in position by dental cement attached to three stainless steel screws placed into the skull. The pump and tubing had previously been filled with either saline vehicle, human recombinant insulin (83.3 mU/ml, Actrapid; Novo Nordisk, Copenhagen, Denmark) or murine recombinant leptin (50  $\mu$ g/ml; PeproTech, London, U.K.). This resulted in delivery rates of 2 mU/24 h and 1.2  $\mu$ g/24 h for insulin and leptin, respectively. Before implantation, the pumps were placed overnight in sterile saline at 37°C to initiate pumping.

On the fourth day following surgery, food was removed from rats in the fasted groups for the last 48 h of the infusion period. Rats in the fed group were allowed free access to food throughout. After 6 days of ICV saline, insulin, or leptin infusion, rats were injected intravenously with either GHRP-6 (50  $\mu$ g/rat; Bachem, St. Helens, U.K.) or isotonic saline. Then, 90 min later rats were terminally anesthetized with sodium pentobarbitone (60 mg/kg i.v.; Sagatal, Rhône Mérieux, Essex, U.K.) and a blood sample was collected into chilled lithium-heparin-coated microcentrifuge tubes (Sarstedt, Leicestershire, U.K.) by cutting the right atrium of the heart. Note that due to sampling problems it was not possible to obtain blood samples from all rats. Rats were then perfused via the aorta with heparinized saline followed by 4% paraformaldehyde in 0.1 mol/l phosphate buffer (pH 7.4). Brains were removed and left overnight in 15% sucrose in 4% paraformaldehyde, a second overnight in 30% sucrose in phosphate buffer, and then frozen on dry ice and stored at –80°C until processed for Fos immunohistochemistry.

**GHRP-6 administration to fed and fasted Zucker rats.** Lean (+/?) and obese (*fa/fa*) Zucker rats were anesthetized with tribromoethanol/amyl hydrate (Avertin, 1 ml/100 g i.p.) for placement of a jugular vein catheter as previously described (15). After 3 days of recovery, food was removed from rats in the fasted groups for 48 h, while those in the fed groups had food available throughout. Fed and fasted Zucker rats were injected intravenously with GHRP-6 (0.4 mg/kg) or saline. Ninety minutes later rats were terminally anesthetized for collection of a blood sample and perfusion as described above.

**Immunohistochemical detection of Fos protein.** Coronal sections (40  $\mu$ m) were cut on a sliding microtome through the arcuate nucleus, and every third section was collected into phosphate buffer. Endogenous peroxidases were deactivated in phosphate buffer containing 20% methanol, 0.2% Triton X-100, and 1.5% hydrogen peroxide for 15 min. Sections were incubated with a rabbit polyclonal anti-Fos antibody (Ab-5, PC-38 Calbiochem; CN Biosciences, Nottingham, U.K.) (1:100,000 diluted in 1% normal sheep serum/0.3% Triton X-100/0.25% BSA/0.1 mol/l phosphate buffer) for 48 h at 4°C. Bound antibody

was localized with a 2-h incubation in peroxidase-labeled goat anti-rabbit IgG (Vector Laboratories, Peterborough, U.K.) (1:200 dilution) and visualized using a nickel-intensified diaminobenzidine reaction (35). Briefly, sections were washed in sodium acetate buffer (0.1 mol/l, pH 6.0) and incubated in the same buffer containing 2.5% nickel sulfate, 0.2% glucose, 0.04% ammonium chloride, 0.025% diaminobenzidine, and ~30 units/ml glucose oxidase (Type VII-S; Sigma, Poole, U.K.). The reaction was observed under a microscope and terminated by washing in 0.1 mol/l acetate buffer.

### Measurement of plasma insulin, leptin, and glucose concentrations.

Blood was obtained just before perfusion of the animal by cutting the right atrium of the heart and collecting it into chilled lithium-heparin-coated microcentrifuge tubes. Plasma was separated by centrifugation, aliquoted, and stored at –80°C until required for assay. Glucose was measured using INFINITY glucose reagent (Sigma, St Louis, MO), and insulin and leptin were assayed using commercially available enzyme-linked immunosorbent assay kits (Crystal Chem, Chicago, IL). All samples from within one study were assayed at the same time.

**Statistical analysis.** For each rat, the number of Fos-positive nuclei per section was counted bilaterally throughout the arcuate nucleus of the hypothalamus (12 sections per brain) and the mean calculated for each experimental group (expressed as mean ± SE nuclei/section). The Kruskal-Wallis test (a nonparametric ANOVA) was used to determine overall differences between more than two experimental groups. Comparisons between individual group means were determined using the nonparametric Mann-Whitney *U* test, with *P* < 0.05 accepted as a statistically significant difference. Differences between plasma insulin, leptin, and glucose levels in fed and fasted animals were compared by one-way ANOVA followed by Newman-Keuls posthoc test.

