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Antagonism between Ghrelin / GH secretagogues and somatostatin in arcuate and periventricular nuclei in prepubertal female rats

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SUMMARY

Combined growth hormone-releasing hormone (GHRH) and growth hormone secretagogues (GHS) have been used alone or in combination with somatostatin (SRIF) to study the pituitary growth hormone (GH) reserve in adults with GH deficiency, but not in children, owing to the possible adverse effects of these treatments. The study aimed to assess the response of the arcuate and periventricular hypothalamic nuclei, using Fos protein as a marker of neuron activity, and to compare that response with the GH response to different stimuli in the presence or absence of SRIF in prepubertal female rats. Rats received the following intraperitoneal stimuli: GHRH (1μg/kg), GHRP-6 (1 μg/kg), ghrelin (1 μg/kg), and GHRH combined with either GHRP-6 or ghrelin, with or without SRIF pretreatment 90 minutes prior to stimulus. The animals were decapitated at different intervals; trunk blood was obtained for the measurement of GH levels using a radioimmunoassay technique, and hypothalamic sections were processed for immunochemical determination of Fos protein expression. In the arcuate nucleus, except for GHRP-6 all stimuli increased Fos protein activity in the absence of SRIF pretreatment; the same effect was achieved by pretreatment with SRIF, although this pretreatment prevented the increase in neuronal activity following all stimuli, except that with GHRH + GHRP-6. In the periventricular nucleus, stimulus with GHRH, GHRP-6, ghrelin and GHRH+ghrelin caused a decline in Fos protein expression in the absence of SRIF, whereas SRIF pretreatment prompted a decrease in Fos protein expression that was counteracted by GHRP6 and GHRH + ghrelin. Peak serum GH values were recorded 15 minutes after the administration of GHRH+ghrelin and GHRH+GHRP-6. SRIH pretreatment inhibited GH release, with a subsequent «escape» and lack of response to stimulation which lasted at least 30 minutes, except following the administration of GHRH. The results suggest that all these actions may be due to functional antagonism between ghrelin/ GH secretagogues and somatostatin.

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Introduction

In clinical practice, combined growth hormone-releasing hormone (GHRH) and GH-secretagogue (GHS) stimuli have been used to assess GH deficiency in adults, exploring both hypothalamic and pituitary GH reserve mechanisms. Owing to possible adverse and health risks early in life, these combined stimuli have not been used for the study of the pituitary GH reserve in children. Little is therefore known about the somatotropic response to them in prepubescent children (Saggese et al., 1998) or about the changes taking place in the hypothalamic nuclei controlling GH secretion, mainly the arcuate and periventricular nuclei.

GHRH and somatostatin (SRIF) are involved in GH regulation, and they are synthesized and secreted by hypothalamic neurons. GHRH-containing neurons are mostly located in the hypothalamic arcuate nucleus, particularly in its ventral portion (Daikoku et al., 1986; Pelletier et al., 1986; Van de Pol et al., 1986; Seoane et al., 2003; Tannenbaum et al., 2003), and they project axons to the median eminence, where they episodically release their synthesis product to stimulate pituitary somatotroph cells. SRIF-neurons are located in the periventricular, arcuate and ventromedial nuclei. Only periventricular SRIF-neurons project axons to the median eminence to release SRIF and inhibit somatotroph cells (Seoane et al., 2003; Elde and Parsons, 1975; Kawano et al., 1982). Somatostatinergic neurons in the arcuate and ventromedial nuclei, and to a lesser extent in the periventricular nucleus, synapse with GHRH-producing neurons to modulate GHRH secretion (Willensen et al., 1999).

After the discovery of a new class of peptides and non-peptide molecules with potent GH release activity, their receptor was cloned (Howard et al., 1996; McKee et al., 1997) but the first natural ligand – called ghrelin – was not isolated until 1999 (Kojima et al., 1999) in the stomach. It has also been shown to be present in the arcuate nucleus (Lu et al., 2002; Mondal et al., 2005). Ghrelin is a potent releaser of GH after systemic or intracerebroventricular administration *in vivo,* but an intact hypothalamic-pituitary connection seems to be required (Tannenbaum et al., 2003).

