

## **Chronic growth hormone (GH) hypersecretion induces reciprocal and reversible changes in mRNA levels from hypothalamic GH-releasing hormone and somatostatin neurons in the rat.**

J Bertherat, ... , C Kordon, J Epelbaum

*J Clin Invest.* 1993;91(4):1783-1791. <https://doi.org/10.1172/JCI116389>.

**Research Article**

Effects of growth hormone (GH) hypersecretion on somatostatin-(SRIH) and GH-releasing hormone (GHRH) were studied by in situ hybridization and receptor autoradiography in rats bearing a GH-secreting tumor. 6 and 18 wk after tumor induction, animals displayed a sharp increase in body weight and GH plasma levels; pituitary GH content was reduced by 47 and 55%, while that of prolactin and thyrotropin was unchanged. At 18 wk, hypothalamic GHRH and SRIH levels had fallen by 84 and 52%, respectively. In parallel, the density of GHRH mRNA per arcuate neuron was reduced by 52 and 50% at 6 and 18 wk, while SRIH mRNA levels increased by 71 and 83% in the periventricular nucleus (with no alteration in the hilus of the dentate gyrus). The numbers of GHRH- and SRIH-synthesizing neurons in the hypothalamus were not altered in GH-hypersecreting rats. Resection of the tumor restored hypothalamic GHRH and SRIH mRNAs to control levels. GH hypersecretion did not modify <sup>125</sup>I-SRIH binding sites on GHRH neurons. Thus, chronic GH hypersecretion affects the expression of the genes encoding for GHRH and SRIH. The effect is long lasting, not desensitizable and reversible.

**Find the latest version:**

<https://jci.me/116389/pdf>



# Chronic Growth Hormone (GH) Hypersecretion Induces Reciprocal and Reversible Changes in mRNA Levels from Hypothalamic GH-releasing Hormone and Somatostatin Neurons in the Rat

Jérôme Bertherat,\* José Timsit,† Marie-Thérèse Bluet-Pajot,\* Jean-Jacques Mercadier,§ Danièle Gourdjii,|| Claude Kordon,\* and Jacques Epelbaum\*

\*Institut National de la Santé et de la Recherche Médicale (INSERM) U 159, Centre Paul Broca, †INSERM U 25, Hôpital Necker, 75014 Paris; ‡Centre National de la Recherche Scientifique (CNRS) URA 1159, Département de Recherche Médicale, Hôpital Marie Lannelongue, Le Plessis Robinson, and || CNRS, UA 1115, Collège de France, Paris, France

## Abstract

Effects of growth hormone (GH) hypersecretion on somatostatin-(SRIH) and GH-releasing hormone (GHRH) were studied by *in situ* hybridization and receptor autoradiography in rats bearing a GH-secreting tumor. 6 and 18 wk after tumor induction, animals displayed a sharp increase in body weight and GH plasma levels; pituitary GH content was reduced by 47 and 55%, while that of prolactin and thyrotropin was unchanged. At 18 wk, hypothalamic GHRH and SRIH levels had fallen by 84 and 52%, respectively. In parallel, the density of GHRH mRNA per arcuate neuron was reduced by 52 and 50% at 6 and 18 wk, while SRIH mRNA levels increased by 71 and 83% in the periventricular nucleus (with no alteration in the hilus of the dentate gyrus). The numbers of GHRH- and SRIH-synthesizing neurons in the hypothalamus were not altered in GH-hypersecreting rats. Resection of the tumor restored hypothalamic GHRH and SRIH mRNAs to control levels. GH hypersecretion did not modify <sup>125</sup>I-SRIH binding sites on GHRH neurons. Thus, chronic GH hypersecretion affects the expression of the genes encoding for GHRH and SRIH. The effect is long lasting, not desensitizable and reversible. (*J. Clin. Invest.* 1993. 91:1783–1791.) Key words: growth hormone • somatostatin • growth hormone-releasing hormone • *in situ* hybridization • SRIH receptor

## Introduction

Growth hormone (GH)<sup>1</sup> secretion by the anterior pituitary is regulated by a complex interplay between two hypothalamic hormones with opposite effects: GH-releasing hormone (GHRH) and somatotropin-releasing inhibitory hormone (SRIH), also named somatostatin (1). GHRH-containing pro-

jections to the median eminence originate almost exclusively from neurons of the arcuate nucleus, while SRIH-containing terminals arise mainly from the hypothalamic periventricular nucleus.

There is considerable evidence that GH regulates its own rhythmic secretion through a negative feedback mechanism (2). Pituitary GH content and release are reduced in rats treated with exogenous GH or bearing ectopic somatotrophic tumors (3–5). The mechanisms of GH feedback control have been investigated by assessing the hypothalamic content or release of GHRH and SRIH, and by measuring the corresponding hypothalamic mRNA levels. GH deprivation by hypophysectomy leads to a reduction in the hypothalamic content and release of SRIH (6–10), but GHRH content is also reduced in the same model (10–14). This has been attributed to an increase in the hypothalamic release of GHRH, although this phenomenon has not been consistently observed (12, 14). These apparent discrepancies underline the difficulty in interpreting changes in peptide content, which reflect the rate of both synthesis and release. With regard to peptide synthesis, GHRH mRNA levels are strongly increased after hypophysectomy (15, 16), while conflicting data have been obtained for SRIH mRNA, the level of which was either reduced (16) or unaltered (13, 15). These data do not permit to conclude on the specific effect of GH since hypophysectomy elicits a multiendocrine deficit and treatment of hypophysectomised rats with tetraiodothyronine, corticosterone, and testosterone is sufficient to reverse the increase in GHRH mRNA levels, even in the absence of GH (15). In addition, exogenous GH treatment in hypophysectomised rats either failed (15) or only partially restored SRIH and GHRH hypothalamic content (12–14). Earlier studies using short-term administration of GH or implantation of GH/prolactin-secreting tumors in normal rats concluded to a feedback effect of GH (6, 9, 14, 16), but more recent ones either failed to demonstrate any effect on SRIH content (17) or showed an effect restricted to male animals (18).

In this study, we used a rat model of GH hypersecretion induced by subcutaneous injection of GH-secreting cells (GC) that rapidly grow as solid, functional tumors (19). We studied the long-term feedback effects of GH hypersecretion on SRIH and GHRH at the hypothalamic level, and the reversibility of the observed changes after tumor resection. Hypothalamic SRIH and GHRH mRNA levels were determined at 6 and 18 wk by *in situ* hybridization in female rats bearing ectopic GH-producing tumors. As SRIH-specific receptors have recently been located on GHRH neurons (20–22), we also used quantitative light microscopic autoradiography (23) to measure <sup>125</sup>I-SRIH binding sites in the arcuate nucleus (22, 23) as a potential regulatory site for SRIH inhibitory tone on GHRH neurons taking part in GH feedback control (24–26).

Part of this work was presented in abstract form at the XXème Colloque de la Société de Neuroendocrinologie Expérimentale, Geneva, Switzerland, 18–20 September 1991.

Address correspondence to J. Epelbaum, INSERM U 159, Centre Paul Broca, 2 ter rue d'Alesia, 75104 Paris, France.

Received for publication 24 June 1992 and in revised form 23 October 1992.

1. Abbreviations used in this paper: GC, GH-secreting cells; GH, growth hormone; GHRH, GH-releasing hormone; PRL, prolactin; prot, protein; SRIH, somatotropin-releasing inhibitory hormone (somatostatin); TSH, thyroid-stimulating hormone.

*J. Clin. Invest.*

© The American Society for Clinical Investigation, Inc.

