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Interactions of Growth Hormone Secretagogues and Growth Hormone-Releasing Hormone/Somatostatin

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The class of novel synthetic compounds termed growth hormone secretagogues (GHSs) act in the hypothalamus through, as yet, unknown pathways. We performed physiologic and histochemical studies to further understand how the GHS system interacts with the well-established somatostatin (SRIF)/growth hormone–releasing hormone (GHRH) neuroendocrine system for regulating pulsatile GH secretion. Comparison of the GH-releasing activities of the hexapeptide growth hormone–releasing peptide-6 (GHRP-6) and GHRH administered intravenously to conscious adult male rats showed that the pattern of GH responsiveness to GHRP-6 was markedly time-dependent, similar to that observed with GHRH. Immunoneutralization of endogenous SRIF reversed the blunted GH response to GHRP-6 at trough times, suggesting that GHRP-6 neither disrupts nor inhibits the cyclical release of endogenous hypothalamic SRIF. By striking contrast, passive immunization with anti-GHRH serum virtually obliterated the GH responses to GHRP-6, irrespective of the time of administration. These findings suggest that the GHSs do not act by altering SRIF release but, rather, stimulate GH release via GHRH-dependent pathways. Our dual chromogenic and autoradiographic *in situ* hybridization experiments revealed that a subpopulation of GHRH mRNA-containing neurons in the arcuate (Arc) nucleus and ventromedial nucleus (VMN) of the hypothalamus expressed the GHS receptor (GHS-R) gene. These results provide strong anatomic evidence that GHSs may directly stimulate GHRH release into hypophyseal portal blood, and thereby influence GH secretion, through interaction with the GHS-R on GHRH- containing neurons. Altogether, these findings support the notion that an additional neuroendocrine pathway may exist to regulate pulsatile GH secretion, possibly through the influence of the newly discovered GHS natural peptide, ghrelin.

Key Words: Pulsatile growth hormone; somatostatin; growth hormone–releasing hormone; growth hormone secretagogue receptor; colocalization.

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Introduction

Regulation of the secretion of growth hormone (GH) from the anterior pituitary gland is under the control of at least two hypothalamic hormones, a stimulatory GH-releasing hormone (GHRH) found in the arcuate (Arc) nucleus, and an inhibitory hormone, somatostatin (SRIF), synthesized in the periventricular nucleus (PeV). In addition to the intricate patterned release of GHRH and SRIF regulating GH directly at the pituitary level, several lines of evidence suggest that SRIF modulates GH secretion indirectly through central regulation of GHRH-containing neurons. The net result of these interactions is a striking pulsatile pattern of GH release (*see* ref. *1* for a review).

In recent years, there has been intense interest in a novel class of peptide and nonpeptidyl synthetic compounds, termed GH secretagogues (GHSs), developed from the prototype hexapeptide GH-releasing peptide-6 (GHRP-6) *(2)* and shown to exert potent GH-releasing activity in multiple species, including human (*see* refs. *3* and *4* for review). Their mechanism of action and functional significance, however, remains obscure. Although GHSs selectively release GH by a direct pituitary action that is distinct from that for GHRH *(2,5,6)*, there is good in vivo evidence that the GHSs also act in the hypothalamus *(7–9)*. Indeed, the hypothalamus, rather than the pituitary, is currently considered as the more important site of action, although the pathways through which GHSs act in the hypothalamus to release GH are not known. Models of GHS action being proposed vary in both type and relationship to SRIF and GHRH.

It had been speculated that the GHSs mimic an as yet unidentified endogenous hormone reflecting the presence

of an additional neuroendocrine system in the brain to regulate pulsatile GH secretion *(10)*. GHS binding sites have been identified in both pituitary and hypothalamic membranes *(11,12)*. Recently, a novel GHS receptor (GHS-R) was cloned from the pituitary of several species, including human *(13)* and rat *(14)*. An initial mapping study identified numerous sites of expression of GHS-R in the brain *(15)*, and GHS-R mRNA was shown to be sensitive to changes in GH status *(16)*. These findings supported the notion that an additional neuroendocrine pathway may exist for the control of pulsatile GH release.

In this article, we report on our physiologic and histochemical studies designed to understand further how the GHS system interacts with the seemingly self-sufficient and well-established GHRH/SRIF neuronal system for the regulation of GH secretion.