A subpopulation of GHRH neurons co-express GH secretagogue receptor mRNA, pointing to their involvement in GH modulation (Tannenbaum et al., 1998). GHS activate GHRH neurons in the arcuate nucleus (Dickson and Luckmann, 1997; Hewson and Dickson, 2000). Somatostatin receptor subtypes 1 and 2 – both involved in the neuroendocrine regulation of GH secretion – are expressed on GHRH neurons in both the arcuate and ventromedial nuclei (Tannenbaum et al., 1998). The GHS receptor is also expressed in a small proportion of somatostatinergic neurons (Tannenbaum et al., 2003). GHS inhibit SRIF activity in these neurons, thus enhancing GHRH release into portal vessels, binding that receptor (Willensen et al., 1999). Chronic GHS administration, however, induces indirect activation of SRIF neurons via an increase in serum GH levels (Bailey et al., 1999). By contrast, SRIF has been shown to act as a physiological GHS antagonist, not only in the pituitary but also in the hypothalamus (Tannenbaum et al., 2003; Dickson et al., 1997).

The hypothalamic and pituitary effects of combined GHRH and GHS/ghrelin during prepubescence are not well known. Therefore, this study sought to monitor neuron activity in the arcuate and periventricular nuclei in female prepuberal rats, following systemic administration of GHRH and GHS, with or without SRIF pretreatment.

Many studies of hypothalamic activation are based on analysing the presence of Fos protein in neurons (Dickson et al., 1995). The c-fos gene is translated into a protein of the same name, which acts as a transcription factor. Expression of Fos protein can be used as a marker of cell activation, making staining of this protein a valuable technique for functional anatomical mapping of neuroendocrine systems (Hoffman et al., 1993). Accordingly, in the present study Fos protein immunostaining was used as a reflection of neuronal activity.

Materials and methods

Animals

Female 26-day-old Wistar rats, kept 3 per cage from weaning onwards, were given free access to food and water; a 12-hour light/dark

cycle was used (0730 h -1930 h). Rats were cared for, and used, in accordance with provisions regarding the care and protection of animals used for experimental and other specific purposes contained in Council of Europe Directive 86/609/EEC (24 november 1986) and in European Treaty Series 125 (November, 1987). These guidelines are reflected in Spanish Royal Decree 223/1988 (14 March, 1989) and Spanish Ministerial Order of 13 October 1989 (as published in the Spanish State Gazette, 18 October 1989)

Experimental design

Rats were divided into two groups: one group received saline pretreatment (saline group) and the other received somatostatin pretreatment (somatostatin group), at -90 minutes. At 0 minutes, both groups received one of the following stimuli: saline, GHRH, GHRP-6, ghrelin, GHRH+GHRP-6, GHRH+ghrelin. All treatments were administered as an intraperitoneal injection of 1 μg/kg weight, in 250 μl saline solution.

Sacrifice and sample collection

Animals were decapitated at -90, 0, 15, 30, 90 minutes. Trunk blood was obtained and after a 24-hour sedimentation period was centrifuged for serum collection. Brains were extracted and the hypothalamus removed and fixed in 6% paraformaldehyde in phosphate buffer (PBS) with 15% sucrose for 48 hours. Fixed samples were embedded in OCT-Tissue Teck®, frozen in isopentane precooled in liquid nitrogen, and 30 μm brain sections were cut on a Brigth cryostat throughout the entire rostrocaudal extent of the arcuate nucleus. Sections were anatomically equivalent between groups. Arcuate and periventricular nuclei were located using Paxinos and Watsons' stereotaxic atlas (Paxinos and Watson, 1998).

GH Radioimmunoassay

Serum GH concentrations were measured by double-antibody RIA using NIDDK kits, as described previously (Jiménez-Reina et al., 2000). All samples from each experiment were measured in the same assay, and GH values were expressed in ng/ml.

