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Activation of Arcuate Nucleus Neurons by Systemic Administration of Leptin and Growth Hormone-Releasing Peptide-6 in Normal and Fasted Rats

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Key Words
Growth hormone · Neuropeptide Y · Leptin · Pro-opiomelanocortin · Growth hormone secretagogues · Fasting

Abstract
Both leptin and growth hormone secretagogues are believed to have stimulatory effects on the hypothalamic growth hormone pulse generator, though whether these are achieved through the same pathway is unknown. Systemic administration of a normally maximal effective dose of the growth hormone secretagogue GHRP-6 to male rats causes the induction of c-Fos protein in the ventromedial aspect of the hypothalamic arcuate nucleus. The effect of the same dose of GHRP-6 is, however, much greater in animals that have been fasted for 48 h, suggesting that in the food-replete rat, arcuate neurons either show reduced sensitivity to endogenous growth hormone secretagogues or they are under the tonic inhibitory influences of other factors. The major populations of arcuate neurons activated by GHRP-6 have been shown to contain neuropeptide Y or growth hormone-releasing factor, while leptin is thought to be inhibitory to neuropeptide Y neurons. Leptin did not alter the response of the rats to GHRP-6. However, it was able by itself to induce c-Fos protein immunoreactivity in the ventral, including the ventrolateral, arcuate nucleus of fasted rats. This is a clear demonstration of the acute activation of arcuate neurons in the rat following systemic leptin injection and suggests that GHRP-6 and leptin act on the growth hormone axis via different pathways.

Introduction

The pulsatile pattern of growth hormone (GH) secretion observed in the male rat is abolished during fasting [1], presumably forming part of the mechanism to help conserve energy stores when adverse metabolic conditions prevail. The mechanism underlying this fasting-induced suppression of GH secretion is not understood, although it seems likely that it involves a changing balance in the output of the two neuroendocrine systems that are primarily involved in the regulation of pulsatile GH secretion: the GH-releasing factor (GRF) neurons of the arcuate nucleus [2] that stimulate GH secretion [3, 4] and the inhibitory somatostatin neurons [5] of the periventricular nucleus [6]. Consistent with this hypothesis, GRF

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mRNA expression is lower in fasted than in fed rats, however, there is no change in the expression of somatostatin mRNA [7]. Despite this, administration of somatostatin antiserum has been shown to restore GH pulses in fasted rats [8], suggesting that increased somatostatin tone is also important for the fasting-induced suppression of pulsatile GH secretion.

Recently, the pulsatile secretion of GH was found to be augmented in both normal and in fasted rats by an intracerebroventricular infusion of leptin [9–11], the so-called satiety hormone produced by white adipose tissue [12–15]. It may be that leptin influences the GH pulse generating mechanism directly since there is immunocytochemical evidence for leptin receptors on subpopulations of GRF and somatostatin neurons, though it is not known which form of the leptin receptor this represents [16]. It should be noted that mRNA for the signal-transducing, long form of the receptor is not present in the periventricular nucleus and this receptor is, therefore, unlikely to be present on native somatostatin neurons [17, 18], even though leptin can inhibit somatostatin release from dispersed hypothalamic cultures [19]. Alternatively, leptin may act indirectly through neurons known to possess the long form of its receptor, including neuropeptide Y (NPY) and pro-opiomelanocortin (POMC) neurons of the hypothalamic arcuate nucleus [20, 21]. NPY neurons participate in a number of important metabolic and neuroendocrine functions, many of which are influenced by circulating levels of leptin [22, 23]. NPY gene expression is upregulated in leptin-deficient *ob/ob* mice [22, 23] and in fasted rats, that have low leptin levels [24]. Conversely, POMC mRNA in the arcuate nucleus is reduced in *ob/ob* mice and fasted rats [25, 26], and at least some of the effects of leptin are mediated through melanocortin receptors [27].