0021-9738/93/04/1783/09 \$2.00

Volume 91, April 1993, 1783–1791

## Methods

### *Rat model of chronic GH hypersecretion*

Animal care and experiments were in accordance with the Helsinki guidelines. GC (27) were cultured in Ham's F10 medium supplemented with 15% horse serum and 2.5% FCS (Gibco-BRL, Cergy-Pontoise, France). A suspension of  $10\text{--}15 \times 10^6$  cells in Hanks' medium (0.3 ml vol) was injected subcutaneously into the flank of 10–12-wk-old female Wistar-Furth rats (Iffa Credo, L'Arbresle, France) under sodium methohexitone anesthesia (40 mg/kg intraperitoneally). The animals were maintained on a regular 12-h light-dark cycle, fed ad libitum, and weighed weekly. For neuroanatomical studies, tumor-bearing rats were divided into three groups. The first group ( $n = 5$ ) was studied 6 wk after GC injection and the second ( $n = 5$ ) at 18 wk; their respective littermates served as controls ( $n = 5$  in each group). The tumors were resected under anesthesia 6 wk after the injection in a third group ( $n = 4$ ), and the animals were studied 12 wk later (i.e., 18 wk after cell injection). For hypothalamic peptide measurements, five additional tumor-bearing rats were killed at 18 wk, as well as their respective control littermates. After decapitation, the brain was rapidly dissected free from the skull, frozen by immersion in isopentane at  $-45^\circ\text{C}$ , and stored in air-tight containers at  $-80^\circ\text{C}$  until use.

### *Hormone radioimmunoassays*

Blood was collected immediately after death into heparinized, chilled tubes, and the plasma was stored at  $-20^\circ\text{C}$  until assay. The anterior pituitary was removed, sonicated in 1 ml of 0.05 M  $\text{NaHCO}_3$  buffer, pH 9.9, and centrifuged at 2,000  $g$  for 30 min at  $4^\circ\text{C}$ ; the supernatants were stored at  $-20^\circ\text{C}$  until hormone measurement. The mediobasal hypothalamus was rapidly dissected from the chilled brains, extracted with 0.2 N acetic acid, and stored at  $-80^\circ\text{C}$  until assay of SRIH and GHRH.

Plasma and pituitary GH, prolactin (PRL), and thyroid-stimulating hormone (TSH) were measured by means of RIA against NIADDK rat RP2, RP3, and RP2 reference standards, respectively (28–30). The detection limit was 1 ng/ml for GH, 1.5 ng/ml for PRL, and 0.1 ng/ml for TSH. Intra- and interassay variations were  $< 5$  and 10%; 6 and 12%; and 15 and 15% for GH, PRL, and TSH, respectively.

SRIH was measured by means of RIA as previously described (31). GHRH was assayed using a double antibody RIA with rat GHRH (Peninsula, Merseyside, UK) as standard and specific antisera kindly provided by C. Rougeot (Institut Pasteur, Paris, France). The detection limit was 2 pg/tube. Intra- and interassay variations were  $< 12$  and 18%, respectively.

### *In situ GHRH and SRIH hybridization*

Serial 20- $\mu\text{m}$  cryostat sections of the hypothalamus at levels A 2.12–A 4.16, according to the atlas of Paxinos and Watson (32), were mounted on 2% gelatin-subbed slides and stored at  $-20^\circ\text{C}$  until use.

In situ hybridization was carried out as described elsewhere (25). Briefly, 45-base oligoprobes (bases 31–75 of rat GHRH cDNA [33] and bases 96–111 of rat SRIH cDNA [34] from Genofit [Geneva, Switzerland]) were 3'-labeled with alpha- $^{35}\text{S}$ -dATP (Amersham, Buckinghamshire, England) using terminal deoxynucleotidyltransferase (Boehringer Mannheim, Meylan, France) at a specific activity of 2,000 Ci/mM. Sections were fixed for 10 min at room temperature in potassium phosphate buffer containing 4% paraformaldehyde and pre-hybridized for 30 min in a solution containing 4 $\times$  SSC and 1 $\times$  Denhardt's solution (Sigma, Saint-Quentin Fallavier, France). They were then rinsed in 4 $\times$  SSC and immersed for 10 min in the same buffer (pH 8) containing triethanolamine (1.33%) and acetic anhydride (0.25%). Hybridization was run for 18 h at  $38^\circ\text{C}$  in the hybridization solution (50% formamide, 4 $\times$  SSC, 1 $\times$  Denhardt's, 1% sarcosyl, 10 mM dithiothreitol, 0.1 M potassium phosphate, pH 7.4, and 100 ng of yeast tRNA, 100 ng of herring sperm DNA) containing the labeled oligo-probe (2 nM). Sections were then rinsed at  $36^\circ\text{C}$  for 30 min in 4 $\times$  SSC, 3 $\times$  15 min in 1 $\times$  SSC and 3 $\times$  15 min in 0.1 $\times$  SSC, dried and coated by dipping in emulsion K5; (Ilford, St. Priest, France) diluted 1:1 with distilled water. Exposure times were 12–14 d and 5–6 wk for SRIH and

GHRH, respectively. Autoradiograms were developed in Dektol (Kodak, Marnes la Vallée, France), stained with cresyl violet, and cover-slipped. The specificity of labelling has been reported elsewhere (25, 35).

### *$^{125}\text{I}$ -SRIH autoradiography*

Monoiodo Tyro DTp8 SRIH14 (Peninsula) was labelled with chloramine T  $^{125}\text{I}$ -SRIH (780 Ci/mM). The labeled tracer was purified on a carboxymethyl cellulose column (CM52; Whatman Inc, Clifton, NJ) by stepwise elution with 2–200 mM ammonium acetate at pH 4.6.

$^{125}\text{I}$ -SRIH binding experiments were performed on series of adjacent coronal sections as previously described (20). Sections were preincubated for 15 min at room temperature in 0.05 M Tris-HCl buffer (pH 7.4) containing 0.25 M sucrose and 0.2% BSA. They were then incubated for 45 min at room temperature in the same medium supplemented with  $^{125}\text{I}$ -SRIH,  $\text{MgCl}_2$   $5 \times 10^{-3}$  M, and bacitracin  $5 \times 10^{-3}$  M. To determine nonspecific binding, sections adjacent to those used for total binding were incubated in the presence of 1  $\mu\text{M}$  nonradioactive SRIH14; specific binding was calculated as the difference between total and nonspecific binding. After incubation, sections were rinsed in two consecutive ice-cold baths of supplemented Tris buffer (5 min/bath) and immediately fixed by immersion in 4% glutaraldehyde in 0.05 M phosphate buffer for 30 min at  $4^\circ\text{C}$  (9). This procedure irreversibly cross-links  $> 90\%$  of  $^{125}\text{I}$ -SRIH molecules to tissue proteins (36). After fixation, sections were dehydrated in a graded ethanol series, defatted in xylene, rehydrated, and coated by dipping in Ilford K5 emulsion. After 4–6 wk exposure, the autoradiograms were developed and stained as for in situ hybridization.

### *Image analysis and quantification*

$^{125}\text{I}$ -SRIH autoradiography. Sections were examined with a Leitz orthoplan microscope coupled to a computerized image analysis system (RAG 200; Biocom, Les Ulis, France). Cells were located with bright field illumination and  $^{125}\text{I}$ -SRIH labeling was quantified under dark field illumination (23). Optical density was converted into radioactivity units (dpm/pixel) with reference to standards prepared from brain pastes with known concentrations of  $^{125}\text{I}$ -SRIH. A series of standards was treated in parallel with the experimental sections in each experiment. Pericellular grains were quantified in the arcuate nucleus by tracing a circle of uniform diameter on the highly labeled perikarya. A minimum of 10 cell bodies were measured on each side of the third ventricle.  $^{125}\text{I}$ -SRIH binding was also quantified on the same sections at the level of the dentate gyrus of the hippocampus, which showed homogenous labeling. A minimum of 10 sections were analyzed for each animal. Specific binding amounted to 70% of total binding in the dentate gyrus of the hippocampus, and 50% in the arcuate nucleus.