Fos protein immunocytochemistry

Brain sections were incubated with rabbit polyclonal Fos antibody (Santa Cruz Biotechnology) diluted 1:500 in Tris-phosphate buffer (TBS) with 1% bovine serum albumin (BSA) and 0.3% Triton X-100 for 48 hours at 4ºC. After washing, sections were reacted for 24 hours with biotinylated swine anti-rabbit serum (1:100) in TBS and 0.3% Triton X-100, and for 24 hours with streptavidin-peroxidase (1:100). The reaction was visualized using nickel-enhanced diaminobenzidine (DAB) (Sigma Chemical Co.) with 0.1% hydrogen peroxide for 10 minutes. Fos-positive cells per section were counted in six sections per animal in the arcuate and periventricular nuclei and these cells were averaged to give values for each stimulus.

Statistical analysis

Results were expressed as means ± SEM number of Fos-positive cells per section. Three animals were used for each stimulus and each administration time in both the somatostatin and saline group. The experiments were repeated three times. For each group, the data were analyzed by ANOVA, using the Holm-Sidak method to test for intra-group differences. Data were considered significant when $P<0.05$.

RESULTS

GH release

a) *Saline group*

There was no change in serum GH levels between -90 minutes and 0 minutes (6.22 ± 0.53 *vs* 5.91 ± 0.34 ng/ml). Following stimulus (time 0') with GHRP-6, ghrelin, GHRH+GHRP-6 and GHRH+ghrelin, peak GH values were reached at 15 minutes ($P<0.0001$) *vs* saline controls; Fig. 1); the most potent stimuli were GHRH+ghrelin and GHRH+GHRP6 (42.44 ± 2.41 and 38.29 ± 2.72 *vs* 5.91±0.34 ng/ml at 0'; $P<0.0001$). With GHRH, the peak GH response was recorded at 30 minutes (20.08 ± 1.25 *vs* 5.91±0.34 ng/ml; $P<0.0001$) (Fig. 1). By 90 minutes after stimulation with GHRH, GHRP-6 and ghrelin, GH levels were higher than at 0 minutes ($P<0.05$), but with GHRH+GHRP-6 and GHRH+ghrelin GH levels were lower (figure 1). The area under the curve (AUC) for GH release was greater with all stimuli than with saline (Fig. 2).

b) *Somatostatin group*

Administration of SRIF at -90 minutes prompted a GH release peak at 0 minutes (24.54 ± 2.08 ng/ml *vs* 6.22 ± 0.53) (Fig. 3).

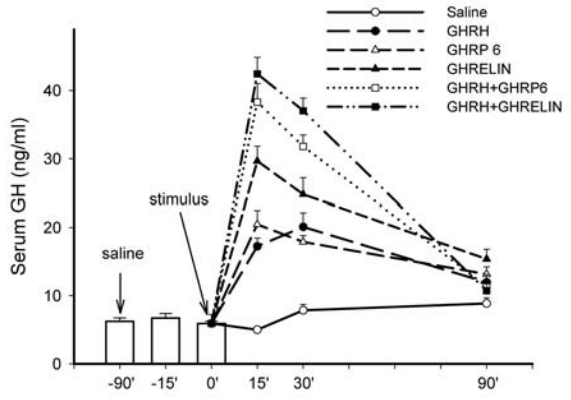


Figure 1. GH serum levels (ng/ml) in the saline-pretreated group.