Another possible mechanism that might account for the suppression of GH secretion in fasting rats is that there is a change in the release and/or actions of an endogenous ligand for the GH secretagogue receptor. The GH secretagogues are a group of compounds, including both peptides and non-peptides, that increase GH release by a direct pituitary action [28] and also by a central mechanism that includes altering the output of the GRF-somatostatin pulse generating system. The secretagogue receptor has been cloned recently by Guan et al. [29] and its distribution mapped in the brain. Messenger RNA for the receptor is present, though not exclusively, in GRF neurons [30]. The GH secretagogues activate a subpopulation of cells in the arcuate nucleus including some that fulfil multiple electrophysiological criteria for identification as GRF neurons [31]. GH secretagogues induce the expression of c-*fos* protein in the arcuate nucleus [31] and c-*fos* mRNA has been localised to a population of GRF neurons [32, 33]. In addition, a major population of neurons activated by the secretagogue GH-releasing peptide (GHRP-6) appear to be NPYergic [32].

We hypothesised, since in the fasted state there is a reduction in circulating levels of leptin [24] and an upregulation of leptin receptors [34–36], that in this situation we might identify an interaction of exogenously administered leptin with neurons activated by GH secretagogues. In order to mimic the natural presentation of leptin to neurons we have chosen to administer the peptide intravenously. The available data suggest that both leptin and the secretagogues are stimulatory on the GH axis [9–11]. However, leptin has an inhibitory action on the synthetic activity of NPY neurons [24], which are a major population activated by the growth hormone secretagogue GHRP-6 [32]. Here, and in a previous abstract [37], we have used c-*fos* immunocytochemistry to measure the activity of hypothalamic neurons in response to leptin and GHRP-6 in normal and fasted rats.

Materials and Methods

Animals and Preparation of Tissue

Adult male rats (total of 63 animals; 310–450 g) of the Porton-Wistar strain were kept under a 14-hour day:10-hour night lighting regime with free access to food and water. All procedures were carried out in accordance to the UK Animal Scientific Procedures Act. On day 1 rats were intravenously (i.v.) cannulated under brief tribromoethanol/amyl hydrate (10 mg/kg, intraperitoneal injection) anaesthesia. A Silastic tube attached to a short length of polyethylene cannula was implanted chronically into the right external jugular vein and exteriorised at the nape of the neck. The cannula was plugged with Teflon-coated, braided silver-coated copper wire (Biomed wire, Cooner Wire Co., USA), attached to a stainless steel pin that fitted the end of the cannula tightly. On day 2 half of the rats had their food removed for 48 h. On the day of experimentation, 4 h after lights on (day 4), the plug was removed and the cannula extended with tubing, that contained heparinised saline. This allowed the animal free movement during i.v. injections. Recombinant murine leptin (1 mg/ml; Pepro Tech EC Ltd., London, UK), or water vehicle, was administered at 2.5 μg/g body weight. This was followed, 15 min later, by 100 μg GHRP-6 (Bachem, Saffron Walden, UK) dissolved in heparinised saline or saline vehicle. This dose of GHRP-6 has been shown to cause maximal c-*fos* protein induction in arcuate nucleus neurons of normal male rats after 90 min [31]. The rats were terminally anaesthetised with sodium pentobarbitone (60 mg/kg body weight, i.v.) 90 min after the second injection and were perfused transcardially with heparinised isotonic saline followed by 4% paraformaldehyde in 0.1 *M* phosphate buffer (PB; pH 7.4). The brains were removed and postfixed for 2 h in the same fixative. They were then transferred to 30% sucrose in 0.1 *M* PB for 24 h for cryoprotection.