*In situ GHRH and SRIH hybridization.* Grain density was quantified using epifluorescence illumination and the Histo program (Biocom), which gives densitometric integration of the number of grains per cell. Labeled cells were identified by cresyl violet staining of the nucleus, associated with a cluster of silver grains. Clusters were counted if the number of grains was above the background level on each section. The number of grains was quantified by tracing a circle of uniform diameter on the perikarya. For both regions and probes, a minimum of six sections were analyzed for each animal.

### *Statistical analysis*

Data are expressed as means  $\pm$  SEM. Groups were compared using one-way ANOVA and a posteriori using Fisher's test to compare tumor-bearing rats to controls, tumor-bearing to tumor-resected rats, and tumor-resected rats to controls.

## Results

### *Effects of tumor growth on rat body weight and plasma GH levels*

The main characteristics of the tumor-bearing and control rats used in the neuroanatomical studies are shown in Table I. Body

Table I. Main Characteristics of Tumor-Bearing and Control Rats

Group (n)	Time of the study	Body weight	Tumor weight	GH plasma levels
	wk	g	g	μg/liter
Control (5)	6	199±3	—	22±14
Tumor (5)	6	304±11*	3.2±0.8	318±52*
Control (5)	18	236±4	—	7.4±4.3
Tumor (5)	18	509±19*	48.2±4.6	5,395±1,327*
Tx (4)	18	279±6 <sup>§</sup>	(5.3±1.0 <sup>  </sup> )	5.1±1.7 <sup>§</sup>

Rats were studied 6 and 18 wk after the subcutaneous injection of GH-secreting cells and compared with control littermates. Tx, tumor-bearing animals were tumorectomized 6 wk after cell injection and were studied 12 wk later.

\*  $P < 0.01$  vs age-matched control rats; †  $P < 0.05$  vs age-matched control rats; §  $P < 0.01$  vs 18-wk tumor-bearing rats. || Tumor weight at 6 wk.

weight increased in tumor-bearing rats 6 and 18 wk after GC cell injection (53 and 116%, respectively, compared to the corresponding controls). This was accompanied by a very strong increase in GH plasma levels. Body weight fell significantly after resection of the tumor, but remained higher than in age-matched controls. Plasma GH levels returned to control values. In the group of rats dissected for hypothalamic peptide measurements, 18 wk after GC cell injection, tumors weighed 38.4±4.3 g. Body weight (tumor-bearing rats [ $n = 5$ ]: 442±22 g; controls [ $n = 5$ ]: 217±6,  $P < 0.01$ ) and GH plasma levels (tumor-bearing rats: 6,953±1,993 μg/liter; controls: 7±1,  $P < 0.01$ ) increases were equivalent to those described in Table I.

#### Hormone content in the pituitary

Pituitary contents of GH, prolactin, and TSH were assessed in 6-wk tumor-bearing rats ( $n = 5$ ) and controls ( $n = 5$ ). The weight of the pituitary was similar in both groups (10.1±1 mg and 11.8±0.4 mg in control and tumor-bearing rats, respectively). Pituitary GH content displayed a twofold decrease in tumor-bearing rats (89±10 μg/mg protein [prot]) as compared to controls (167±9 μg/mg prot,  $P < 0.001$ ). In contrast, there was no difference in prolactin content (65±11 μg/mg prot vs 78±18 μg/mg prot) or TSH content (5.2±0.4 mg/mg prot vs 6.0±1.0 mg/mg prot). GH content in pools of four pituitaries obtained from 18-wk tumor-bearing rats (38 μg/mg prot) were lower than control values (84 μg/mg prot) and returned to control values after resection of the tumor (80 μg/mg prot).

#### GHRH and SRIH content in the hypothalamus

GHRH peptide levels were very strongly reduced in the hypothalamus of 18-wk tumor-bearing rats (43.6±15.4 pg/mg prot,  $n = 5$ ) compared to controls (277.2±51.7 pg/mg prot,  $n = 5$ ,  $P < 0.02$ ). SRIH peptide levels were also reduced (16.5±1.4 vs 34.1±3.6 ng/mg prot,  $P < 0.02$ ).

#### In situ hybridization

**GHRH.** In the tumor-bearing animals, the mean number of grains per cell in the arcuate nucleus fell by 52 and 50%, 6 and 18 wk after GC cell injection (Figs. 1 and 2). The density of GHRH mRNA labeling returned to control levels following tumorectomy. The numbers of GHRH-hybridizing cells in the arcuate nucleus were similar between groups (at 6 wk, controls: 15±2 cells/hemisection, tumor-bearing rats: 11±1, NS; at 18

wk, controls: 14±1; tumor-bearing rats: 12±1, and tumor-resected rats: 14±1, NS).

**SRIH.** In the periventricular nucleus, the mean number of grains per cell increased by 71 and 83% at 6 and 18 wk, respectively, relative to the controls (Fig. 3 and 4). After tumorectomy, SRIH mRNA labeling was similar to control values, and fell by 56% relative to the tumor-bearing rats. In this nucleus, the numbers of SRIH-hybridizing cells were not significantly different between the controls and the experimental animals (at 6 wk, controls: 40±6 cells/hemisection, tumor-bearing rats: 46±4, NS; at 18 wk, controls: 38±5, tumor-bearing rats: 49±4, and tumor-resected rats: 46±6, NS). SRIH mRNA levels were also measured in the dentate gyrus to check the regional specificity of the changes in the hypothalamus. No difference was found between the various experimental groups: The number of grains per cell was 30±5 in the controls and 34±3 in the tumor-bearing rats at 6 wk; the values were, respectively, 23±3 and 24±4 at 18 wk; the value in the tumorectomized animals was 18±3.

**<sup>125</sup>I-SRIH binding.** Within the arcuate nucleus, no difference was observed in pericellular specific <sup>125</sup>I-SRIH binding in the tumor-bearing rats at 6 or 18 wk relative to their respective controls and to values after tumorectomy (Fig. 5). Representative sections are shown in Fig. 6. Similarly, no differences were observed within the dentate gyrus in the various treatment groups compared to their controls: 6 wk after cell injection, specific <sup>125</sup>I-SRIH binding was 2,326±229 dpm in the controls and 2,721±609 dpm in the tumor-bearing rats. At 18 wk, values were 2,833±239 and 2,506±367 dpm, respectively, and 2,572±408 dpm after tumorectomy.

## Discussion

The data reported herein point to persistent effects of GH hypersecretion on the GHRH/SRIH hypothalamic network during chronic GH hypersecretion in the rat.

We used GC tumor-bearing rats to study the specific effects of chronic GH hypersecretion on the regulation of the growth hormone axis. Other cell lines used to induce ectopic somatotrophic tumors (4, 18, 37, 38) usually secrete both GH and prolactin in vitro, and, occasionally, in vivo. In contrast, GC cells do not secrete prolactin in vitro (39) and prolactin plasma levels are not increased in GC tumor-bearing rats (19).

There was a very strong increase in GH plasma levels and body weight 6 wk after GC cell injection, with a further increase at 18 wk. Removal of the tumor led to a fall in GH plasma levels to the normal range. Pituitary GH content was reduced by 54–64% in the tumor-bearing animals, supporting the negative feedback effect of chronically increased circulating GH levels. In contrast, GH hypersecretion did not affect pituitary prolactin or TSH content.