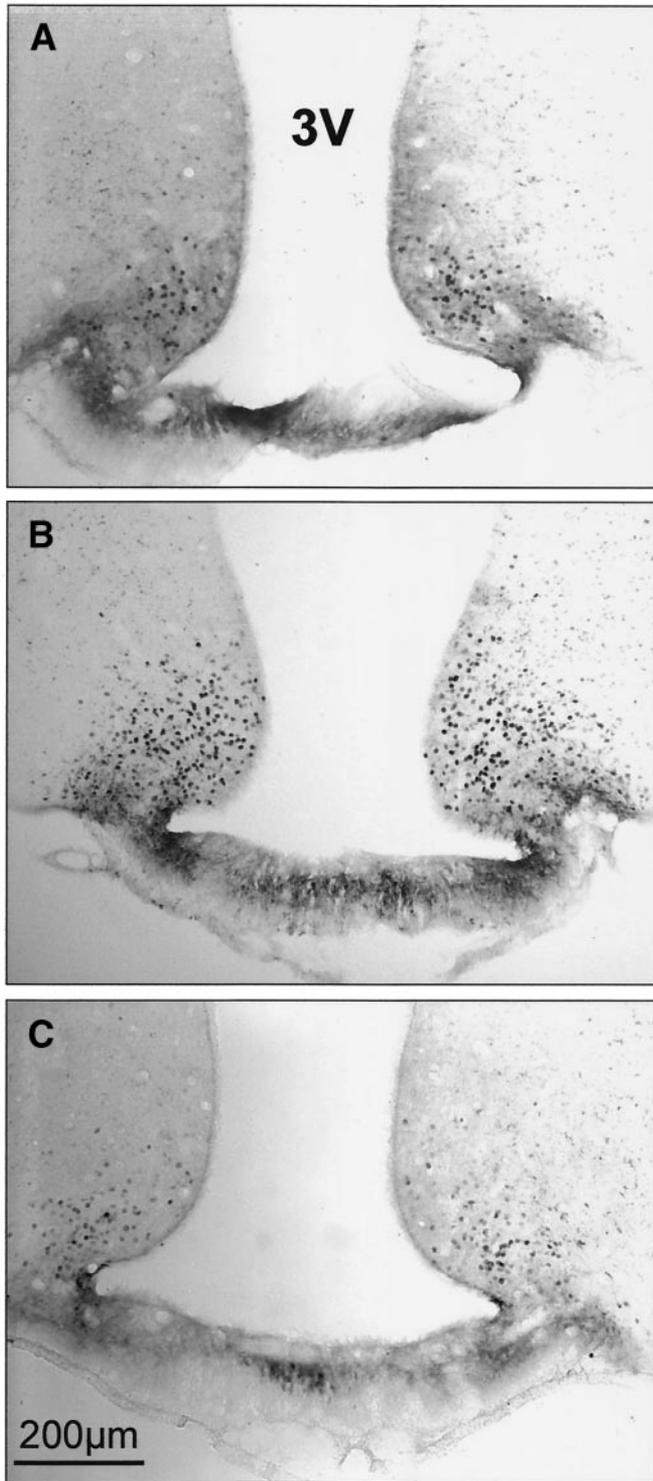
## RESULTS

### Effect of chronic central infusion of insulin or leptin on arcuate nucleus Fos response to GHRP-6 in fasted rats.

In agreement with previous data (15,16), 48-h fasted rats showed a threefold increase in the number of cells detected that expressed Fos protein after GHRP-6 administration compared with ad libitum-fed controls (Fig. 1A, B). These data were obtained from rats infused centrally with saline for 6 days (and fasted for the last 48 h as appropriate), indicating that the infusion protocol did not interfere with central GHS-responsiveness. In two separate studies, the Fos response to GHRP-6 in fasted rats infused centrally with saline (100 ± 12 and 98 ± 12 cells/section) was significantly greater than that in fed controls (30 ± 3 and 35 ± 6 cells/section, *P* = 0.004 and 0.001, respectively; Fig. 2A and B). Importantly, both central insulin infusion (Figs. 1C and 2A) and central leptin infusion (Fig. 2B) to fasted rats significantly suppressed this potentiated Fos response to GHRP-6. While central insulin infusion during fasting significantly reduced the GHRP-6-induced Fos response from 100 ± 12 to 55 ± 6 cells/section (*P* = 0.01), this was still significantly greater than that in fed controls (*P* = 0.01; Fig. 2A). However, infusion of leptin during fasting significantly reduced the Fos response to GHRP-6 from 98 ± 12 to 30 ± 5 cells/section (*P* = 0.001), and this was not different from the response seen in fed controls (Fig. 2B).

It is important to note that arcuate nucleus Fos expression was less than or equal to four cells per section in fasted animals infused centrally with either insulin or leptin and injected intravenously with saline (Fig. 2A and B), indicating that neither fasting nor central infusion for 6 days leads to induction of Fos protein. In agreement with previous reports (15,16), the arcuate nucleus was the only hypothalamic area to express Fos protein in response to GHRP-6 in both fed and fasted rats.

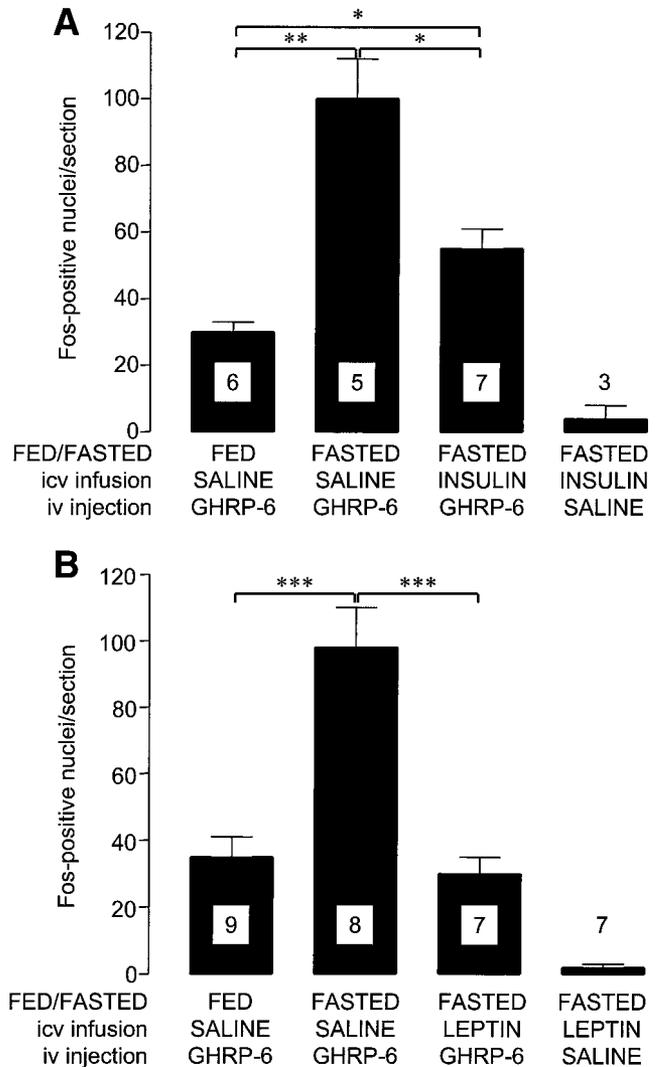
**Effect of chronic central infusion of insulin or leptin on plasma insulin, leptin, and glucose concentrations.** Based on data from two separate studies, a signif-



**FIG. 1.** Photomicrographs showing GHRP-6-induced Fos protein immunoreactivity in the arcuate nucleus of Wistar rats fed ad libitum and infused centrally with saline for 6 days (A), fasted for the last 48 h of ICV saline infusion (B), and fasted for the last 48 h of ICV insulin infusion (C). 3V, 3rd ventricle.

icant reduction in plasma insulin, leptin, and glucose was found in ICV saline-infused rats following a 48-h fast compared with ad libitum-fed controls. Furthermore, this reduction was unaffected by central infusion of either insulin or leptin (Fig. 3A–F).