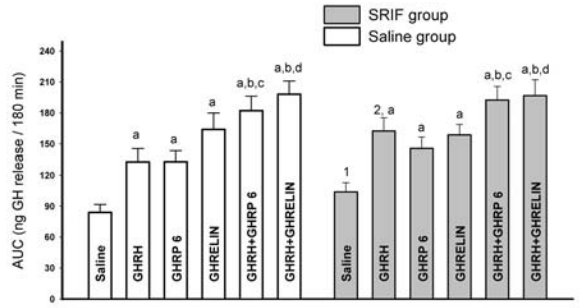
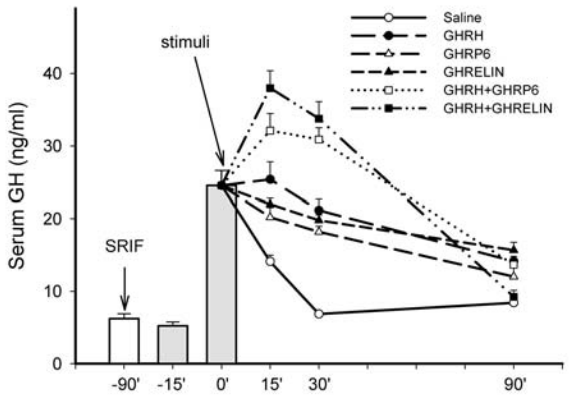


Figure 2. Area under the curve (AUC) for GH released over 180 minutes in the saline-pretreated group (white bars) and in the SRIF-pretreated group (grey bars). 1: P<0.05 vs. Saline of saline group; 2: P<0.05 vs. GHRH of saline group; **a**: P<0.05 vs. saline homonymous group; **b**: P<0.05 vs. GHRH homonymous group; **c**: P<0.05 vs. GHRP 6 homonymous group; **d**: P<0.05 vs. GHRELIN homonymous group.



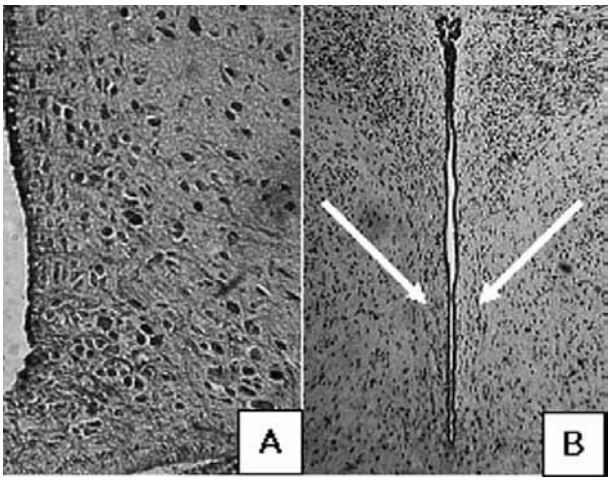


Figure 4. Fos protein immunostaining of neurons in the arcuate (A) and periventricular (B) nuclei.

At this time, stimuli were administered (saline, GHRH, GHRP-6, ghrelin, GHRH+GHRP-6 and GHRH+ghrelin). Only GHRH+GHRP6 and GHRH+ghrelin were able to increase GH values at 15 minutes (32.11 ± 2.36 and 37.98 ± 2.39 respectively *vs* 24.54 ± 2.08 ng/ml at 0'; P<0.05), and keep them similar to those recorded following administration of the same stimuli in the saline group (Fig. 3). The remaining stimuli did not prompt any increase in GH levels (Fig. 3). At 90 minutes, serum GH levels were similar to those observed in the saline group (Fig. 3). The AUC for GH release was greater with all stimuli than with saline (Fig. 2) and higher with combined stimuli than with isolated stimuli (Fig. 2). The AUC for GH release of saline and GHRH stimuli were greater in the SRIF-pretreated group than in the saline-pretreated group (P<0.05; Fig. 2).

Fos protein immunochemistry in hypothalamic nuclei

Figure 4 shows neurons exhibiting Fos protein immunostaining in the arcuate (Fig. 4A) and periventricular nuclei (Fig. 4B).

a) *Saline group*

Arcuate nucleus

The number of neurons exhibiting Fos protein immunostaining at 90 minutes was 70.25 ± 5.87 and 71.75 ± 3.11 in animals treated with saline and GHRP-6, respectively; i.e. similar to those recorded at 0 minutes (68.25 ± 4.85). The remaining treatments prompted an increase in the number of Fos-immunostained neurons (GHRH (109 ± 9.88), ghrelin (134.75 ± 4.93), GHRH + GHRP-6 (161.25 ± 8.78) and GHRH + ghrelin (155.50 ± 18.70) after 90 minutes (P<0.05) (Fig. 5A).