Results

Temporal Pattern of GH Responsiveness to GHRP-6 and GHRH

Figure 1 illustrates the mean plasma GH responses evoked by GHRP-6 (20 μg) administered during peak and trough periods of GH secretion. Administration of GHRP-6 during a spontaneous GH secretory episode (1100 h) resulted in a marked increase in plasma GH levels, whereas injection during a trough period (1300 h) had only a minimal effect. The mean plasma GH responses at 1100 h were significantly greater than those at 1300 h (694.5 ± 104.8 vs 205.5 ± 35.8 ng/mL; p <0.02).

Figure 2 summarizes the temporal pattern and magnitude of GH responsiveness to various doses of GHRP-6, as compared with those observed after similar doses of GHRH. GHRP-6 caused potent dose- and time-dependent GH release. The magnitude and pattern of GH responses to GHRP-6 were similar to those induced by GHRH, except at the lowest dose tested (1 μg), at which GHRH-induced GH release was significantly ($p < 0.001$) greater than that of GHRP-6. At all doses examined, for both peptides, the mean plasma GH responses at times of spontaneous GH peaks were greater than those during GH trough periods (Fig. 2).

Effects of Passive Immunization with GHRH and SRIF Antisera on Pattern and Magnitude of GH Responsiveness to GHRP-6

Normal sheep serum–treated control rats exhibited high GHRP-6-induced GH release at GH peak times (1100 h) and a lesser response during GH trough periods (Fig. 3A). Immunoneutralization of endogenous GHRH virtually obliterated the GH responses to GHRP-6 at both time points (Fig. 3B). By contrast, the administration of SRIF antiserum reversed the blunted GHRP-6 response observed during trough periods (1300 h) in normal sheep serum–treated controls and restored GH responsiveness to GHRP-6 to

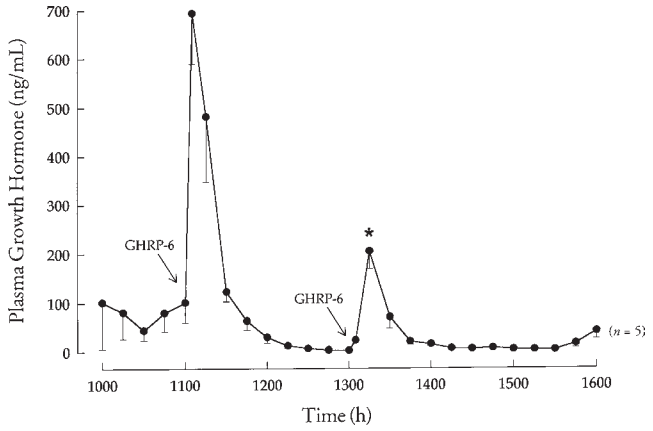

Fig. 1. Mean plasma GH responses to GHRP-6 (20 μg) intravenously administered during spontaneous peak (1100 h) and trough (1300 h) periods of GH secretion. High GH responsiveness to GHRP-6 was observed at peak times, whereas injection of GHRP-6 during a trough period had a minimal effect. Arrows indicate the times of injections, vertical lines represent the SE, and the number of animals is shown in parentheses. $^{*}p < 0.02$ compared with GHRP-6-induced GH release at 11:00 AM.

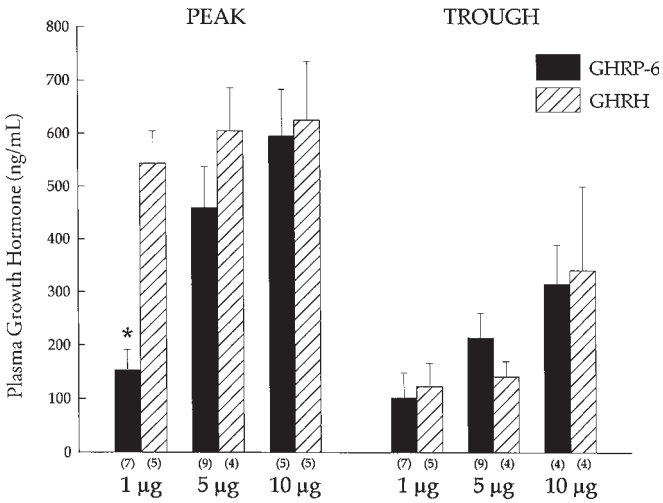

Fig. 2. Comparison of the magnitude and pattern of GH responses to GHRP-6 and GHRH (at doses of 1, 5, and 10 μg) administered during spontaneous peaks and troughs of GH secretion. Each bar represents the mean + SE; the number of animals in each group is shown in parentheses. $^{*}p < 0.001$ compared with GH response to GHRH at the same dose.

levels as high or higher than those observed at 1100 h (Fig. 3C).