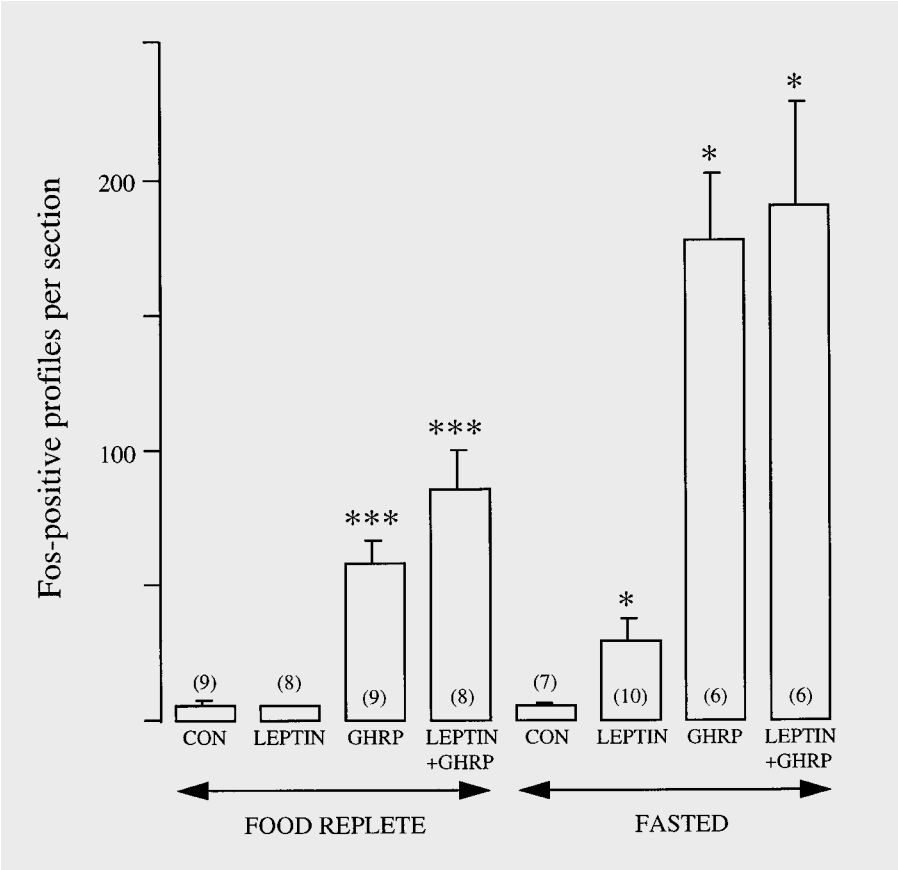

Fig. 1. The number of c-Fos protein-positive profiles per section in the arcuate nucleus of food-replete and fasted rats. The control group (CON) received two injections of vehicle. Bars represent the mean ± S.E.M. (figures in brackets show the number of animals per group). *** $p < 0.0001$ versus food-replete control group; * $p < 0.005$ versus fasted control group.

Immunocytochemistry

Brains were frozen and 30-μm equidistant sections were cut throughout the arcuate nucleus using a sledge microtome. Endogenous peroxidases were deactivated by treatment of the sections with 20% methanol, 1.5% hydrogen peroxide and 0.2% Triton X-100 in 0.1 *M* PB for 30 min. Sections were preincubated for 1 h in 0.1 *M* PB containing 0.3% Triton X-100 and 1% normal sheep serum at room temperature and then in the same buffer containing a rabbit polyclonal anti-Fos antibody (Ab-2, PC05, Oncogene Science Inc., New York), diluted 1:1,000, overnight at 4°C. Following washing the sections were incubated in a peroxidase-labelled antirabbit IgG antibody (Vector Laboratories Ltd., Peterborough, UK), diluted 1:500, overnight at 4°C. Black nuclear c-*fos* protein immunoreactive staining was visualised using nickel intensified diaminobenzidine. Briefly, sections were washed in sodium acetate buffer (0.1 *M*, pH 6.0) before incubation in the colour reaction solution: 2.5% nickel sulphate, 0.2% glucose, 0.04% ammonium chloride, 0.025% diaminobenzidine, approximately 30 units/ml glucose oxidase (Type VII-S; Sigma, Poole, UK) in the same buffer. The reaction was followed using a microscope and terminated with 0.1 *M* acetate buffer. In control experiments, using sections from GHRP-6-treated animals, c-*fos* protein immunostaining was prevented by omission of either the primary or secondary antibodies (results not shown). The specificity of the primary antibody has been shown by liquid-phase adsorption with N-terminal c-*fos* protein [38].