Hypothalamic GHRH content was reduced in 18 wk tumor-bearing rats, suggesting a long term negative feedback effect of GH plasma levels, but hypothalamic SRIH content also fell in the same animals. Studies on the effect of GH on GHRH and SRIH hypothalamic contents yielded conflicting results. In intact rats, GH administration for 1 or 2 wk led to either a moderate decrease (18) or no apparent change of GHRH contents (17), while SRIH content was not modified. Hypophysectomy resulted in a considerable decrease in SRIH hypothalamic levels (6, 7, 9, 17) but also in GHRH content (12, 13, 17), a result that mirrors our observations of the effect of

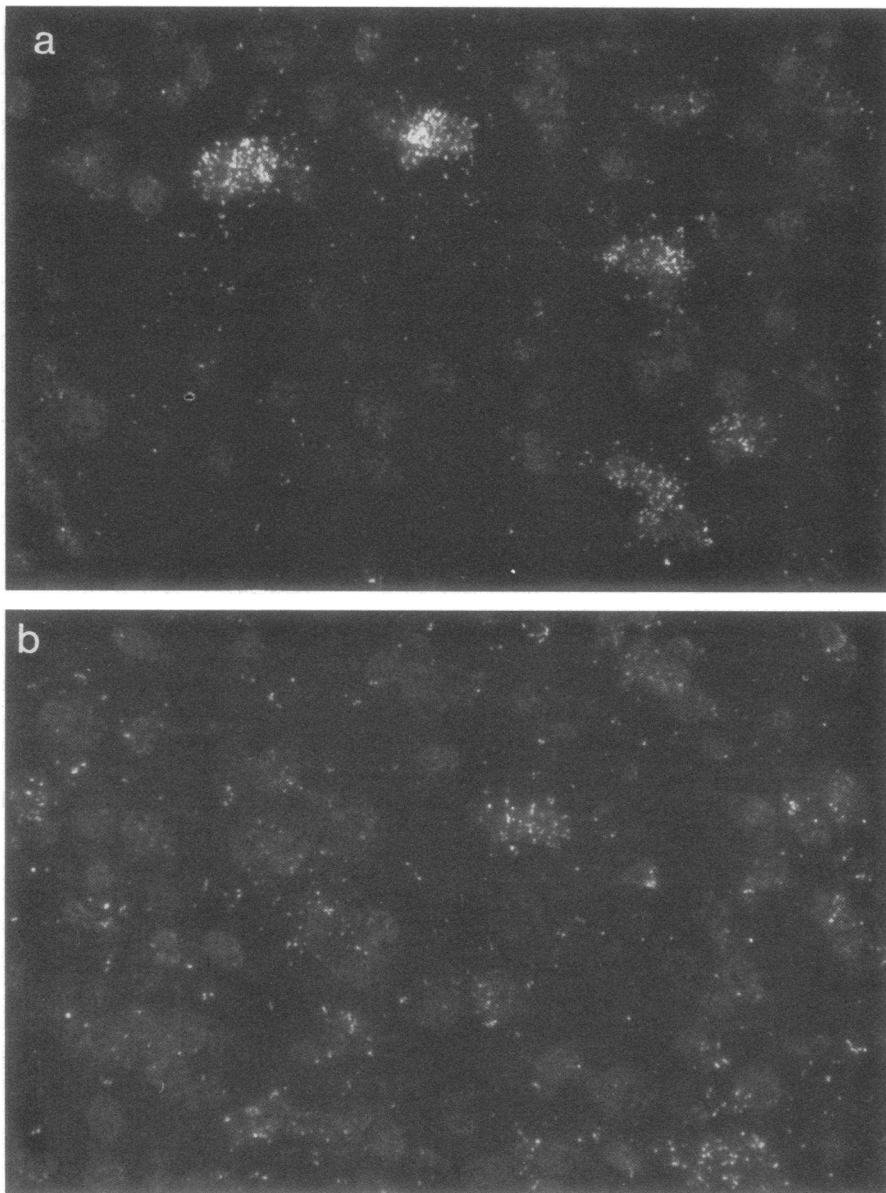


Figure 1. Autoradiograms of in situ GHRH hybridization. In situ GHRH hybridization in the arcuate nucleus of a control (a) and a 6-wk tumor-bearing rat (b). Dark field illumination  $\times 25$ .

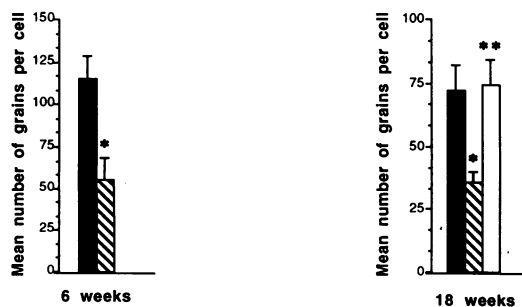
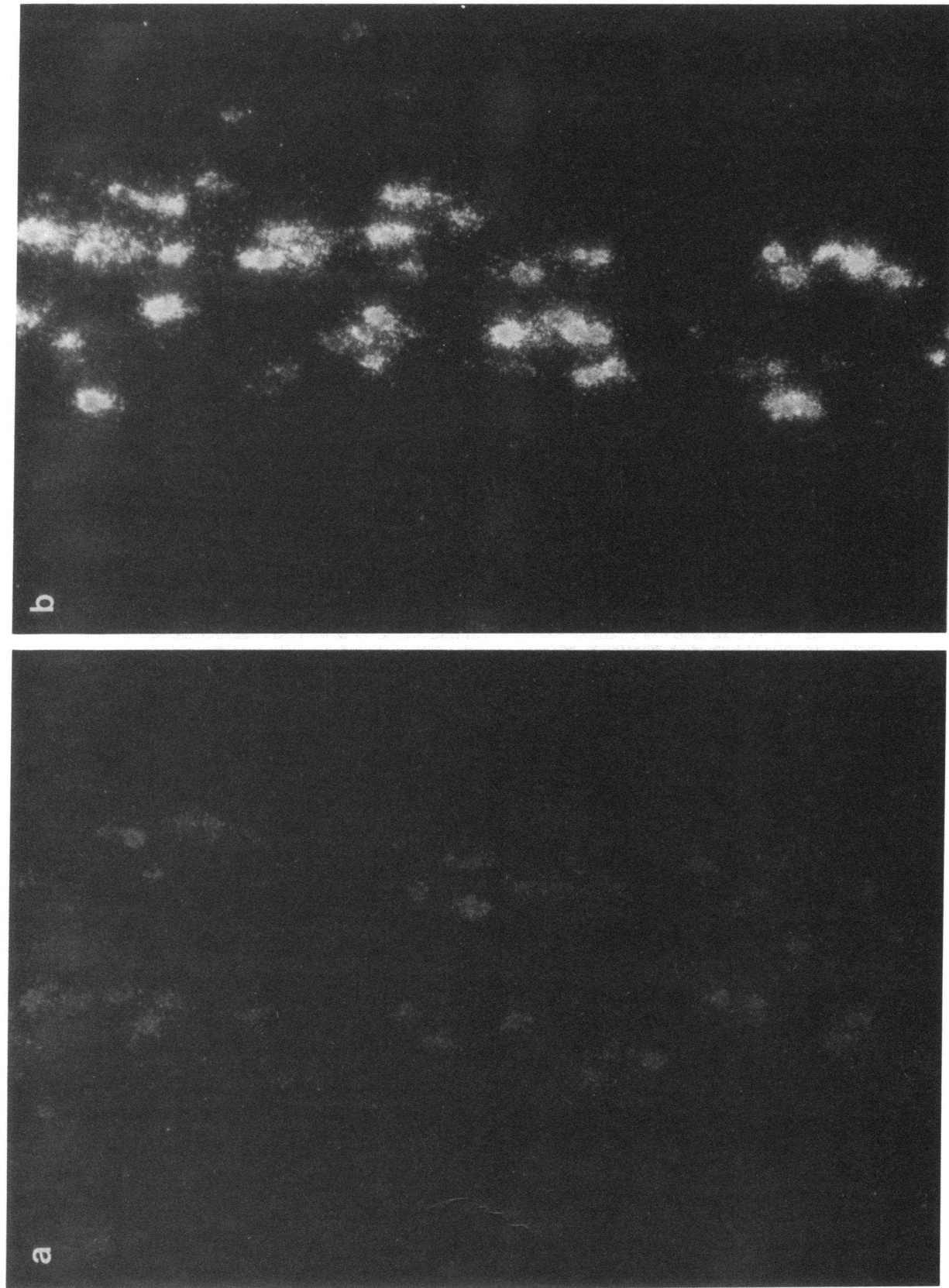
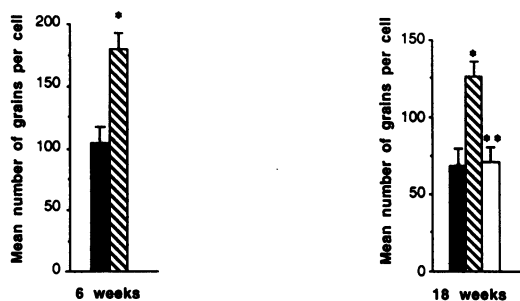