Expression of GHS-Rs by GHRH Neurons

Light microscopic examination of coronal sections taken through the rat brain revealed moderate to strong autoradiographic GHS-R hybridization signal in several hypothalamic as well as extrahypothalamic regions. Within the hypothalamus, numerous intensely labeled cells were detected throughout the ventromedial nucleus (VMN), as well as within the Arc and lateral mammillary nuclei; more

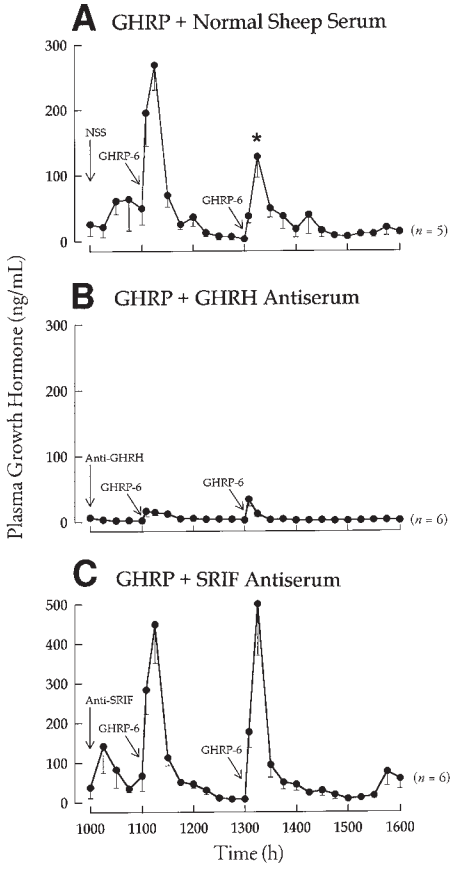

Fig. 3. Effects of immunoneutralization with GHRH and SRIF antisera on mean plasma GH responses to GHRP-6 (5 µg) administered during peak (1100 h) and trough (1300 h) periods of GH secretion. **(A)** Normal sheep serum-treated control rats exhibited high GHRP-6-induced GH release at 1100 h and a lesser response at 1300 h. **(B)** Administration of anti-GHRH serum virtually obliterated the GH responses to GHRP-6 at both time points. **(C)** Immunoneutralization of endogenous SRIF reversed the blunted GH response to GHRP-6 observed at 1300 h in normal sheep serum–treated controls to levels as high or higher than those observed at 1100 h (note different *y*-axis in [C]). Arrows indicate the times of injections, vertical lines represent the SE, and the number of animals in each group is shown in parentheses. $^{*}p < 0.02$ compared with GHRP-6-induced GH release at 1100 h.

sparsely distributed or less intensely labeled neurons were also evident in the anterior hypothalamic nucleus; retrochiasmatic area; and paraventricular, dorsomedial, and medial tuberal nuclei (Table 1). Moderate to strong GHS-R hybridization signal was also present in several extrahypothalamic regions, including over pyramidal cells in the CA2/CA3 subfields of the hippocampus, over granule cells in the dentate gyrus, over the granular layer of the retrosplenial cortex, in the medial amygdaloid nucleus, in the pars compacta of the substantia nigra, and in the interstitial nucleus of Cajal (Table 1).

Within the Arc nucleus and VMN, GHS-R hybridizing cells showed substantial overlap with GHRH-expressing neurons labeled with digoxigenin (DIG) in the same sections (Fig. 4). In the Arc nucleus, this overlap was most prevalent ventrolaterally, dorsal to the infundibular recess of the third ventricle. In the VMN, GHS-R hybridizing neurons were evident throughout the nucleus and predominated in its dorsomedial aspect, whereas GHRH mRNA-stained neurons were mainly found in the regions bordering it ventrally and dorsally (Fig. 4).