Analysis

Analysis was carried out blind on coded slides; c-*fos*-positive nuclear profiles were counted bilaterally in 15–20 sections per animal throughout the entire rostrocaudal extent of the arcuate nucleus according to the atlas of Swansson [39]. Sections were equivalent anatomically and in number between all groups. The number of c-*fos*-positive profiles per section was calculated for each animal and these figures were meaned to give values for each experimental group. GHRP-6- or leptin-treated groups were compared to their relevant control groups by the Mann-Whitney U test. Variability across treatment groups was analysed by non-parametric analysis of variance (Kruskal-Wallis test). Interactions between treatments were assessed using the Satterthwaite technique to determine degrees of freedom.

Results

Analysis of variance detected a highly significant variation between the number of c-*fos*-positive profiles in the arcuate nucleus of different experimental groups (Kruskal-Wallis test, $p < 0.0001$; fig. 1). In normal, food-replete male rats there were 6 ± 2 Fos-positive profiles per

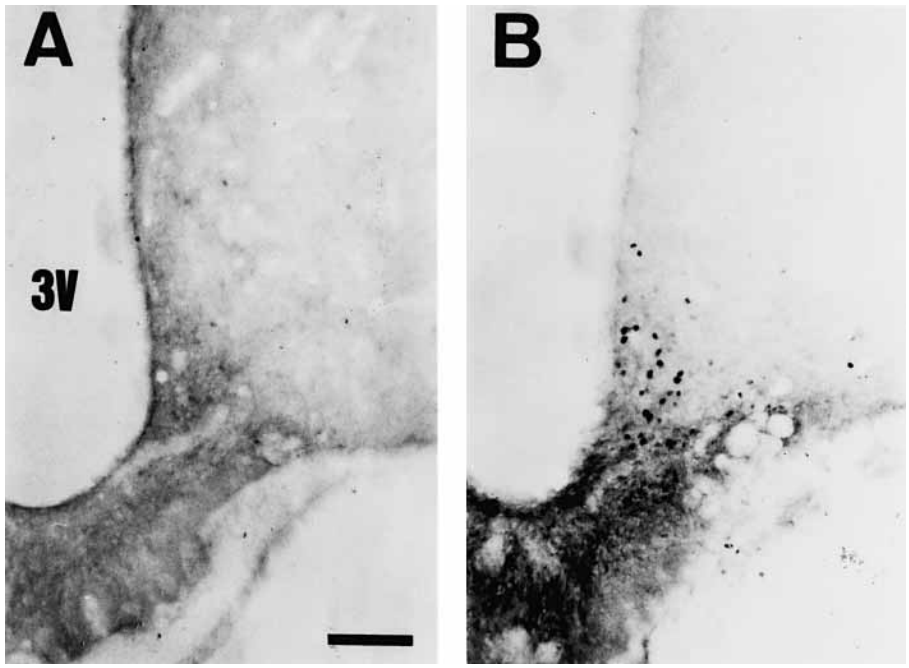

Fig. 2. The expression of Fos immunoreactivity in the hypothalamic arcuate nucleus of food-replete rats. **A** Leptin injection did not induce c-Fos immunoreactivity. **B** GHRP-6 caused a significant induction of c-Fos protein mainly in neurons of the ventromedial ARC. Bar represents 100 μm. 3V = Third ventricle. Sections are equivalent to plate 27 in Swanson [39].

arcuate nucleus section and this was not changed by systemic administration of leptin (6 ± 1 profiles per section; two-way Mann-Whitney U test, $p > 0.05$). Intravenous injection of GHRP-6 caused a significant induction of c-*fos* protein in the arcuate nucleus (58 ± 8 profiles per section; $p < 0.0001$; fig. 2), as previously reported [31]. C-*fos*-positive neurons were present throughout the rostrocaudal extent of the arcuate nucleus, but were absent from other hypothalamic nuclei. In the rostral arcuate nucleus there was staining throughout the nucleus, while in the middle sections there was a definite clustering of responsive neurons in the ventromedial arcuate with few in the ventrolateral or dorsomedial aspects of the nucleus. In more caudal sections there were c-*fos*-positive neurons in the ventromedial and ventrolateral regions, but with few in the dorsomedial arcuate. Administration of leptin 15 min before GHRP-6 did not significantly alter the response to GHRP-6 alone (leptin + GHRP-6, 85 ± 15 profiles per section).