Plasma insulin levels in 48-h-fasted rats infused cen-



**FIG. 2.** Mean  $\pm$  SE number of Fos-positive cells in the arcuate nucleus of Wistar rats injected intravenously with saline or GHRP-6 after central infusion of saline and insulin (2 mU/day) (A) or leptin (1.2  $\mu$ g/day) (B) for 6 days and allowed free access to food throughout (FED) or fasted for the last 48 h of the infusion period (FASTED). \* $P = 0.01$ , \*\* $P = 0.004$ , and \*\*\* $P = 0.001$ , Mann-Whitney  $U$  test. Numbers within or above columns indicate the number of animals in each group.

trally with either saline or insulin ( $0.4 \pm 0.1$  and  $0.3 \pm 0.05$  ng/ml, respectively) were significantly lower than ICV saline-infused fed controls ( $2.5 \pm 0.2$  ng/ml,  $P < 0.01$ ; Fig. 3A). Similarly, a 90% reduction in circulating leptin levels was observed in fasted rats infused with saline or insulin ( $0.2 \pm 0.1$  and  $0.35 \pm 0.1$  ng/ml, respectively) compared with fed controls ( $2.4 \pm 0.5$  ng/ml,  $P < 0.01$ ; Fig. 3B), and plasma glucose levels were reduced by 43% (fasted/ICV saline:  $84 \pm 8$ ; fasted/ICV insulin:  $73 \pm 6$  mg/dl) compared with fed rats ( $147 \pm 22$  mg/dl,  $P < 0.01$ ; Fig. 3C).

Similarly, plasma insulin levels were significantly lower in saline- or leptin-infused fasted rats ( $0.8 \pm 0.1$  and  $0.6 \pm 0.1$  ng/ml, respectively) compared with fed controls ( $1.8 \pm 0.3$  ng/ml,  $P < 0.05$ ; Fig. 3D)—plasma leptin levels were 84% lower in fasted rats (fed/ICV saline:  $0.9 \pm 0.2$ ; fasted/ICV saline:  $0.14 \pm 0.02$ ; fasted/ICV leptin:  $0.13 \pm 0.02$  ng/ml,  $P < 0.05$ ; Fig. 3E), and there was a 47% decrease in plasma glucose levels (fed/ICV saline:  $241 \pm 19$ ; fasted/ICV

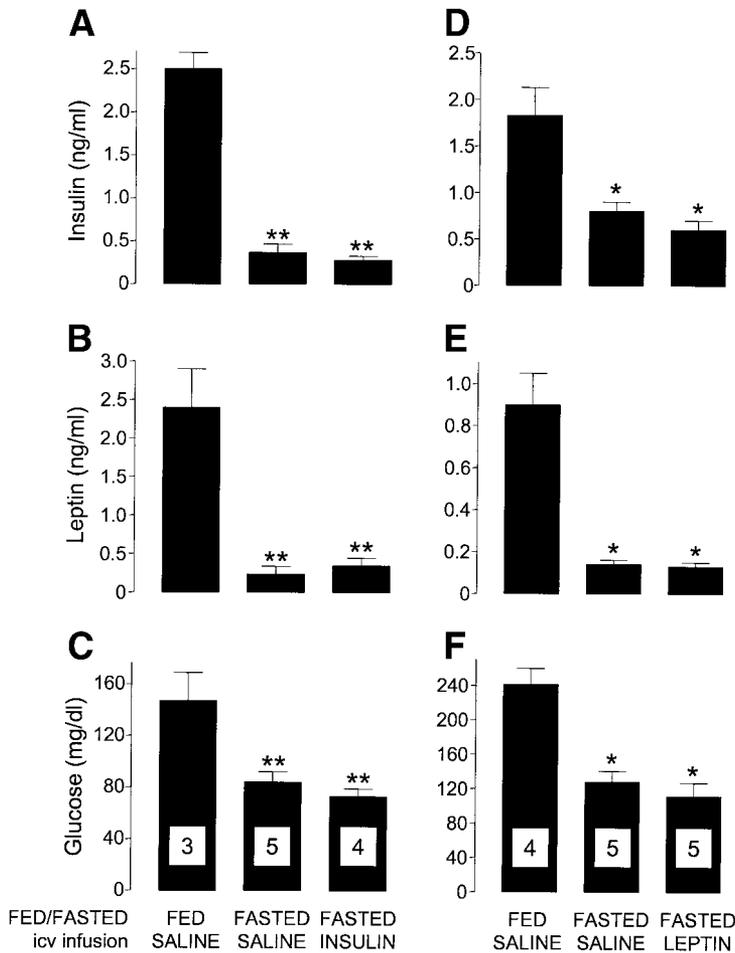


FIG. 3. Mean  $\pm$  SE plasma insulin, leptin, and glucose concentrations in male Wistar rats infused centrally for 6 days with saline, insulin (A–C), or leptin (D–F) and fasted for the last 48 h of the infusion (FASTED) or allowed free access to food throughout (FED). \* $P < 0.05$  and \*\* $P < 0.01$  vs. fed rats, ANOVA followed by Newman-Keuls posthoc test. Number of samples assayed for data in A–C and D–F is shown within the columns in C and F, respectively.

saline:  $128 \pm 12$ ; fasted/ICV leptin:  $111 \pm 16$  mg/dl,  $P < 0.05$ ; Fig. 3F).