Table 1
Expression of GHS-R mRNA in Rat Brain

Area	Hybridization levels
Hypothalamus	
Anterior nucleus	+
Paraventricular nucleus	+
Arcuate nucleus	++
Ventromedial nucleus	+++
Dorsomedial nucleus	+
Medial tuberal nucelus	+
Lateral mammillary nucleus	+++
Extrahypothalamic areas	
Hippocampus (CA2/CA3)	++
Dentate gyrus (granular layer)	+++
Medial amygdaloid nucleus	+
Retrosplenial cortex (granular layer)	+
Zona incerta	+
Substantia nigra, pars compacta	+++
Interstitial nucleus of Caja	++
Periaqueductal grey	+

At high magnification, there was a clear-cut colocalization of cells located in the zones of the Arc nucleus and VMN in which the two populations overlapped. Quantitative analysis of double-labeled cells revealed that 27% of GHRH-expressing neurons detected in the Arc nucleus and 22% of those detected in the VMN coexpressed GHS-R (Table 2). Yet, these double-labeled cells represented only a small subpopulation of GHS-R-expressing neurons in either the Arc nucleus or the VMN. In both of these regions, double-labeled cells were detected among neurons expressing either GHRH or GHS-R alone.

Discussion

The physiologic results reported herein clearly demonstrate that the hexapeptide GHRP-6 causes potent, dose-dependent GH release in conscious adult male rats; the magnitude of the GH response to GHRP-6 was similar to that induced by GHRH, except at the lowest dose tested. Of importance, GH responsiveness to GHRP-6 was found to be markedly time-dependent, with high GH release observed when GHRP-6 was administered during a spontaneous GH secretory episode and a lesser response when injected during a GH trough period. Similar variation in GH responsiveness to GHRH was also observed. We have previously shown that the weak GH response to GHRH during GH trough periods due to antagonism by the cyclical

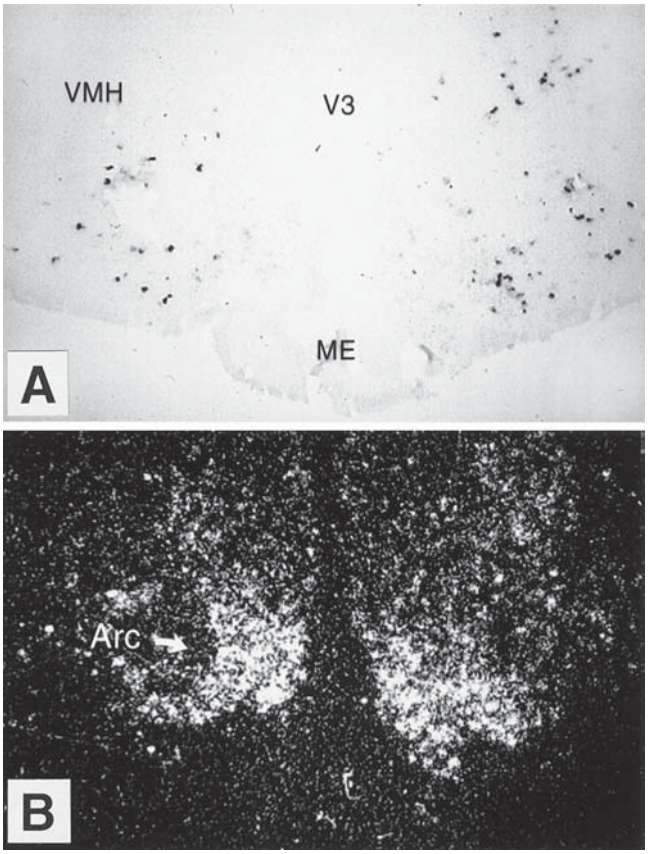

Fig. 4. Comparative distribution of GHRH **(A)** and GHS-R **(B)** mRNA in the caudal pole of the rat Arc nucleus. GHRH mRNA was labeled with DIG and illustrated in bright field. GHS-R mRNA was revealed by autoradiography and illustrated in dark field. GHRH-hybridizing neurons are evident in the Arc as well as along the ventral and dorsal borders of the VMH. GHS-R-expressing cells overlap extensively with GHRH-expressing ones in both the Arc and VMN. V3, third ventricle; ME, median eminence.

Table 2
Estimated Percentage of GHRH Neurons Coexpressing GHS-R mRNA in Arcuate and Ventromedial Nuclei

	Cell number[a]	Percentage[b]
Arcuate nucleus	178 ± 92	26.9 ± 22.4
Ventromedial nucleus	68 ± 35	21.8 ± 5.1

[a]Number of GHRH-expressing cells examined; values are the mean ± SEM of four animals.