Systemic administration of leptin caused a significant increase in the number of neurons expressing c-*fos* protein in the arcuate nucleus of fasted rats (5 ± 2 to 29 ± 9; $p < 0.005$; fig. 1, 3). Though most of the neurons activated by leptin were in the ventromedial arcuate, there was a more even distribution than that seen with GHRP-6 alone, with a higher proportion in ventrolateral aspects of the nucleus. The GHRP-6 ($p < 0.005$) and leptin + GHRP-6 ($p < 0.005$) groups both had significantly more c-*fos*-positive neurons in the arcuate nucleus compared to the fasted control group.

There was no significant interaction between GHRP-6 and leptin treatments in either the normal or fasted groups (both $p > 0.05$). By contrast, GHRP-6 caused the induction of c-*fos* protein in significantly more neurons in fasted rats than it did in normal rats ($p < 0.005$; fig. 1). This was true also when GHRP-6 was administered after an injection of leptin ($p < 0.05$).

Discussion

Previously, we have shown that a single systemic injection of GHRP-6 causes the induction of the immediate-early gene, c-*fos*, in subpopulations of neurons of the hypothalamic arcuate nucleus, including those that contain NPY and GRF [31, 32]. Here, in male rats deprived of food for 48 h, there is a much greater effect of the same dose of GHRP-6 on arcuate neurons, though it should be noted that GHRP-6 still did not cause the induction of c-*fos* protein in other areas of the hypothalamus (results not shown), even though its receptors have been located outside the arcuate nucleus [29].

The increased activation seen in the arcuate nucleus in the fasted state might be explained, firstly, due to the activation of other neuron subpopulations, such as those that contain tyrosine hydroxylase or POMC-derived peptides

