Central Administration of a Growth Hormone (GH) Receptor mRNA Antisense Increases GH Pulsatility and Decreases Hypothalamic Somatostatin Expression in Rats

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To test the hypothesis of the involvement of centrally expressed rat growth hormone receptors (rGH-R) in the ultradian rhythmicity of pituitary GH secretion, adult male rats were submitted to a 60 hr intracerebroventricular infusion of an antisense (AS) oligodeoxynucleotide (ODN) complementary to the sequence of rGH-R mRNA. Eight hour (10 A.M.–6 P.M.) GH secretory profiles, obtained from freely moving male rats infused with 2.0 nmol/hr of rGH-R AS, revealed a marked increase in GH peak amplitude (150 ± 12 vs 101 ± 10 ng/ml), trough levels (16.2 ± 3.0 vs 5.4 ± 1.4 ng/ml), and number of peaks (2.9 ± 0.3 vs 1.8 ± 0.2). No change was observed in rats treated with an ODN complementary to the prolactin receptor mRNA sequence (2.0 nmol/hr). Infusion of increasing ODN concentrations resulted in a dose-dependent stimulation of GH release. In par-

allel, somatogenic binding sites in the choroid plexus were decreased by 40%, and levels of rGH-R mRNA were increased in the periventricular nucleus (PeV) but unchanged in the arcuate nucleus (ARC). Levels of somatostatin mRNA, in the PeV but not in the ARC, were lowered by the treatment. Levels of GH-releasing hormone mRNA in the ARC were not affected. These data suggest that GH negative feedback results from a direct effect on central GH receptors and a subsequent activation of hypophysiotropic somatostatin neurons located in the anterior periventricular hypothalamus.

Key words: antisense oligonucleotides; growth hormone receptor; growth hormone pulsatility; somatostatin; growth hormone releasing hormone; *in situ* hybridization; male rat

In the male rat, growth hormone (GH) release displays a typical ultradian rhythm, with high amplitude GH secretory bursts occurring at 3.00–3.30 hr intervals throughout the nycthemeron (Tannenbaum and Martin, 1976). In addition to GH releasing hormone (GHRH) and somatostatin (SRIH), the two hypothalamic neurohormones controlling GH secretion, several other factors also participate in GH regulation (for review, see Bertherat et al., 1995). In particular, GH itself elicits a “short,” hypothalamic, negative feedback on its own secretion. Intravenous (Willoughby et al., 1980; Clark et al., 1988) and intracerebroventricular (Tannenbaum, 1980; Abe et al., 1983) administrations of human GH strongly decrease GH plasma levels, an effect that has been attributed to inhibition of GHRH synthesis (Chomczynski et al., 1988) and release (Clark et al., 1988), increase in SRIH neuronal activity (Rogers et al., 1988; Lanzi and Tannenbaum, 1992), or both (Miki et al., 1989). Sensitivity of both SRIH and GHRH neurons to GH feedback is also suggested by experiments on hypersomatotropic rats, because subcutaneous grafts of tumoral GH cells result in increased SRIH mRNA in the periventricular nucleus (PeV) and decreased GHRH mRNA in the arcuate nucleus (ARC) (Bertherat et al., 1993).

Mechanisms involved in these GH effects are not completely elucidated as yet. Nevertheless, recent data indicate that the GH receptor gene is expressed in the rat hypothalamus (Hasegawa et al., 1993), and GH receptor mRNA-containing cells have been visualized in the PeV and the ARC, the major respective locations of SRIH and GHRH neurons (Burton et al., 1992; Minami et al., 1993; Burton et al., 1995). A comparable distribution has also been reported on the basis of immunohistochemical studies (Lobie et al., 1993).

These anatomical observations, however, are not sufficient to demonstrate that hypothalamic GH receptors actually mediate the feedback effects of the hormone. The capacity of GH to cross the blood–brain barrier is not well validated, and several actions of the hormone are relayed by other moieties, such as the insulin-like growth factors (IGFs). In the present work, we examined whether centrally located GH receptors are involved in the ultradian rhythmicity of pituitary GH secretion. For that purpose, we attempted to block GH receptor synthesis in the CNS by infusing intracerebroventricularly an antisense (AS) oligodeoxynucleotide (ODN) complementary to a portion of the coding sequence of the rat GH receptor (rGH-R) messenger ODN. GH secretory profiles were monitored in rats treated with the AS and compared with values obtained in control animals or in rats treated with an ODN directed against the prolactin receptor, a related yet distinct receptor species. In parallel, brain somatogenic and lactogenic binding sites were monitored by autoradiography and rGH-R, GHRH, and SRIH mRNA levels measured by semiquantitative *in situ* hybridization.

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MATERIALS AND METHODS

AS ODNs

Eighteen base AS ODNs were synthesized. They correspond to the sequence overlapping the initiation codon of the rGH-R mRNA (rGH-R AS ODN: 5'-CAC-CCG-CCA-AAG-ATC-CAT-3') and of the rat PRL receptor (rPRL-R) mRNA (rPRL-R AS ODN: 5'-AGC-AAG-TGC-AGA-TGG-CAT-3'), respectively (Genosys). AS ODNs were dissolved in saline and infused to the animals as described below.

Animals

Four weeks before the experiments, adult male Wistar rats weighing 100–150 gm (Charles River, St. Albin les Elbeuf, France) were housed individually in a room with controlled temperature (22–24°C) and illumination (12 hr light/dark schedule with lights on at 1 A.M.). They had free access to food and water. Rats were handled regularly to minimize stress effects.