[b]Percentage of GHRH neurons coexpressing GHS-R mRNA.

increased release of endogenous SRIF in the male rat *(17)*. In the present study, immunoneutralization of endogenous SRIF reversed the blunted GH response to GHRP-6 at trough times and, in fact, enhanced GH responsiveness to GHRP-6, as compared to normal sheep serum-treated controls. Taken together, these findings strongly suggest that GHRP-6 neither inhibits nor disrupts the cyclical release of endogenous hypothalamic SRIF. Furthermore, they clearly indicate that GHRP-6 can effectively stimulate GH release in the absence of SRIF.

Although some investigators have suggested that GHSs may release GH by inhibiting the cyclical release of hypothalamic SRIF in vivo *(7)*, and both an increase *(18)* and decrease *(19)* in GHS-induced SRIF secretion in the rat have been postulated, the administration of GHSs to conscious sheep does not influence SRIF secretion into hypophyseal portal blood *(9)* nor do GHSs alter SRIF release from hypothalamic cultures *(20)*.

In striking contrast to the effects of anti-SRIF serum, immunoneutralization of endogenous GHRH virtually obliterated the GH responses to GHRP-6 irrespective of the time of administration. This finding is congruent with previous reports indicating that GHS-induced GH release is attenuated by anti-GHRH serum *(7,21,22)*. Together, these results implicate GHRH cells as targets for GHSs. Indeed, there is convincing evidence that GHSs stimulate GH release via GHRH-dependent pathways. GHSs have been shown to activate a subpopulation of Arc neurons *(8)* and to induce c-*fos* expression in GHRH mRNA-containing cells in the Arc nucleus *(23)*. Moreover, the administration of GHSs provokes the release of GHRH into hypophyseal portal blood *(9)*. We interpret all these findings to indicate that the GHSs do not act by altering SRIF release but, rather, stimulate GH release via GHRH-dependent pathways.

Our dual chromogenic and autoradiographic *in situ* hybridization experiments provide strong anatomic evidence to support this notion. High concentrations of GHS-R-expressing cells were detected within the ventral aspect of the Arc nucleus as well as throughout the VMN. Although the distribution of GHRH-expressing neurons was considerably more restricted than that of GHS-R hybridizing cells, it overlapped significantly with the latter both in the ventral aspect of the Arc and along the dorsal and ventral borders of the VMN. Quantification of dually-stained neurons indicated that the proportion of double-labeled cells was, in fact, quite similar in these two nuclei, suggesting that GHSs may equally affect the activity of these two cell populations. The localization of the GHS-R to Arc GHRH neurons, as demonstrated here, provides compelling evidence that the GHSs may directly modulate GHRH release into hypophyseal portal blood, and thereby influence GH secretion, through interaction with the GHS-R on GHRH-containing neurons.

The finding that the proportion of GHRH/GHS-R dually-hybridizing cells was as high in the VMN as in the Arc nucleus was surprising because VMN GHRH neurons reportedly do not project to the median eminence and are therefore presumably not directly involved in the control of GH secretion (reviewed in ref. *24*). Nonetheless, the present findings, together with the earlier demonstration that GHS-R expression in both the VMN and Arc nucleus is

sensitive to changes in GH status *(16)*, suggest that GHSs may control GH secretion, not merely through the Arc but also through VMN GHRH neurons. Clearly, however, GHRH/GHS-R expressing neurons form but a small subpopulation of GHS-R-expressing cells in the VMN, implying that the GHSs acting in this nucleus are also likely to exert effects on functions other than GH.

In keeping with this observation, GHS-R hybridizing cells were also detected in a number of extrahypothalamic areas, some (but not all) of which had previously been shown to contain GHS-R mRNA *(15)*. New sites of GHS-R mRNA reported here include the retrosplenial cortex, amygdala, and interstitial nucleus of Cajal. These findings support the view that the GHSs may be involved in a variety of limbic and motor functions in addition to promoting GH release.