Figure 2. GHRH mRNA levels in the arcuate nucleus. Mean ( $\pm$ SD) number of grains per cell quantified by in situ hybridization, in control rats, rats studied 6 (left panel) and 18 (right panel) wk after injection of GH-secreting cells, and tumorectomized animals. \* $P < 0.01$  tumor-bearing vs control rats. \*\* $P < 0.01$  tumor-bearing vs tumorectomized rats. ■, Control; ▨, tumor bearing; □, tumorectomized.

chronic GH hypersecretion. Also, in hypophysectomized animals, treatment with GH either did not modify (17) or only partially restored GHRH concentrations (12, 13, 17) and had only minimal effects on SRIH hypothalamic contents (6, 7). Moreover, the effects of GH appeared to be sex dependent, being more pronounced in males than in females, as well as time dependent (18). Indeed no changes in GHRH and SRIH contents were observed 2 wk after implantation of the GH/PRL-secreting MtTW15 tumor. By contrast, at 4 wk, a decrease in GHRH content was noticed, but of a smaller extent (18%) than in our study (84%), and no effect was apparent on SRIH content (18). It is therefore tempting to speculate that the differences observed in the effects of high GH in these various studies may be caused by the different time course of the experiments, as well as a sex difference. At the opposite, in conditions of life-long GH deficiency, such as in the Lewis Dw/Dw dwarf rat, GHRH contents are increased 1.45-fold and SRIH contents are decreased by 74% as compared to con-

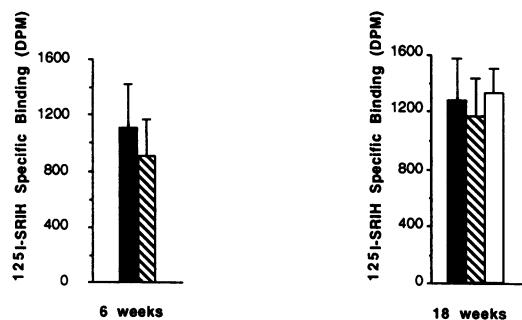


**Figure 3.** Autoradiograms of in situ SRIH hybridization in the periventricular nucleus in a control (a) and a 6-wk tumor-bearing rat (b). Dark field illumination  $\times 12.5$ .



**Figure 4.** SRIH mRNA levels in the periventricular nucleus. Mean ( $\pm$ SD) number of grains per cell quantified by in situ hybridization in control rats, rats studied 6 (left panel) and 18 (right panel) wk after injection of GC cells, and tumorectomized animals. \* $P < 0.01$  tumor-bearing vs control rats. \*\* $P < 0.01$  tumor-bearing vs tumorectomized rats. ■, Control; ■, tumor bearing; □, tumorectomized.

controls (40). However, in another dwarf rat strain, the SDRs, the number of GHRH-containing cells are doubled as compared to controls, while SRIH-containing cells are only minimally affected, and median eminence terminals containing both peptides seems unchanged (41, 42). It is therefore difficult to conclude about GH feedback actions only from the measurement of peptide contents, since the latter reflects variations in the rates of both synthesis and release. We thus measured peptide mRNA levels using a quantitative in situ hybridization method. GHRH mRNA levels in the arcuate nucleus were decreased in tumor-bearing rats. This observation is in keeping with the changes previously reported in normal rats after hypophysectomy and GH replacement (13, 15), as well as in rats bearing GH-prolactin-secreting MtTW15 tumors for 4 wk (38), and in the dwarf lit/lit mouse (43). In contrast to the effect observed on arcuate GHRH mRNA-containing neurons, SRIH mRNA levels were increased in the periventricular nucleus. Conflicting data have been reported concerning the effects of GH on SRIH mRNA. A decline in hypothalamic SRIH mRNA levels after hypophysectomy and partial restoration after a short term (5 d) treatment with supra-physiologic doses of GH were observed by one group (16) but not by others (13, 15). These differing results might be explained by the use of Northern blot analysis (13, 15) compared to in situ hybridization (16). Indeed, SRIH-synthesizing cells are widely distrib-