Periventricular nucleus

In all treated animals, except those receiving the saline stimulus (70.50 ± 4.46 *vs* 63.00 ± 4.12 at time 0') and the GHRH+GHRP-6 treated animals (69,17 ± 3,35), a decrease was recorded in the number of Fos protein-immunostained neurons after stimulus administration (GHRH: 36.33 ± 1.96; GHRP-6: 21.33 ± 1.84; ghrelin: 49.83 ± 1.05; GHRH+ghrelin: 28.33 ± 1.60; *vs* 63.00 ± 4.12 at 0', P<0.05) (Fig. 5B).

b) *Somatostatin group*

Arcuate nucleus

After somatostatin administration, an increase was noted in the number of Fos protein immunostained neurons (110.75 ± 6.44 *vs* 68.25 ± 4.85 at -90 minutes) (P<0.05) (Fig. 6A). Subsequent administration of all treatments did not increase the number of Fos protein-expressing neurons with respect to saline-treated animals (GHRH: 111.50 ± 8.74; GHRP-6: 135.75 ± 9.52; GHRH+ghrelin: 116.50 ± 9.11) (99 ± 7.85), except in the GHRH+GHRP-6 stimulus (210.25 ± 7.19) (P<0.05) (Fig. 6A).

Periventricular nucleus

Somatostatin administration prompted a decrease in the number of Fos protein immunostained neurons in this nucleus (38.17 ± 2.90 vs 46.67 ± 2.58 at -90 minutes; P<0.05).

In treated animals, increase was noted in Fos protein expression with respect to controls (38.17 ± 2.90) in saline (49.83 ± 2.36), GHRP-6 (56.83 ± 4.22) and GHRH+ghrelin (55 ± 1.78) stimuli (P<0.05; Fig. 6B).

Discussion

A female prepuberal animal model was used to study the activation of the hypothalamic arcuate and periventricular nuclei following combined treatments with GHRH, GH secretagogues and ghrelin, and at the same time to examine GH release into the bloodstream. Serum GH levels were measured, and the response to different stimuli was compared in order to identify the most potent combination of GH release. SRIF pretreatment was used to inhibit endogenous somatostatinergic tone, thus facilitating the GH cell response.

In the saline group, both GHRH and GHS stimulated GH release, ghrelin displaying a more marked effects than GHRH (Arvart et al., 2001). Combined stimuli act synergically (Arvart et al., 2001), as is apparent from the AUC for GH release over 180 minutes. Pretreatment with SRIF prompted an "escape" of GH release ninety minutes after administration (Jiménez-Reina et al., 2006), and only stimulus with GHRH+GHRP6 or GHRH+ghrelin – in combination, though not alone (Tzanela et al.,