In more recent experiments (data not shown, Tannenbaum and Epelbaum, in preparation), we have investigated other neurochemical cell types that might express the GHS-R. A very weak hybridization signal was detected in SRIF mRNA-containing neurons in the PeV, the location of the major hypophysiotropic SRIF cells, suggesting that PeV SRIF cells are not major direct targets for GHSs. The largest proportion (48%) of GHS-R-expressing cells in the Arc nucleus was colocalized in neuropeptide Y (NPY) mRNA-containing neurons, a hypothalamic pathway known to influence GH *(25,26)*. Similar findings were recently reported *(27)*, although the percentages of SRIF and NPY neurons coexpressing GHS-R mRNA in that study differed significantly from ours.

Altogether, the presence of GHS-R on GHRH-, SRIF-, and NPY-expressing neurons, all known to impact the GH axis, supports the notion that an additional neuroendocrine pathway may exist to regulate pulsatile GH release. Recently, an endogenous ligand specific for the GHS-R, designated ghrelin, was isolated from rat stomach *(28)*. This novel, GH-releasing acylated peptide (the probable natural GHS hormone) was shown to be expressed, albeit weakly, in the ventrolateral border of the hypothalamic Arc nucleus in addition to the stomach. The challenge ahead is to determine whether ghrelin plays a physiologically important role in the regulation of pulsatile GH secretion, and how it might interact with the classic GHRH/SRIF neuronal system for GH control.

Materials and Methods

Animals and Experimental Procedures

All animal-based procedures were approved by the McGill University Animal Care Committee.

Adult male Sprague-Dawley rats (225–300 g) were purchased from Charles River Canada (St. Constant, Canada) and individually housed on a 12-h light, 12-h dark cycle (lights on, 6:00 AM to 6:00 PM) in a temperature (22 ± 1°C)- and humidity-controlled room. Purina rat chow (Ralston Purina, St. Louis, MO) and tap water were available ad libitum.

For the physiologic studies, chronic intracardiac venous cannulae were implanted under sodium pentobarbitol (50 mg/kg, intraperitoneally) anesthesia using a previously described technique *(29)*. After surgery, the rats were placed directly in isolation test chambers with food and H_2O freely available until body weight returned to preoperative levels (usually within 5–7 d). On the test day, food was removed 1.5 h before the start of sampling and was returned at the end.

In the first experiment, we compared the temporal pattern and magnitude of GH responsiveness to GHRP-6 and GHRH. Free-moving chronically cannulated rats were administered various doses of either rat GHRH(1-29)NH_2 or GHRP-6 intravenously at two different time points during a 6-h sampling period. The times of 1100 h and 1300 h were chosen because these times reflect typical peak and trough periods of GH secretion, as previously documented *(17,29)*. Both the GHRH peptide (kindly provided by Dr. P. Brazeau, Notre Dame Hospital, Montreal) and GHRP-6 were diluted in normal saline just before use.

Blood samples (0.35 mL) were withdrawn every 15 min over the 6-h sampling period (1000 h to 1600 h) from all animals. To document the rapidity of the GH responses to the two secretagogues, an additional blood sample was obtained 5 min after each injection of the peptides. All blood samples were immediately centrifuged, and the plasma was separated and stored at –20°C for subsequent assay of GH. To avoid hemodynamic disturbance, the red blood cells were resuspended in normal saline and returned to the animal after removal of the next blood sample.

In the second series of experiments, designed to assess the roles of endogenous GHRH and SRIF in mediating the GH responses to GHRP-6, two groups of rats were administered 1 to 2 mL of specific GHRH or SRIF antisera intravenously, at 1000 h, after removal of the first blood sample. The GHRP-6 peptide (5 µg) was subsequently intravenously injected at 1100 h and 1300 h. A third group of rats served as controls and received 1 to 2 mL of normal sheep serum and 5 µg of GHRP-6 intravenously at the same time points. Blood samples were withdrawn from 1000 h to 1600 h, as already described. The SRIF and GHRH antisera were the same as described in our previous passive immunization studies *(17,30)*.

For the histochemical studies, adult male Sprague-Dawley rats were killed by decapitation between 1100 h and 1115 h AM. The brains were snap-frozen in isopentane at –40°C for 1 min and stored at –80°C. They were coronally sectioned through the hypothalamus, at 20-µm thickness, from the optic chiasm rostrally, to the mammillary bodies caudally. Sections were collected on poly-L-lysine (50 µg/mL)-coated slides, dried for 2 min at 37°C, and stored at –70°C until *in situ* hybridization was performed.