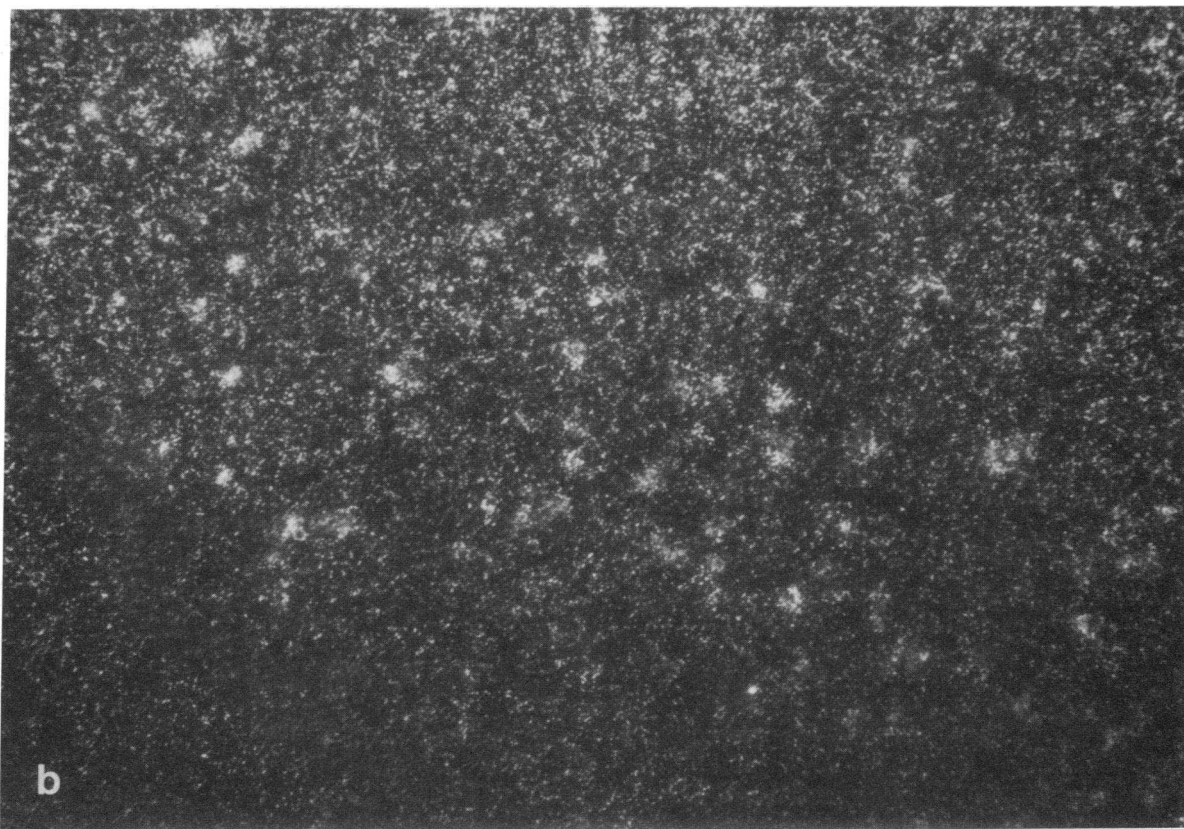
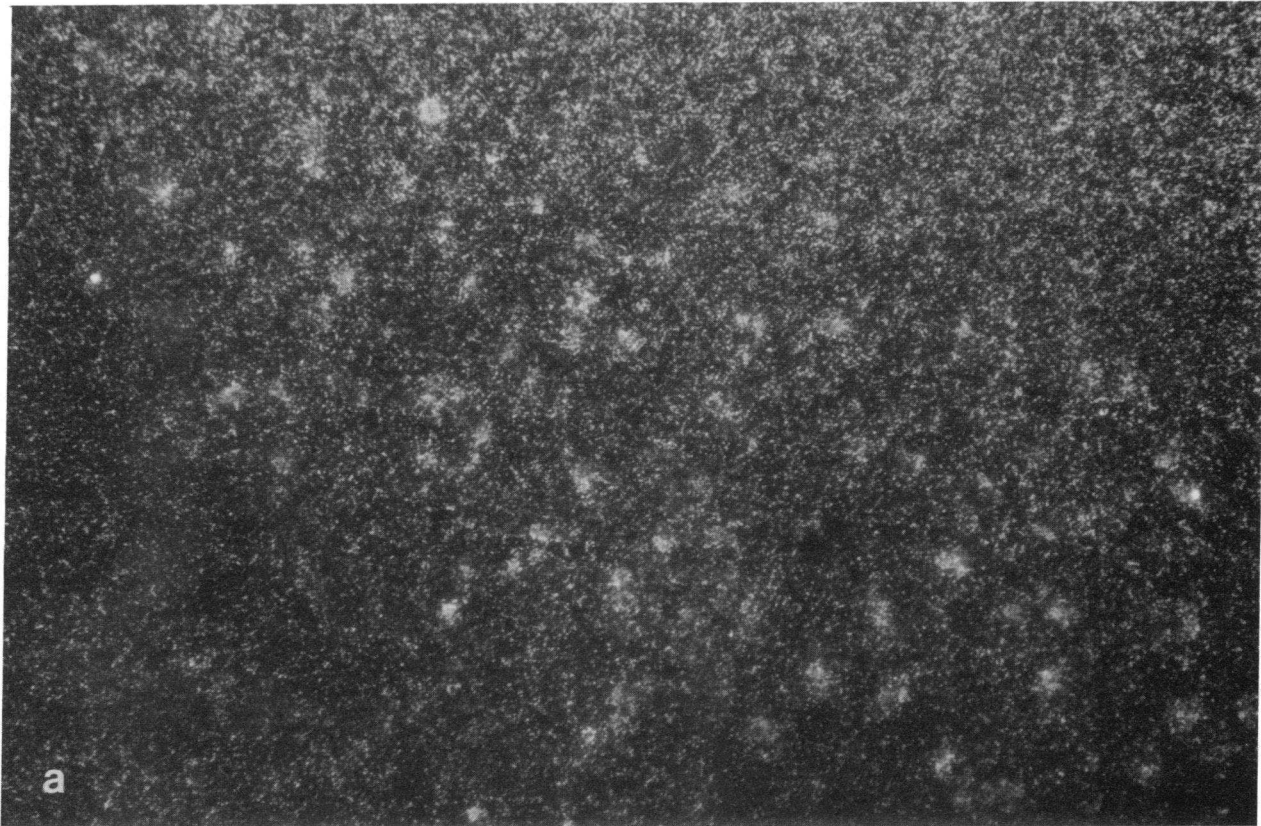
**Figure 5.** Pericellular  $^{125}\text{I}$ -SRIH specific binding levels in the arcuate nucleus.  $^{125}\text{I}$ -SRIH specific binding, expressed as dpm, was not different in the arcuate nucleus of control rats and tumor-bearing rats 6 (left panel) and 18 (right panel) wk after injection of the GC cells (left panel). ■, Control; ■, tumor bearing; □, tumorectomized.

uted in the hypothalamus and Northern blot analysis is unlikely to detect an area-restricted change. The regional specificity of the effect is supported by our observation of an increase in SRIH mRNA levels within the periventricular nucleus but not in the dentate gyrus, an extrahypothalamic region. Alternatively, the short term duration of the GH treatment might also explain such a discrepancy since our results demonstrate a positive effect on SRIH mRNA levels in the periventricular nucleus after 6 and 18 wk of GH hypersecretion. A recent study in transgenic mice (44) also demonstrated that a life-long excess in endogenous GH results in a similar stimulation of hypothalamic SRIH mRNA levels. In that respect, it can be postulated that the decrease in SRIH hypothalamic content observed 18 wk after GC implantation, concomitant to the increased SRIH mRNA levels, reflects an increased release of the peptide as also observed in the case of GHRH in conditions of GH deficiency in hypophysectomized rats (13) and lit/lit mouse (43). Alternatively, the discordant effects observed on SRIH peptide contents and SRIH mRNA levels could be related to an impairment of posttranscriptional and/or translational mechanisms during chronic GH hypersecretion.

The tumors were fully functional at 18 wk, as evidenced by the good correlation between tumor weight and GH plasma levels (data not shown), and we could thus study the very long-term effects of GH hypersecretion. The maximal GH feedback on GHRH and SRIH mRNAs was reached within the first 6 wk of GH hypersecretion and persisted for the following 12 wk in spite of a further increase in GH plasma levels. These effects were completely reversed after removal of the tumor. This indicates the persistence of GH feedback during chronically high GH plasma levels. However, this long-lasting feedback action of GH on SRIH and GHRH mRNAs was still reversible after normalization of GH plasma levels.

The finding that in presence of high GH levels, SRIH mRNA levels are increased in parallel with a decrease in GHRH mRNA levels, is consistent with several lines of evidence indicating that SRIH could inhibit GHRH synthesis within the arcuate nucleus (see reference 20 for review) through specific receptors located on GHRH neurons (23, 25). Recently, it has been shown that SRIH inhibits GHRH release in vitro on rat hypothalamic explants (45) and in vivo in conscious sheep (46). Interestingly, high GH levels also increase hypothalamic SRIH release (6, 9). We thus investigated whether SRIH receptors located on GHRH neurons were also affected by GH hypersecretion. The fact that  $^{125}\text{I}$ -SRIH specific binding on arcuate nucleus perikarya was not altered might indicate that SRIH receptors on GHRH neurons are not desensitized in the presence of high SRIH levels resulting from the stimulation of periventricular somatostatinergic neurons stimulated by increased GH secretion. Alternatively, SRIH fibers innervating GHRH arcuate neurons might originate from another source than the GH-regulated periventricular hypothalamic system, and this could also explain the lack of modification in  $^{125}\text{I}$ -SRIH specific binding on arcuate nucleus perikarya. At any rate, these observations suggest that the ability of SRIH to inhibit GHRH arcuate neurons by acting on specific receptors is maintained during GH hypersecretion. The decrease in GHRH mRNA levels might thus be mediated by direct SRIH inhibition within the arcuate nucleus.

The mechanisms of GH feedback control of SRIH and GHRH synthesis are still unknown, although a direct effect of GH at the hypothalamic level through a short-loop mechanism



*Figure 6.* Autoradiograms of <sup>125</sup>I-SRIH binding in the arcuate nucleus. (*a*) control rat, (*b*) 6-wk tumor-bearing rat. Dark field illumination  $\times 12.5$ .



has been suggested. In the rat, central administration of GH led to a decrease in GH plasma levels (3). In the same respect, transgenic mice that selectively express the GH gene in the central nervous system exhibit low plasma levels of GH (47). GH receptor mRNAs have been evidenced by in situ hybridization in the arcuate and periventricular nucleus with a distribution similar to that of GHRH and SRIH neurons (48). However, the presence of GH receptor mRNAs is not always associated with that of functional GH binding sites (49). Alternatively, GH feedback could be explained by an indirect pathway through a long-loop mechanism involving intermediate factors such as insulin-like growth factors (50), since binding sites for insulin-like growth factors 1 and 2 have been described in the hypothalamus (51). However, it has recently been shown that short-term GH hypersecretion exerts negative feedback without modifying IGF-I plasma levels (52). On the other hand, in the GH-deficient Lewis Dw/Dw rat, GH regulates GHRH mRNA levels independently of IGF-I, while SRIH mRNA modulation is dependent on the latter only (53). Thus, the respective roles of GH and insulin-like growth factors in these feedback mechanisms remain to be clarified.