**GHRP-6–induced Fos response in fed and fasted Zucker rats.** Very few Fos-positive cells were seen in the arcuate nucleus of Zucker rats after the administration of saline (lean/fed:  $3 \pm 1$  cells/section,  $n = 3$ ; lean/fasted:  $6 \pm 3$ ,  $n = 3$ ; obese/fed:  $3.6$ ,  $n = 2$ ; obese/fasted:  $8 \pm 5$ ,  $n = 3$ ). In agreement with the response seen in fed Wistar rats (15,16), administration of GHRP-6 to ad libitum–fed lean (+/?) Zucker rats resulted in the activation of  $45 \pm 8$  cells/section in the arcuate nucleus (Fig. 4). A significant increase in the Fos response to GHRP-6 was seen in lean (+/?) Zucker rats after a 48-h fast ( $110 \pm 20$  cells/section,  $P = 0.02$ ; Fig. 4). Interestingly, the Fos response to GHRP-6 in normally fed obese (*fa/fa*) Zucker rats ( $83 \pm 15$  cells/section) was almost twice that seen in fed lean (+/?) Zucker rats ( $P = 0.03$ ) and was not significantly different to the response seen in fasted lean (+/?) Zucker rats ( $P = 0.2$ ; Fig. 4). Furthermore, in obese (*fa/fa*) Zucker rats fasting did not further increase the Fos response to GHRP-6 ( $83 \pm 9$  cells/section; Fig. 4).

**Plasma insulin, leptin, and glucose concentrations in Zucker rats.** Obese Zucker rats displayed hyperinsulinemia and hyperleptinemia, whereas plasma glucose levels were similar in both lean and obese Zucker rats (Fig. 5A–C). Plasma insulin levels in ad libitum–fed Zucker rats were significantly lower in lean ( $5.7 \pm 0.8$  ng/ml) compared with obese rats ( $8.1 \pm 0.5$  ng/ml,  $P < 0.05$ ) and fell significantly in both lean ( $1.2 \pm 0.3$  ng/ml,  $P < 0.01$ ) and

obese ( $5.7 \pm 0.9$  ng/ml,  $P < 0.05$ ) rats following a 48-h fast (Fig. 5A). Obese Zucker rats had extremely elevated leptin levels compared with lean controls (lean/fed:  $4.4 \pm 0.7$  ng/ml; obese/fed:  $112.7 \pm 18.3$  ng/ml,  $P < 0.01$ ), which were not significantly altered after a 48-h fast ( $90.1 \pm 20.2$  ng/ml). A marked decrease in leptin levels was seen in lean Zucker rats following a 48-h fast ( $1.1 \pm 0.2$  ng/ml,  $P < 0.01$ ; Fig. 5B). Plasma glucose levels were not significantly different in ad libitum–fed lean and obese Zucker rats ( $405.5 \pm 30.6$  and  $461.7 \pm 65.1$  mg/dl, respectively; Fig. 5C). Following a 48-h fast, a significant decrease in glucose levels was seen in lean rats ( $311.9 \pm 18.3$  mg/dl,  $P < 0.05$ ) but not in obese rats ( $351.4 \pm 19.6$  mg/dl; Fig. 5C).

## DISCUSSION

Our first published reports of the central actions of synthetic GHS (now known to be ghrelin mimetics) described the effect of GHRP-6 to induce Fos protein expression in the arcuate nucleus of ad libitum–fed rats (36). It now seems clear, however, that this nucleus also contains a discrete subpopulation of neurons that only respond to these compounds in the fasting state. Thus, when the same dose of GHS (which was maximally effective in fed rats) was administered to 48-h–fasted rats, we saw a threefold increase in the number of cells expressing Fos protein compared with ad libitum–fed controls (15,16). We hypothesized that this increased responsiveness may reflect changes in circulating satiety factors. In the present study,

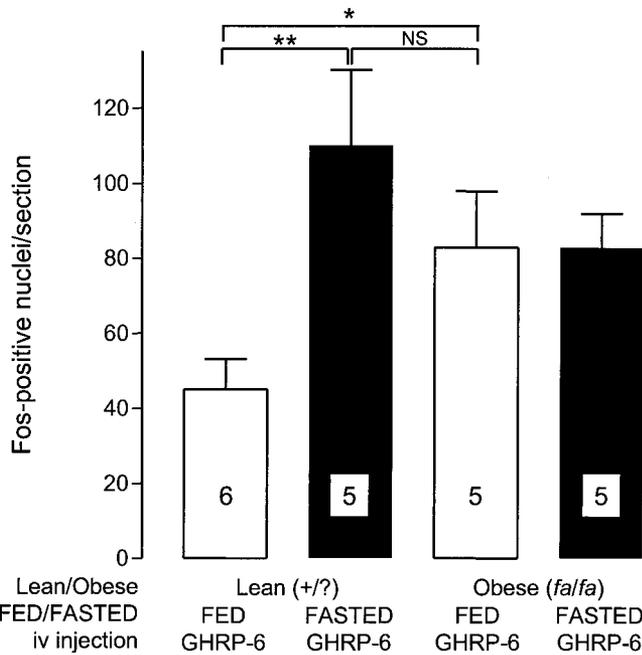


FIG. 4. Mean  $\pm$  SE number of Fos-positive cells in the arcuate nucleus of fed and 48-h fasted lean (+/?) and obese (*fa/fa*) Zucker rats following intravenous administration of GHRP-6. \* $P = 0.03$  and \*\* $P = 0.02$ , Mann-Whitney  $U$  test (NS, not significant). Numbers within columns indicate the number of animals in each group.

we show that chronic central infusion of either insulin or leptin to 48-h-fasted rats suppressed the potentiation of the Fos response to a maximally effective dose of GHRP-6 normally seen in the fasted state. Furthermore, leptin/insulin-insensitive obese Zucker rats showed a twofold greater Fos response to GHRP-6 compared with lean controls, despite manifest hyperleptinemia and hyperinsulinemia, and the Fos response was not further potentiated following a 48-h fast. In contrast, the marked decrease in leptin and insulin levels in lean Zucker rats was associated with a 2.5-fold increase in GHRP-6-induced Fos expression. These data suggest that a subpopulation of ghrelin-responsive cells in the arcuate nucleus is subject to inhibition by a central action of the satiety hormones, insulin and leptin.