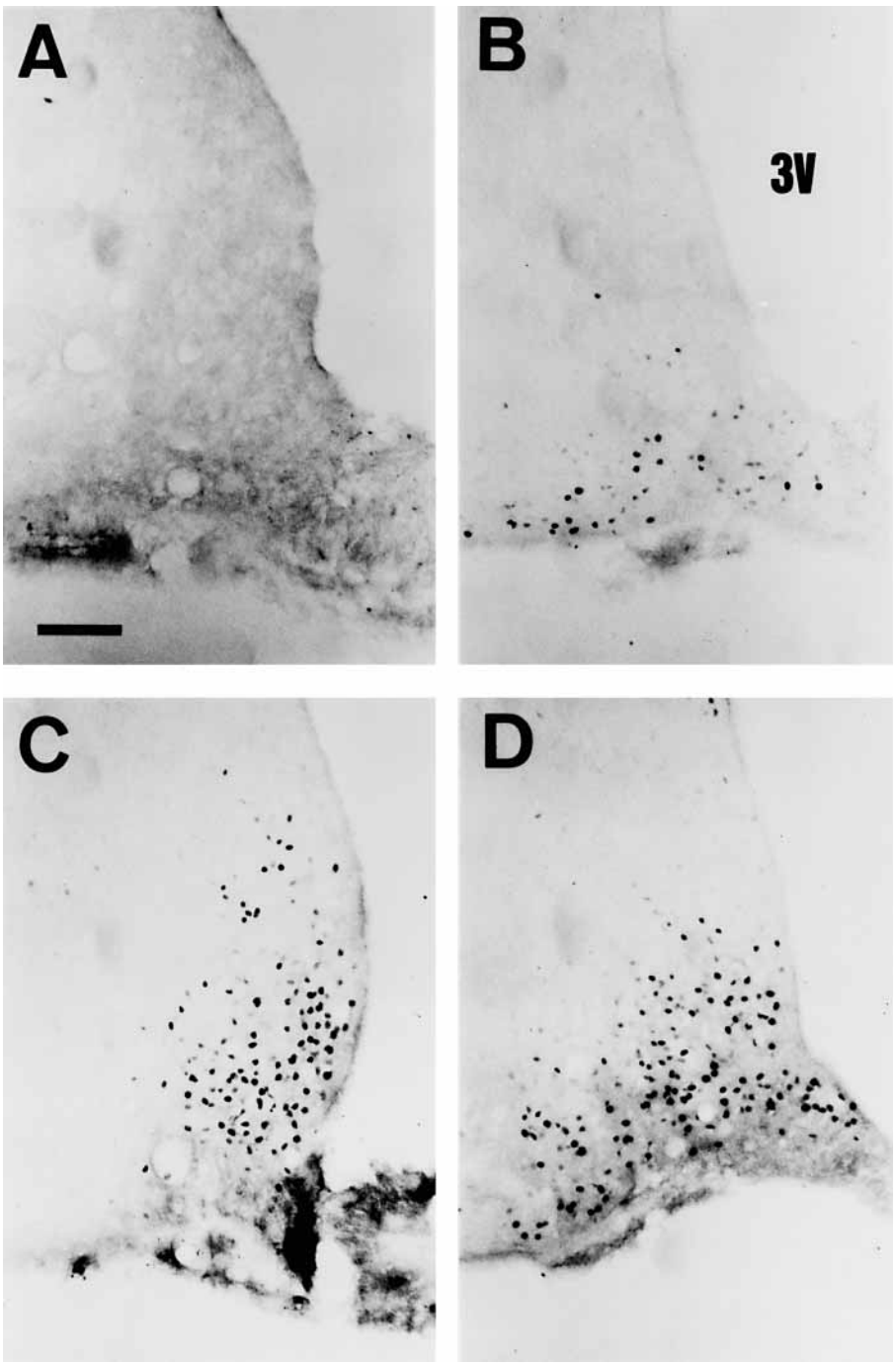

Fig. 3. The expression of c-Fos protein immunoreactivity in the ARC of fasted rats following injection of vehicle only (**A**), leptin only (**B**), GHRP-6 only (**C**) or leptin and GHRP-6 (**D**). Bar represents 100 µm. 3V = Third ventricle. Sections are equivalent to plate 28 in Swanson [39].

[32]. This may be the least likely explanation since the distribution of c-*fos* staining was not different in food-replete and fasted animals following GHRP-6 treatment. Secondly, due to an upregulation of GH secretagogue receptors following the removal of an, as yet unidentified, endogenous ligand by fasting. However, no significant differences in mRNA for the GH secretagogue receptor have been measured between fed and fasted rats [P.A. Bennett and I.C.A.F. Robinson, personal commun.]. Lastly, the increased response to GHRP-6 may be due to the removal of inhibitory influences allowing the recruitment of more NPY and GRF neurons. Various factors may be responsible for tonic inhibitory effects on arcuate neurons, including GH itself [1], insulin and glucocorticoids [40]. GH

feeds back on the hypothalamus to have inhibitory influences on GRF neurons [41], but stimulatory effects on NPY neurons [42]. Thus, a reduction in GH feedback would have opposing effects on GRF and NPY neurons. Furthermore, the administration of GHRP-6 to *dw/dw* rats that lack significant GH did not lead to abnormally elevated c-*fos* protein induction in the arcuate nucleus compared to that observed in normal animals [43].