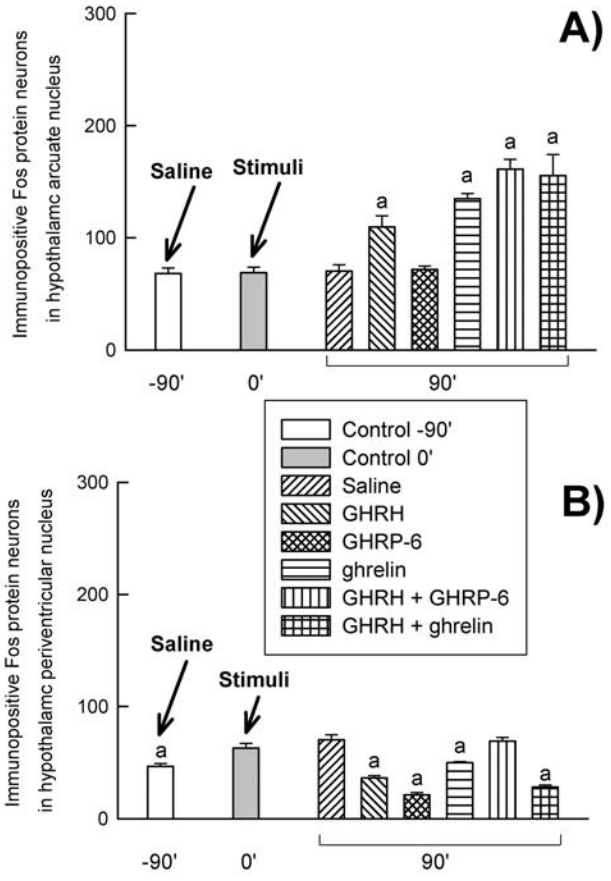


Figure 5. Fos protein-immunopositive neurons in the arcuate nucleus (A) and in the periventricular nucleus (B) of prepuberal Wistar female rats 90 minutes after stimulus administration in the saline pretreated group. **a**: P< 0,05 vs. control 0'.

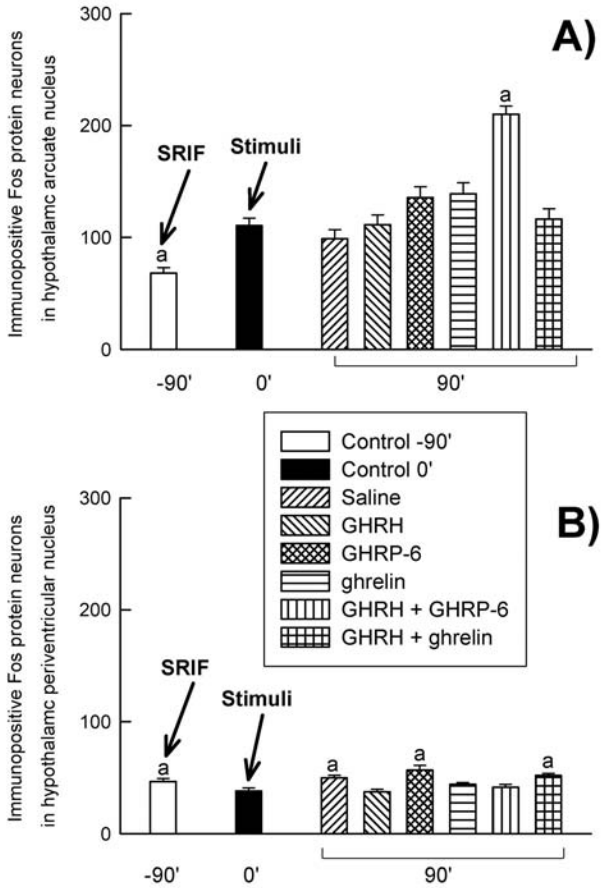


Figure 6. Fos protein-immunopositive neurons in the arcuate nucleus (A) and in the periventricular nucleus (B) of prepuberal Wistar female rats 90 minutes after stimulus administration in the SRIF pretreated group. **a**: P< 0,05 vs. control at 0'.

1996) – elicited an increase in GH release. In the somatostatin group, the total amount of GH released following administration of GHRH and GHRP6 was greater than in the saline group, indicating that somatostatin pretreatment is a predisposing factor for increased GH release (Dickerman et al., 1993; Tzanela et al., 1996).

Fos protein expression was also studied as a marker of neuron activation (Hoffman et al., 1993), in the main hypothalamic regions involved in GH regulation: the arcuate and periventricular nuclei. The GH secretagogue receptor (GHS-R) is strongly expressed in the hypothalamic neurons of the arcuate, paraventricular, ventromedial, supraoptic, tuberomammillary and suprachiasmatic nuclei. There is no GHS-R expression in periventricular nuclei, so changes in neuron activity at this level have been seen as neuronal connexion-mediated (Tannenbaum et al., 2003) or by indirect activation of SRIF neurons via modifications in GH serum levels.