As expected, circulating levels of insulin and leptin decreased significantly in rats fasted for 48 h, and this was associated with a large increase in the number of arcuate nucleus cells activated (i.e., expressed Fos) after systemic GHRP-6 administration. These peripherally produced hormones are thought to enter the central nervous system (CNS) via selective (receptor-mediated) transport mechanisms (37,38). This transport across the blood-brain barrier is affected both by fasting and obesity, resulting in decreased entry of these hormones into the CNS (39–41). By infusing insulin or leptin directly into the cerebral ventricles, we sought to circumvent this central decrease and indeed were able to suppress the increase in hypothalamic responsiveness to GHRP-6 normally seen in the fasted state. It seems likely that this is brought about by a direct central inhibitory action of these hormones on GHS-responsive circuits, as the low circulating levels of insulin and leptin achieved during fasting remained suppressed in rats infused centrally with either hormone.

While peripheral replacement of insulin or leptin might

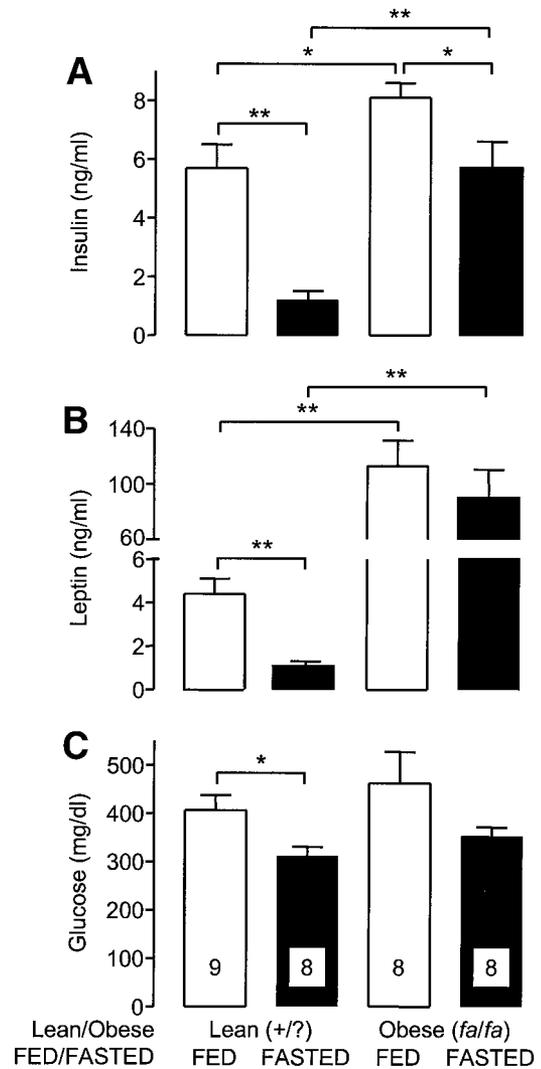


FIG. 5. Mean  $\pm$  SE plasma insulin (A), leptin (B), and glucose (C) concentrations in fed and 48-h-fasted lean (+/?) and obese (*fa/fa*) Zucker rats. \* $P < 0.05$  and \*\* $P < 0.01$ , ANOVA followed by Newman-Keuls posthoc test. Number of samples assayed for each group is shown within the columns in C.

be more a physiological approach to study the central effects of circulating satiety factors, it was important to avoid peripheral actions of these hormones (e.g., on blood glucose), as this could in itself alter central responsiveness to GHS. Therefore, we adapted a previously published protocol in which chronic ICV infusion of insulin prevented the increase in NPY mRNA in the arcuate nucleus of fasted rats without affecting plasma insulin or glucose levels (26) and used this for both insulin and leptin infusion. It is important to note that replacement of physiological levels of circulating leptin by continuous subcutaneous infusion has been shown to prevent the rise in NPY mRNA associated with a 70-h fast (42), and it therefore seems possible that this regimen could also prevent the increased activation of arcuate neurons by GHRP-6 during fasting.

The increased Fos response to GHRP-6 was not completely restored to fed control levels by ICV insulin infusion, and one possible explanation is that insulin-infused fasted rats may have had lower cerebrospinal fluid (CSF) insulin levels than fed (saline-infused) rats. Alternatively

insulin may act together with other circulating factors that are suppressed by fasting such as leptin or glucose to regulate the activity of GHS-responsive neurons. Certainly both insulin and leptin have receptors in the arcuate (18,19), have similar effects to suppress food intake (20,21) and appear to activate a common intracellular signaling pathway (phosphatidylinositol-3-OH kinase) (31,43). Whether coinfusion of the current doses of leptin and insulin to fasted rats would reveal some additive suppressive effects of these peptides on the central actions of GHS or merely reflect the "fed" phenotype remains to be seen.

The dose of leptin used probably produced CSF levels in excess of normal physiological values, since this dose has previously been shown to reduce food intake and body weight in rats (33,44). However, even chronic ICV infusion of this dose of leptin did not lead to a complete suppression of hypothalamic responsiveness to GHRP-6, as the Fos response in leptin-infused fasted rats was identical to that seen in normally fed rats. Furthermore, this action of leptin to reduce the number of arcuate neurons activated by GHS is only seen in fasted rats, as we previously found no difference in Fos response to GHS administered to ad libitum-fed rats infused centrally for 1 week with either saline or leptin (33). We cannot rule out the possibility that the inhibitory effect of leptin and insulin on the Fos response is not a direct action on arcuate neurons. Thus, we have previously demonstrated that somatostatin reduced the Fos response to GHS administration in fed rats (45), and leptin administration to fasted rats has been shown to increase somatostatin mRNA expression in the periventricular nucleus, probably by enhancing growth hormone feedback (46). It is not known whether insulin exerts a similar effect on somatostatin. Nonetheless, if this increased synthesis of somatostatin is matched by increased release in the arcuate nucleus, it might account, at least in part, for the reduced Fos response to GHRP-6 administration seen in fasted rats infused with leptin or insulin.