In conclusion, GH feedback controls hypothalamic peptide synthesis through an inhibition of GHRH mRNA and a stimulation of SRIH mRNA, and persists during chronic exposure to high levels of growth hormone in the rat. Despite the long-term GH hypersecretion, the changes observed in SRIH and GHRH mRNA levels are reversible after normalization of GH plasma levels. <sup>125</sup>I-SRIH specific binding sites are not altered in the arcuate nucleus, suggesting that SRIH may still act at this level as an inhibitory factor in the complex interplay between the two neurohormones.

## Acknowledgments

We wish to thank Pr. J. Lubetzki for his constant support and interest in this work, C. Rougeot for the gift of specific GHRH antiserum, F. Mounier and C. Videau for their expert technical assistance, and D. Young for critical review of the manuscript.

This work was supported in part by the Institut National de la Santé de la Recherche Médicale and by a Contrat de Recherche Clinique (No. 101) from the Assistance Publique des Hôpitaux de Paris. J. Bertherat is a recipient of a grant from the Fonds d'Etude du Corps Médical des Hôpitaux de Paris.

## References

- Brazeau, P., W. Vale, R. Burgus, N. Ling, M. Butcher, J. Rivier, and R. Guillemin. 1973. Hypothalamic polypeptide that inhibits the secretion of immunoreactive pituitary growth hormone. *Science (Wash. DC)*. 179:77-79.
- Müller, E. E., V. De Gennaro Colonna, S. G. Cella, A. Torsello, E. Higo, S. Loche, V. Arce, D. Cocchi, and V. Locatelli. 1991. Autoregulation of the growth hormone axis. In *Molecular and Clinical Advances in Pituitary Disorders. Current Issues in Endocrinology and Metabolism*. S. Melmed and R. Robbins, editors. Blackwell Scientific Publications, Inc., Cambridge, MA, 177-194.
- Tannenbaum, G. S. 1980. Evidence for autoregulation of growth hormone secretion via the central nervous system. *Endocrinology*. 107:2117-2120.
- MacLeod, R. M., and A. Abad. 1968. On the control of prolactin and growth hormone synthesis in rat pituitary glands. *Endocrinology*. 83:799-806.
- Porter, T. E., T. T. Chen, and L. S. Frawley. 1989. Pituitaries transplanted under the renal capsule contain functional growth hormone (GH) secretors and suppress GH and prolactin release from individual eutopic pituitary cells. *Endocrinology*. 125:3059-3067.
- Patel, Y. C. 1979. Growth hormone stimulates hypothalamic Somatostatin. *Life Sci*. 24:1589-1594.
- Kanatsuka, A., H. Makino, Y. Matsushima, M. Osegawa, M. Yamamoto, and A. Kumagai. 1979. Effect of hypophysectomy and growth hormone administration on somatostatin content in the rat hypothalamus. *Neuroendocrinology*. 29:186-190.
- Terry, L. C., and W. R. Crowley. 1980. The effect of hypophysectomy on somatostatin-like immunoreactivity in discrete hypothalamic and extrahypothalamic nuclei. *Endocrinology*. 107:1771-1775.
- Berelowitz, M., S. L. Firestone, and L. A. Frohman. 1981. Effects of growth hormone excess and deficiency on hypothalamic somatostatin content and release and on tissue somatostatin distribution. *Endocrinology*. 109:714-719.
- Merchenthaler, I., and A. Arimura. 1985. Effects of hypophysectomy on immunocytochemically demonstrated growth-hormone releasing factor (GHRF) in the rat brain. *Peptides*. 6:865-867.
- Ganzetti, I., V. de Gennaro, M. Redaelli, E. E. Müller, and D. Cocchi. 1986. Effects of hypophysectomy and growth hormone replacement on hypothalamic GHRH. *Peptides*. 7:1011-1014.
- Katakami, H., T. R. Downs, and L. A. Frohman. 1987. Effect of hypophysectomy on hypothalamic Growth-hormone releasing factor content and release in the rat. *Endocrinology*. 120:1079-1082.
- Chomczynski, P., T. R. Downs, and L. A. Frohman. 1988. Feedback regulation of growth hormone (GH)-releasing hormone gene expression by GH in rat hypothalamus. *Mol. Endocrinol.* 2:236-241.
- De Gennaro Colonna, V., E. Cattaneo, D. Cocchi, E. E. Müller, and A. Maggi. 1988. Growth Hormone regulation of growth hormone-releasing hormone gene expression. *Peptides*. 9:985-988.
- Wood, T. L., M. Berelowitz, M. C. Gelato, C. T. Roberts, D. LeRoith, W. J. Millard, and J. F. McKelvy. 1991. Hormonal regulation of rat hypothalamic neuropeptides mRNAs: effect of hypophysectomy and hormone replacement on growth-hormone-releasing factor, somatostatin and the insulin-like growth factors. *Neuroendocrinology*. 53:298-305.
- Rogers, K. V., L. Vician, R. A. Steiner, and D. K. Clifton. 1988. The effect of hypophysectomy and growth hormone administration on pre-prosomatostatin messenger ribonucleic acid in the periventricular nucleus of the rat hypothalamus. *Endocrinology*. 122:586-591.
- Leidy, W., M. T., McDermott, and R. J. Robbins. 1990. Effect of hypophysectomy and growth hormone administration on hypothalamic growth hormone-releasing hormone and somatostatin content: relationship to age-related growth rate. *Neuroendocrinology*. 51:400-405.
- Maiter, D. M., S. M. Gabriel, J. I. Koenig, W. E. Russel, and J. B. Martin. 1990. Sexual differentiation of growth hormone feedback effects on hypothalamic growth hormone-releasing hormone and somatostatin. *Neuroendocrinology*. 51:174-180.
- Timits, J., B. Riou, J. Bertherat, C. Wisniewsky, N. S. Kato, A. S. Weisberg, J. Lubetzki, Y. Lecarpentier, S. Winegrad, and J. J. Mercadier. 1990. Effects of chronic growth hormone hypersecretion on intrinsic contractility, energetics, isomyosin pattern, and myosin adenosine triphosphatase activity of rat left ventricle. *J. Clin. Invest.* 86:507-515.
- Epelbaum, J., E. Moyse, G. S. Tannenbaum, C. Kordon, and A. Beaudet. 1989. Combined autoradiographic and immunohistochemical evidence for an association of somatostatin binding sites with growth hormone-releasing factor-containing nerve cell bodies in the rat arcuate nucleus. *J. Neuroendocrinol.* 1:109-115.
- Bertherat, J., P. Dournaud, A. Berod, E. Normand, B. Bloch, W. Rostène, C. Kordon, and J. Epelbaum. 1992. Growth hormone releasing hormone synthesizing neurons are a subpopulation of somatostatin receptor labelled cells in the rat arcuate nucleus: a combined in situ hybridization and receptor light microscopic radioautographic study. *Neuroendocrinology*. 56:25-31.
- McCarthy, G. F., A. Beaudet, and G. S. Tannenbaum. 1992. Colocalization of somatostatin receptors and growth-hormone-releasing factor immunoreactivity in neurons of the rat arcuate nucleus. *Neuroendocrinology*. 56:18-24.
- Bertherat, J., A. Slama, C. Kordon, C. Videau, and J. Epelbaum. 1991. Characterization of pericellular [<sup>125</sup>I]-Tyr0 dTrp8 Somatostatin binding sites in the rat arcuate nucleus by a newly developed method: quantitative high-resolution light microscopic radioautography. *Neuroscience*. 41:571-579.
- Tannenbaum, G. S., G. F. McCarthy, P. Zeitler, and A. Beaudet. 1990. Cysteamine-Induced enhancement of growth hormone-releasing factor (GRF) immunoreactivity in arcuate neurons: morphological evidence for putative somatostatin/GRF interactions within hypothalamus. *Endocrinology*. 127:2551-2560.
- Bertherat, J., A. Berod, E. Normand, B. Bloch, W. Rostene, C. Kordon, and J. Epelbaum. 1991. Somatostatin depletion by cysteamine increases somatostatin binding and growth hormone-releasing factor messenger ribonucleic acid in the arcuate nucleus. *J. Neuroendocrinol.* 3:115-118.
- Daikoku, S., and Y. Tsutuo. 1990. Neuron systems regulating growth hormone releasing hormone (GRF)-containing neurons in the rat hypothalamus. Second International Congress of Neuroendocrinology, Bordeaux, France, 24-29 June. *Neuroendocrinol.* 52, S1:121. (Abstr.)
- Evans, G. A., and M. G. Rosenfeld. 1979. Regulation of prolactin mRNAs analyzed using a specific cDNA probe. *J. Biol. Chem.* 254:8023-8026.
- Bluet-Pajot, M. T., D. Durand, F. Mounier, J. F. Leonard, and C. Kordon. 1989. Differential response of lactotrophs and somatotrophs to a joined application of vasoactive intestinal peptide and thyrotrophin-releasing hormone in the rat. *J. Neuroendocrinol.* 1:135-139.