Though published data suggest that leptin is stimulatory to the GH axis [9–11], leptin has an inhibitory action on the electrical activity of hypothalamic neurons [44, 45], and on the synthetic activity of NPY neurons [24]. Thus, leptin may be a factor that normally inhibits GHRP-sensitive arcuate neurons. A single dose of leptin given i.v. 15 min before GHRP-6, or concomitantly (results not shown), was unable to block the increased effectiveness of GHRP-6 in fasted rats. Neither did it significantly potentiate the effect of GHRP-6. Though this dose of leptin can have an effect on arcuate nucleus neurons (see below) it may be acting on other subpopulations or it may be required at a higher dose, perhaps administered as an infusion, if it is to reverse the effects of fasting.

We have shown that leptin given systemically to fasted rats can cause significant induction of c-*fos* protein in arcuate nucleus neurons. It is assumed that the reduction of endogenous leptin by fasting [24] allows the response in arcuate neurons to be detected, which is otherwise masked due to receptor downregulation. Indeed, an increase in mRNA for the long form of the leptin receptor in the arcuate nucleus following 48 h of fasting has been reported [34–36]. Previous results on the acute positive effects of systemic leptin on arcuate nucleus neurons are scarce. Woods and Stock [46] reported small numbers of c-*fos*-positive neurons in the arcuate nucleus of *ob/ob* mice treated with leptin, though this was not quantified or compared to controls. Recently, Elias et al. [47] reported c-*fos* expression in the arcuate nucleus of normally fed rats. Such staining in the arcuate nucleus has not been noted previously by the same group, though it does report extensive staining in other parts of the mediobasal hypothalamus [48, 49]. Leptin is thought to have a negative effect on the transcriptional activity of NPY neurons and, therefore, it might be unusual for it to induce a positively acting transcription factor, such as c-*fos*, in these cells. This and other evidence suggests that the neurons activated by systemic leptin are not NPYergic. Firstly, Glaum et al. [44] noted that leptin reduced the potassium-evoked rise of intracellular calcium in some, but not all, acutely isolated arcuate neurons. It is assumed that the neurons that are inhibited contain NPY, while those that are stimulated are of another phenotype. Secondly, the long form of the leptin receptor is present on POMCergic neurons as well as NPYergic neurons in the arcuate nucleus [20, 21] and the neurons described here as being activated by leptin have a distribution that includes the ventrolateral and ventromedial arcuate nucleus, more similar to the distribution of POMC or GRF neurons in the rat [32]. Thirdly, leptin can regulate the expression of POMC mRNA [26] and is thought to have some of its effects on feeding mediated by POMC-derived peptides [27]. Fourthly, food deprivation causes a decrease in POMC mRNA, suggesting that this treatment removes a positively acting input to these neurons [50]. Finally, as direct evidence, Elias et al. [47] describe c-*fos* staining in cocaine- and amphetamine-regulated transcript (CART)-positive neurons, that are shown in separate, non-treated animals to colocalise POMC mRNA.

In summary, our results suggest that GH secretagogues and leptin do not act on the same neurons in the hypothalamic arcuate nucleus. There is growing evidence that leptin modulates GH secretion by an effect on periventricular somatostatin neurons, either directly or indirectly [10, 11, 19]. However, mRNA for the signal-transducing, long form of the receptor is not present in the periventricular nucleus [17, 18], which would suggest an indirect action. The removal of somatostatinergic tone would result in the release of GH, and perhaps could also lead to the acute activation of GRF neurons in the arcuate nucleus. The recent interest in interactions between leptin and alpha melanocyte-stimulating hormone (α-MSH) [27, 51, 52] indicates a role for POMC/α-MSH neurons of the arcuate nucleus in mediating some of the effects of leptin. Furthermore, POMC colocalises with the anorectic peptide CART [47, 53]. Certainly, the distribution of neurons activated by systemic leptin, in both medial and lateral aspects of the ventral arcuate nucleus is compatible with this possibility. In the present study we were unable to carry out double immunocytochemistry since colchicine treatment, that would be required for the chemical identification of NPY and GRF neurons, is incompatible with c-*fos* activity mapping. A full survey of the neuronal phenotypes activated by each of the treatments will require further extensive study using alternative technologies.

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