Systemic administration of release stimuli induced an increase in Fos protein expression in the arcuate nucleus, as reported in adult rats (Dickson et al., 1996; Dickson and Luckman, 1997; Luckman et al., 1999; Wang et al., 2002); the administration of somatostatin had a similar effect. This finding may be due to the presence in this nucleus of several neuron types involved in other functions. Thus, GHRH induces SRIF modulator neuron activation (Willensen et al., 1999), and GH secretagogues act through GHRH neurons to enhance GH release, although they also induce activation of other neurons in this nucleus, such as AgrP and NPY neurons involved in appetite regulation (Seoane et al., 2003; Dickson and Luckman, 1997; Wang et al., 2002). The GH-secretagogues + GHRH combination, as well as ghrelin alone, increased Fos expression in the arcuate nucleus as compared with GHRH treatment alone. Also, serum GH levels were higher in animals treated with combined stimuli. This may in part be due to the existence of different forms of somatotroph cell stimulation, and in part to different effects on neurons in the arcuate nuclei. Thus, ghrelin seems to play a major role in hypothalamic regulation in prepuberal animals, as it does in adults (Seoane et al., 2003). After somatostatin pretreatment, Fos protein expression was only increased at 90 minutes with combined GHRH and GHRP-6. The high Fos protein expression recorded in GHRH+GHRP-6 treated animals could be due to the recruitment of a larger neuron population not inhibited by somatostatin. Ghrelin-induced activation was blocked because of SRIF functional antagonism (Tannenbaum et al., 2003), a finding also recorded 90 minutes after somatostatin administration and in combined treatment with GHRH; a blunted response has been reported to GHRP-6 following high doses of somatostatin (Dickson et al., 1997).

In the periventricular nucleus, the increase in Fos protein expression was probably prompted by handling stress during injection (Chen and Du, 2002; Reyes et al., 2003). Although the GH secretagogue receptor is not present in this nucleus, Fos expression decreased after the administration of all stimuli except combined GHRH+GHRP-6. If most of the neuron population is somatostatinergic, secreting SRIF at the median eminence (Ishikawa et al., 1987), it is likely that the inhibition of somatostinergic tone would help to maintain GH synthesis and release in somatotroph cells. Somatostatin also prompted a decline in Fos expression in the periventricular nucleus, and no subsequent stimulus enabled a recovery of neuron activity, perhaps because somatostatin blocked inhibitory afferences. An inhibitory effect of GH secretagogues on the periventricular nucleus has been reported by Hewson et al. (1999) in rats, although no secretagogue receptor has been located in this nucleus. It has been suggested that this effect may be due to a presynaptic action in GHRH and NPY neurons in the arcuate nucleus connected with the periventricular nucleus. Thus, a greater decrease in Fos expression in rats treated with a combination of GHRH and GH secretagogues might be attributable to the activation of a larger number of neurons with a greater aggregate inhibitory effect in the periventricular nucleus.

To conclude, in this study of hypothalamic involvement in the response to several GH-release stimuli in prepuberal rats we found a higher GH-release response following the administration of combined GHRH+GH secretagogue stimuli. Moreover, the hypothalamic neuron population in the arcuate nucleus displayed an enhanced expression of Fos protein as a marker of greater neuron activation. SRIF pretreatment did not increase GH release, although a greater proportion of somatotroph cells storing larger amounts of hormone has

been reported in earlier studies (Jiménez-Reina et al., 2006). At hypothalamic level, somatostatin blocked the stimulant effect for at least 90 minutes, perhaps via neuromodulator neuron inhibition, except in the GHRH+GHRP-6 treated animals. In the periventricular nucleus, somatostatin likely acted to block the inhibitory afferences evoked by GH release stimuli. All these actions may be due to functional antagonism between ghrelin/ GH secretagogues and somatostatin.

Future research should examine the nature of the neuronal connections in the hypothalamus following administration of combined GHRH and GH secretagogues.

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