GH Assay

Plasma GH concentrations were measured in duplicate by double antibody radioimmunoassay (RIA) using materials supplied by the NIDDK (National Institute of Diabetes and Digestive and Kidney Diseases) Hormone Distribution Program (Bethesda, MD). The averaged plasma GH values are reported in terms of the rat GH reference preparation (rGH RP-2). The standard curve was linear between 0.62 and 320 ng/mL; the least detectable concentration of plasma GH under the conditions used was 1.2 ng/mL. All samples with values above 320 ng/mL were reassayed at dilutions ranging from 1:2 to 1:10. The intra- and interassay coefficients of variation were 7.7 and 10.7%, respectively, for duplicate samples of pooled plasma containing a mean GH concentration of 60.7 ng/mL.

Preparation of Probe

Growth Hormone Secretagogue Receptor

Single-stranded sense and antisense RNA probes were generated from constructed full-length rat GHS-R type 1a cDNA *(14)* inserted into the mammalian expression vector pcDNA3 (Invitrogen, Carlsbad, CA). To obtain GHS-R antisense probes, the cDNA templates were produced by linearization of the vector with *Eco*RI and transcription with the Gemini II system (Promega Biotec, Madison, WI) using SP6 RNA polymerase and [^{35}S]-uridine 5'-[α-thio]triphosphate (DuPont-New England Nuclear, Boston, MA). Sense probes were prepared from *Not*I-linearized plasmid DNA in the presence of T7 RNA polymerase. Aliquots were stored at –70°C. Before use, the identity and integrity of the probes were verified by polyacrylamide gel electrophoresis against known standards. The final probe specific activity was *approx* 1.6×10^9 dpm/μg.

Growth Hormone–Releasing Hormone

The prghrf-2 plasmid was obtained from Dr. Kelly Mayo, Northwestern University, Evanston, IL. A 217-bp fragment including the entire GHRH-43 coding sequence was subcloned into the transcription vector pGEM-4 (Promega Biotec), and a DIG-labeled antisense cRNA probe was made in vitro using the DIG RNA Labeling Mix (Boehringer Mannheim, Laval, Quebec) containing 3.5 m*M* DIG-II-UTP; 6.5 m*M* unlabeled UTP; 10 m*M* GTP, ATP, and CTP; and T7 RNA polymerase.

Double-Labeled In Situ *Hybridization*

We performed dual chromogenic and autoradiographic *in situ* hybridization using a protocol described previously *(31,32)*. Briefly, processed sections were hybridized with ^{35}S-labeled antisense GHS-R probe (3 to 6×10^6 cpm/mL) and 3.75 μL/mL of DIG-labeled antisense GHRH probe in hybridization buffer. Overnight hybridization at 60°C was followed by RNase treatment, a series of stringent saline sodium citrate washes, and a wash at 60°C. The slides were then blocked with 2% normal sheep serum and incubated overnight at room temperature with 150 μL of anti-DIG antibody conjugated to alkaline phosphatase (1:1000) (Boehringer Mannheim). Slides were rinsed in buffer before applying 150 μL of chromogen and incubated at 37°C for 7 h until color development. They were then washed in TE, dehydrated in 70% ethanol, air-dried, dipped in 3% parlodion, and dried overnight. All slides were coated with NTB2 photographic emulsion (Eastman Kodak, Rochester, NY) diluted 1:1 with distilled H_2O and exposed for 5 to 6 wk at 4°C.

Image Analysis

Light microscopic autoradiograms were analyzed using a computer-assisted image analysis system (Biocom, Les Ulis, France) coupled to a Leitz Diaplan microscope (Leitz, Rockleigh, NJ). Twenty tissue sections for each rat were analyzed. First, purple-stained DIG-labeled GHRH cells were outlined and counted under bright-field illumination. Second, the number of silver grains overlying individual DIG-labeled GHRH cells was counted. Cells were considered double labeled if the density of silver grains counted over them was at least three times higher than background (determined in another area of the hypothalamus). Results were expressed as a percentage (mean ± SE) of GHRH mRNA-positive cells dually stained for GHS-R mRNA in the Arc and VMN of the basal hypothalamus.

Statistical Analyses

Analysis of variance and student's *t*-tests for unpaired and paired data, as appropriate, were used for statistical comparisons between and within experimental groups. The results are expressed as the mean ± SE and $p < 0.05$ was considered significant.

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