Another important observation of the present study is the demonstration of increased hypothalamic responsiveness to GHRP-6 in obese (*fa/fa*) Zucker rats. The *fa* mutation causes a defect in the leptin receptor (leptin resistance) and subsequently the development of insulin resistance. Certainly, neither central nor peripheral leptin or insulin administration to obese Zucker rats reduces food intake, body weight, or hypothalamic NPY gene expression at doses that are effective in lean controls (20,28,30). The most likely explanation for the almost twofold increase in the Fos response to GHRP-6 seen in obese Zucker rats is the inability of leptin and insulin to exert an inhibitory effect on arcuate neurons, despite the high concentrations found in the circulation. Fed obese Zucker rats display a Fos response to GHRP-6, similar to that seen in lean Zucker rats following a 48-h fast. Since in obese Zucker rats leptin and insulin cannot signal information to the hypothalamus about the size of the energy store, it is perceived to be in a fasted state (even when fed) and the hypothalamus displays increased responsiveness to GHS administration. Perhaps not surprisingly, the Fos response to GHRP-6 in obese Zucker rats was not further increased after a 48-h fast in direct contrast to the marked increase seen in lean Zucker rats, which was associated

with clear decreases in plasma insulin and leptin levels. Similarly, no change in arcuate preproneuroptide Y mRNA levels was seen in obese Zucker rats following a 72-h fast, while an almost twofold increase in was reported in lean Zucker rats (47).

The neurochemical identity of the cells recruited by fasting (or leptin/insulin resistance) to become activated by GHSs remains to be determined. In ad libitum-fed rats, NPY and growth hormone-releasing hormone (GHRH) cells represent the major populations of cells activated by GHRP-6 (51 and 23%, respectively), although it is clear that these are not the only cells activated (13). Although it is well established that GHRH neurones are targets for GHS (12,13,48,49), it seems unlikely that these are recruited to activity during fasting. In male rats, arcuate GHRH mRNA is decreased during 48-h fasting (50), and this is associated with suppressed pulsatile growth hormone (GH) secretion (51). Furthermore, the stimulatory effect of GHRP-6 on GH release is blunted in fasted rats (52). Thus, the paradox of increased GHS-induced neuronal activation in the arcuate nucleus together with decreased GH output suggests that additional GHRH neurons are not activated by GHS during fasting. Rather, the neurons recruited by fasting to activation by GHS may release an inhibitor of GH secretion such as NPY (53). In support of this, fasting increases the number of cells expressing NPY/AgRP mRNA levels in the arcuate nucleus (54), and these increases can be prevented by insulin or leptin replacement (26,42). It is well established that, at least in fed rats, NPY neurons express GHS-R mRNA (12) and that GHS/ghrelin administration increases NPY and AgRP mRNA expression (9–11,14) and induces *c-fos* in NPY cells (9,13), indicating that these cells are central targets for GHSs/ghrelin. Taken together with our data showing that the number of cells activated by GHSs/ghrelin is increased by fasting and suppressed by central insulin or leptin infusion, strongly suggests that a greater number of NPY cells could be activated by GHSs/ghrelin in fasted rats.

The present findings, that the hypothalamic circuits through which GHRP-6 (and hence, ghrelin) acts are sensitive to perturbations in energy balance (fasting and leptin/insulin resistance) and subject to inhibitory control by insulin and leptin, lend further support to the hypothesis that ghrelin may play a physiological role in the regulation of food intake as well as an adaptive role in the response to negative energy balance.

#### ACKNOWLEDGMENTS

This research was supported by the EC Fifth Framework (QLRT-1999-02038) and the MRC (U.K.).

The authors thank Professor Claes Ohlsson and Maud Petersson at the Department of Clinical Pharmacology, Sahlgrenska University Hospital, Gothenburg, Sweden, for performing the insulin, leptin, and glucose assays.

#### REFERENCES

1. Kojima M, Kosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K: Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature* 402:656–660, 1999
2. Howard AD, Feighner SD, Cully DF, Arena JP, Liberatore PA, Rosenblum CI, Hamelin M, Hreniuk DL, Palyha OC, Anderson J, Paresse PS, Diaz C, Chou M, Liu KK, McKee KK, Pong S-S, Chaung L-Y, Elbrecht A, Dashkevich M, Heavens R, Rigby M, Sirinathsinghji DJS, Dean DC, Melillo DG, Patchett AA, Nargund R, Griffin PR, DeMartino JA, Gupta SK, Schaeffer JM, Smith