29. Niswender, G. D., C. L. Chen, A. R. Migdley, J. Ellis, and J. Meites. 1969. Radioimmunoassay for rat prolactin. *Proc. Exp. Biol. Med.* 130:793-799.
30. Kieffer, J. D., B. D. Weintraub, W. Brigelman, S. Leeman, and F. Maloof. 1974. Homologous RIA of thyrotropin rat plasma. *Acta Endocrinol. (Copenh.)*. 76:495-505.
31. Rorstad, O. P., J. Epelbaum, P. Brazeau, and J. B. Martin. 1979. Chromatographic and immunological properties of immunoreactive somatostatin in hypothalamic and extrahypothalamic brain regions of the rat. *Endocrinology*. 105:1083-1092.
32. Paxinos, G., and C. Watson. 1986. The rat brain on stereotaxic coordinates. Academic Press Ltd. London.
33. Mayo, K. E., G. M. Cerelli, M. G. Rosenfeld, and R. M. Evans. 1985. Characterization of cDNA and genomic clones encoding the precursor to rat hypothalamic growth hormone-releasing factor. *Nature (Lond.)*. 314:464-467.
34. Funckes, C. L., C. D. Minth, R. Deschenes, M. Magazin, M. A. Taviani, M. Sheets, K. Collier, H. L. Weith, D. C. Aron, B. A. Roos, and J. E. Dixon. 1983. Cloning and characterization of mRNA encoding rat preprosomatostatin. *J. Biol. Chem.* 258:8781-8787.
35. Llorens-Cortes, C., J. Bertherat, C. Jomary, C. Kordon, and J. Epelbaum. 1992. Regulation of somatostatin synthesis by GABA<sub>A</sub> receptor stimulation in mouse brain. *Mol. Brain Res.* 13:277-281.
36. Moyses, E., A. Beaudet, J. Bertherat, and J. Epelbaum. 1992. High resolution autoradiographic localization of somatostatin binding sites in rat brain. *J. Chem. Neuroanat.* 5:75-84.
37. Seo, H., S. Refetoff, and V. S. Fang. 1977. Induction of hypothyroidism and hypoprolactinemia by growth hormone producing rat pituitary tumors. *Endocrinology*. 100:216-226.
38. Maiter, D., J. I. Koenig, and L. M. Kaplan. 1991. Sexually dimorphic expression of the growth hormone-releasing hormone gene is not mediated by circulating gonadal hormones in the adult rat. *Endocrinology*. 128:1709-1716.
39. Bancroft, F. C. 1981. GH-cells: functional clonal lines of rat pituitary tumor cells. In *Functionally Differentiated Cell Lines*. G. Sato, editor. Alan R. Liss, New York. 47-60.
40. Bilezikjian, L. M., S. W. Sutton, S. Otto, A. L. Bolount, and P. M. Plotsky. 1989. Characterization of the hypothalamic-somatotrope axis in the dwarf rat. 72nd Annual Meeting of the Endocrine Society, Atlanta, GA, 20-23 June 1989. 129:57. (Abstr.)
41. Sakuma, S., K. Maebeura, and H. Ishihara. 1989. Immunohistochemical studies on the somatostatin and growth hormone-releasing factor (GRF) neurons in the hypothalamus of the novel dwarf rat. *J. Endocrinol.* 127:69-75.
42. Sakuma, S., H. Ishikawa, and S. Okuma. 1990. The cell population of somatostatin and growth hormone-releasing factor using quantitative immunohistochemistry in the isolated GH deficient dwarf rat. *Brain Res.* 506:307-310.
43. Frohman, M. A., T. R. Downs, P. Chomczynski, and L. A. Frohman. 1989. Cloning and characterization of mouse growth hormone-releasing hormone (GRH) complementary DNA: increased GRH messenger RNA levels in the growth hormone-deficient lit/lit mouse. *Mol. Endocrinol.* 3:1529-1536.
44. Hurley, L. D., and C. J. Phelps. 1992. Hypothalamic preprosomatostatin messenger ribonucleic acid expression in mice transgenic for excess or deficient endogenous growth hormone. *Endocrinology*. 130:1809-1815.
45. Yamauchi, N., T. Shibasaki, N. Ling, and H. Demura. 1991. In vitro release of growth hormone-releasing factor (GRF) from the hypothalamus: somatostatin inhibits GRF release. *Regul. Pept.* 33:71-78.
46. Magnan, E., V. Guillaume, B. Conte-Devolx, M. Cataldi, F. Thomas, and C. Oliver. 1991. Effets de l'administration aigüe d'un analogue de la somatostatine, le BIM 23014 sur les taux de GH-RH et de la somatostatine dans la circulation porte hypophysaire du mouton. Xième congrès français d'endocrinologie. *Ann. Endocrinol. (Paris)*. 52:167. (Abstr.)
47. Hollingshead, P. G., L. Martin, S. L. Pitts, and T. A. Stewart. 1989. A dominant phenocopy of hypopituitarism in transgenic mouse resulting from central nervous system synthesis of human growth hormone. *Endocrinology*. 125:1556-1564.
48. Burton, K. A., E. B. Kabingtin, D. K. Clifton, and R. A. Steiner. 1991. Distribution of GH receptor mRNA in the adult male rat brain. 73rd Annual Meeting of the Endocrine Society. Washington, D.C. 19-22 June 1991. 419:1555. (Abstr.)
49. Fraser, R. A., K. Siminovski, and S. Harvey. 1991. Growth hormone receptor gene: novel expression in pituitary tissue. *J. Endocrinol.* 128:R9-R11.
50. Berelowitz, M., M. Szabo, L. A. Frohman, S. Firestone, I. Chu, and R. L. Hintz. 1981. Somatomedin-C mediates growth hormone negative feedback by effects on both the hypothalamus and the pituitary. *Science (Wash. DC)*. 212:1279-1281.
51. Bohannon, N. J., E. S. Corp, B. J. Wilcox, D. P. Figlewicz, D. M. Dorsa, and D. G. Baskin. 1988. Localization of binding sites for insulin-like growth factor-I (IGF-I) in the rat brain by quantitative autoradiography. *Brain Res.* 444:205-213.
52. Lanzi, R., and G. S. Tannenbaum. 1992. Time course and mechanism of growth hormone's negative feedback effect on its own spontaneous release. *Endocrinology*. 130:780-788.
53. Sato, M., and L. A. Frohman. 1992. Differential effects of central and peripheral administration of GH and IGF-I on hypothalamic GH-releasing hormone (GRH) and somatostatin (SRIH) gene expression in the GH-deficient dwarf (dw) rat. 74th Annual Meeting of the Endocrine Society. San Antonio, TX, 24-27 June 1992. 446:1580. (Abstr.)