- RG, Van der Ploeg LHT: Receptor in pituitary and hypothalamus that functions in growth hormone release. *Science* 273:974–977, 1996
3. Wren AM, Small CJ, Ward HL, Murphy KG, Dakin CL, Taheri S, Kennedy AR, Roberts GH, Morgan DGA, Ghatei MA, Bloom SR: The novel hypothalamic peptide ghrelin stimulates food intake and growth hormone secretion. *Endocrinology* 141:4325–4328, 2000
  4. Locke W, Kirgis HD, Bowers CY, Abdoh AA: Intracerebroventricular growth hormone-releasing peptide-6 stimulates eating without affecting plasma growth hormone responses in rats. *Life Sci* 56:1347–1352, 1995
  5. Tschöp M, Smiley DL, Heiman ML: Ghrelin induces adiposity in rodents. *Nature* 407:908–913, 2000
  6. Lall S, Tung LYC, Ohlsson C, Jansson J-O, Dickson SL: Growth hormone (GH)-independent stimulation of adiposity by GH secretagogues. *Biochem Biophys Res Comm* 280:132–138, 2001
  7. Wren AM, Small CJ, Abbott CR, Dhillo WS, Seal LJ, Cohen MA, Batterham RL, Teheri S, Stanley SA, Ghatei MA, Bloom SR: Ghrelin causes hyperphagia and obesity in rats. *Diabetes* 50:2540–2547, 2001
  8. Cummings DE, Purnell JQ, Frayo RS, Schmidova K, Wisse BE, Weigle DS: A preprandial rise in plasma ghrelin levels suggests a role in meal initiation in humans. *Diabetes* 50:1714–1719, 2001
  9. Nakazato M, Murakami N, Date Y, Kojima M, Matsuo H, Kangawa K, Matsukura S: A role for ghrelin in the central regulation of feeding. *Nature* 409:194–198, 2001
  10. Asakawa A, Inui A, Kaga T, Yuzuriha H, Nagata T, Ueno N, Makino S, Fujimiya M, Nijima A, Fujino MA, Kasuga M: Ghrelin is an appetite-stimulatory signal from stomach with structural resemblance to motilin. *Gastroenterology* 120:337–345, 2001
  11. Shintani M, Ogawa Y, Ebihara K, Aizawa-Abe M, Miyanaga F, Takaya K, Hayashi T, Inoue G, Hosoda K, Kojima M, Kangawa K, Nakao K: Ghrelin, an endogenous growth hormone secretagogue, is a novel orexigenic peptide that antagonizes leptin action through the activation of hypothalamic neuropeptide YY1 receptor pathway. *Diabetes* 50:227–232, 2001
  12. Willesen MG, Kristensen P, Rømer J: Co-localization of growth hormone secretagogue receptor and NPY mRNA in the arcuate nucleus of the rat. *Neuroendocrinology* 70:306–316, 1999
  13. Dickson SL, Luckman SM: Induction of *c-fos* mRNA in neuropeptide Y and growth hormone-releasing hormone neurons in the rat arcuate nucleus following systemic injection of the growth hormone secretagogue, GHRP-6. *Endocrinology* 138:771–777, 1997
  14. Kamegai J, Tamura H, Shimizu T, Ishii S, Sugihara H, Wakabayashi I: Central effect of ghrelin, an endogenous growth hormone secretagogue, on hypothalamic peptide gene expression. *Endocrinology* 141:4797–4800, 2000
  15. Luckman SM, Rosenzweig I, Dickson SL: Activation of arcuate nucleus neurones by systemic administration of leptin and growth hormone-releasing peptide-6 in normal and fasted rats. *Neuroendocrinology* 70:93–100, 1999
  16. Hewson AK, Dickson SL: Systemic administration of ghrelin induces Fos and Egr-1 proteins in the hypothalamic arcuate nucleus of fasted and fed rats. *J Neuroendocrinol* 12:1047–1049, 2000
  17. Baskin DG, Figlewicz Lattemann D, Seeley RJ, Woods SC, Porte D Jr, Schwartz MW: Insulin and leptin: dual adiposity signals to the brain for the regulation of food intake and body weight. *Brain Res* 848:114–123, 1999
  18. Marks JL, Porte Jr D, Stahl WL, Baskin DG: Localization of insulin receptor mRNA in rat brain by in situ hybridization. *Endocrinology* 127:3234–3236, 1990
  19. Mercer JG, Hoggard N, Williams LM, Lawrence CB, Hannah LT, Trayhurn P: Localization of leptin receptor mRNA and the long form splice variant (Ob-Rb) in mouse hypothalamus and adjacent brain regions by in situ hybridization. *FEBS Lett* 387:113–116, 1996
  20. Ikeda H, West DB, Pustek JJ, Figlewicz DP, Greenwood MRC, Porte D Jr, Woods SC: Intraventricular insulin reduces food intake and body weight of lean but not obese Zucker rats. *Appetite* 7:381–386, 1986
  21. Halaas JL, Boozer C, Blair-West J, Fidathusein N, Denton DA, Friedman JM: Physiological response to long-term peripheral and central leptin infusion in lean and obese mice. *Proc Natl Acad Sci USA* 94:8878–8883, 1997
  22. Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM: Positional cloning of the mouse obese gene and its human homologue. *Nature* 372:425–432, 1994
  23. Lee GH, Proenca R, Montez JM, Carroll KM, Darvishzadeh JG, Lee JI, Friedman JM: Abnormal splicing of the leptin receptor in diabetic mice. *Nature* 379:632–635, 1996
  24. Phillips MS, Liu QY, Hammond HA, Dugan V, Hey PJ, Caskey CT, Hess JF: Leptin receptor missense mutation in the fatty Zucker rat. *Nat Genet* 13:18–19, 1996
  25. Brüning JC, Gautam D, Burks DJ, Gillette J, Schubert M, Orban PC, Klein R, Krone W, Müller-Wieland D, Kahn CR: Role of brain insulin receptor in control of body weight and reproduction. *Science* 289:2122–2125, 2000
  26. Schwartz MW, Sipols AJ, Marks JL, Sanacora G, White JD, Scheurink A, Kahn SE, Baskin DG, Woods SC, Figlewicz DP, Porte D Jr: Inhibition of hypothalamic neuropeptide Y gene expression by insulin. *Endocrinology* 130:3608–3616, 1992
  27. Marks JL, Waite K, Li M: Effects of streptozotocin-induced diabetes mellitus and insulin treatment on neuropeptide Y mRNA in the rat hypothalamus. *Diabetologia* 36:497–502, 1993
  28. Schwartz MW, Marks JL, Sipols AJ, Baskin DG, Woods SC, Kahn SE, Porte D Jr: Central insulin administration reduces neuropeptide Y mRNA expression in the arcuate nucleus of food-deprived lean (Fa/Fa) but not obese (fa/fa) Zucker rats. *Endocrinology* 128:2645–2647, 1991
  29. Stephens TW, Basinski M, Bristow PK, Bue-Valleskey JM, Burgett SG, Craft L, Hale J, Hoffmann J, Hsiung HM, Kriauciunas A, McKellar W, Rosteck PR Jr, Schoner B, Smith D, Tinsley FC, Zhang X-Y, Heiman M: The role of neuropeptide Y in the antiobesity action of the obese gene product. *Nature* 377:530–532, 1995
  30. Cusin I, Rohner-Jeanrenaud F, Stricker-Krongrad A, Jeanrenaud B: The weight-reducing effect of an intracerebroventricular bolus injection of leptin in genetically obese *fa/fa* rats: reduced sensitivity compared with lean animals. *Diabetes* 45:1446–1450, 1996
  31. Spanswick D, Smith MA, Mirshamsi S, Routh VH, Ashford MLJ: Insulin activates ATP-sensitive K<sup>+</sup> channels in hypothalamic neurons of lean, but not obese rats. *Nature Neuroscience* 3:757–758, 2000
  32. Spanswick D, Smith MA, Groppi VE, Logan SD, Ashford MLJ: Leptin inhibits hypothalamic neurons by activation of ATP-sensitive potassium channels. *Nature* 390:521–525, 1997
  33. Tung YCL, Hewson AK, Dickson SL: Actions of leptin on growth hormone secretagogue-responsive neurones in the rat hypothalamic arcuate nucleus recorded in vitro. *J Neuroendocrinol* 13:209–215, 2001
  34. Paxinos G, Watson C: *The Rat Brain in Stereotaxic Coordinates*. London, Academic Press, 1992
  35. Shu A, Ju G, Fan L: The glucose oxidase-DAB-nickel method in peroxidase histochemistry of the nervous system. *Neurosci Lett* 85:169–171, 1988
  36. Dickson SL, Leng G, Robinson ACAF: Systemic administration of growth hormone-releasing peptide (GHRP-6) activates hypothalamic arcuate neurones. *Neuroscience* 53:303–306, 1993
  37. Schwartz MW, Sipols A, Kahn SE, Lattemann DF, Taborsky GJ Jr, Bergman RN, Woods SC, Porte D Jr: Kinetics and specificity of insulin uptake from plasma into cerebrospinal fluid. *Am J Physiol* 259:E378–E383, 1990
  38. Banks WA, Kastin AJ, Huang W, Jaspan JB, Maness LM: Leptin enters the brain by a saturable system independent of insulin. *Peptides* 17:305–311, 1996
  39. Steffens AB, Scheurink AJW, Porte D Jr, Woods SC: Penetration of peripheral glucose and insulin into cerebrospinal fluid in rats. *Am J Physiol* 255:R200–R204, 1988
  40. Kastin AJ, Akerstrom V: Fasting, but not adrenalectomy, reduces transport of leptin into the brain. *Peptides* 21:679–682, 2000
  41. Burguera B, Couce ME, Curran GL, Jensen MD, Lloyd RV, Cleary MP, Poduslo JF: Obesity is associated with a decreased leptin transport across the blood-brain barrier in rats. *Diabetes* 49:1219–1223, 2000
  42. Ahima RS, Kelly J, Elmquist JK, Flier JS: Distinct physiologic and neuronal responses to decreased leptin and mild hyperleptinemia. *Endocrinology* 140:4923–4931, 1999
  43. Niswender KD, Morton GJ, Stearns WH, Rhodes CJ, Myers MG Jr, Schwartz MW: Key enzyme in leptin-induced anorexia. *Nature* 413:794–795, 2001
  44. Tannenbaum GS, Gurd W, Lapointe M: Leptin is a potent stimulator of spontaneous pulsatile growth hormone (GH) secretion and the GH response to GH-releasing hormone. *Endocrinology* 139:3871–3875, 1998
  45. Dickson SL, Viltart O, Bailey AR, Leng G: Attenuation of the growth hormone secretagogue induction of Fos protein in the rat arcuate nucleus by central somatostatin action. *Neuroendocrinology* 66:188–194, 1997
  46. Carro E, Señaris RM, Seoane LM, Frohman LA, Arimura A, Casanueva FF, Diéguez C: Role of growth hormone (GH)-releasing hormone and somatostatin on leptin-induced GH secretion. *Neuroendocrinology* 69:3–10, 1999
  47. Sanacora G, Kershaw M, Finkelstein JA, White JD: Increased hypothalamic content of prepro-neuropeptide Y messenger ribonucleic acid in genetically obese Zucker rats and its regulation by food deprivation. *Endocrinology* 127:730–737, 1990
  48. Kamegai J, Hasegawa O, Minami S, Sugihara H, Wakabayashi I: The growth hormone-releasing peptide KP-102 induces *c-fos* expression in the arcuate nucleus. *Mol Brain Res* 39:153–159, 1996
  49. Tannenbaum GS, Lapointe M, Beaudet A, Howard AD: Expression of

- growth hormone secretagogue-receptors by growth hormone-releasing hormone neurons in the mediobasal hypothalamus. *Endocrinology* 139: 4420–4423, 1998
50. Bruno JF, Olchovsky D, White J, Leidy JW, Song J, Berelowitz M: Influence of food deprivation in the rat on hypothalamic expression of growth hormone-releasing factor and somatostatin. *Endocrinology* 127:2111–2116, 1990
51. Tannenbaum GS, Rorstad O, Brazeau P: Effects of prolonged food deprivation on the ultradian growth hormone rhythm and immunoreactive somatostatin tissue levels in the rat. *Endocrinology* 104:1733–1738, 1979
52. Carro E, Seoane LM, Señaris R, Casanueva FF, Dieguez C: Leptin increases in vivo GH responses to GHRH and GH-releasing peptide-6 in food-deprived rats. *Eur J Endocrinol* 142:66–70, 2000
53. Catzeflis C, Pierroz DD, Rohner-Jeanrenaud F, River JE, Sizonenko PC, Aubert ML: Neuropeptide Y administered chronically into the lateral ventricle profoundly inhibits both the gonadotropic and somatotrophic axis in adult female rats. *Endocrinology* 132:224–234, 1993
54. Hahn TM, Breininger JF, Baskin DG, Schwartz MW: Coexpression of Agrp and NPY in fasting-activated hypothalamic neurons. *Nature Neuroscience* 1:271–272, 1998