Surgery procedures and blood sampling

Nine days before the experiment, a chronic intracerebroventricular (ICV) cannula (Alzet, Brain Infusion Kit; Alza, Palo Alto, CA) was inserted into the lateral ventricle of the brain under pentobarbital anesthesia (35 mg/kg body weight, i.p.). The ICV cannula was stereotactically positioned in the lateral ventricle according to the atlas of Paxinos and Watson (1986) at the following coordinates: anterior, –0.8 mm to the Bregma; lateral, –1.5 mm to the midline; ventral, –3.8 mm to the Bregma, with the incisor bar set at –3.3 mm below the interaural line. The cannula was secured to the skull of the animal with stainless steel screws and dental cement. The ICV cannula was connected to a miniosmotic pump (Alzet 2002; Alza) by means of a polyethylene tube catheter, which was introduced under the dorsal skin of the animal. The flow moderator of the pump (delivery rate of 0.5 μ l/hr) was connected with the polyethylene catheter containing 85 μ l of saline, which was administered for 7 d. A small air bubble was inserted to separate this saline solution from an additional 30 μ l of saline or ASs at the required concentrations, to be delivered for an additional 60 hr (48 hr before and 12 hr during the entire GH sampling period). A control group, consisting of rats that were anesthetized but not implanted with chronic ICV cannula, was also included in all experiments. After surgery, animals were allowed to recover in individual chambers.

GH sampling experiments were performed on freely moving rats. Two days before the experiments, an indwelling cannula was inserted into the right atrium under ether anesthesia as described previously (Bluet-Pajot et al., 1986). The cannula was filled with a 250 IU/ml heparinized physiological solution, and the animals were returned to their individual cages. On the day of the experiments, 2 hr before the sampling period, the distal extremity of the cannula was connected to a polyethylene catheter filled with 25 IU/ml heparinized saline. Blood samples (0.5 ml) were taken every 20 min, from 10 A.M. to 6 P.M. After each sample, red blood cells were centrifuged, resuspended in saline, and reinjected at the next sampling to attenuate hemodynamic modifications. Plasma was stored at –20°C until GH radioimmunoassay (RIA).

At the end of the experiments, the animals were killed, and brains were removed, frozen in isopentane (–40°C) for 30 sec, and kept at –80°C. For autoradiography and *in situ* hybridization, serial 14 μ m cryostat sections at the level of the PeV from interaural 7.70 to 6.70, according to the atlas of Paxinos and Watson (1986), and the ARC from interaural 6.44 to 5.40, according to the atlas of Paxinos and Watson (1986), were mounted on 2% gelatin-subbed slides and stored at –20°C until use. Microscopic examination of toluidine blue-stained sections allowed for an appropriate anatomical match across animals and groups.

GH receptor autoradiography

Autoradiography was carried out as described previously (Bick et al., 1989). Tissue sections were preincubated in cold 25 mM Tris-HCl buffer, pH 7.4, containing 10 mM MgCl₂ and 1% BSA (w/v) for 30 min and then incubated with the radiolabeled ligand (2 nM [¹²⁵I]hGH; specific activity 78 μ Ci/ μ g; purchased from Dupont NEN, Les Ulis, France) in the above buffer for 18 hr at 4°C. Nonspecific binding and specific binding of somatogenic and lactogenic receptors were assessed, respectively, by coincubation of tissue sections with the same concentration of radiolabeled ligand plus a 500-fold excess of unlabeled hGH, rGH, or oPRL. After incubation, the tissue sections were rinsed twice

with cold buffer and twice with distilled water, dried, and tightly juxtaposed to tritium-sensitive films (Hyperfilm ³H, Amersham, Buckinghamshire, UK) for 72 hr at –20°C; films then were developed in Dektol (Kodak, Marnes la Vallée, France). Binding was quantitated in disintegrations per minute/surface unit by reference to iodinated standards prepared from brain paste with the help of a computer-assisted image analyzer using a video camera and the RAG program (Biocom, Les Ulis, France), which allows for the conversion of optical densities into radioactivity units.

In situ hybridization

SRIH, GHRH. *In situ* hybridization was carried out as described elsewhere (Bertherat et al., 1993). Briefly, a 45-base oligoprobe (corresponding to amino acid 96–111 of the prepro-SRIH cDNA) (Genofit, Geneva, Switzerland) was 3'-labeled with α^{35} S-dATP (Amersham) using terminal deoxynucleotidyl transferase (Boehringer Mannheim, Meylan, France) at a specific activity of 2000 Ci/mM. Sections were fixed for 10 min at room temperature in potassium phosphate buffer containing 4% paraformaldehyde. Then they were prehybridized for 30 min in a solution containing 4 $\times$ SSC and 1 $\times$ Denhardt's solution (Sigma, Saint-Quentin Fallavier, France) and for 10 min in 4 $\times$ SSC containing triethanolamine (1.33%) and anhydrous acetic acid (0.25%), pH 8.0. Hybridization was run for 18 hr at 38°C in the hybridization solution (50% formamide, 4 $\times$ SSC, 1 $\times$ Denhardt's, 1% sarcosyl, 10 mM dithiothreitol, 100 mM potassium phosphate, pH 7.4, and 100 ng of yeast tRNA and 100 ng of herring sperm DNA) containing the labeled oligoprobe (2 nM). Sections were rinsed at 36°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 RPN40 LM1 emulsion (Amersham). Exposure time was 7 and 20 d at 4°C for the detection of SRIH mRNA in the PeV and ARC, respectively. Autoradiograms were developed in Dektol (Kodak), stained with toluidine blue, and coverslipped.

A similar method was used for GHRH mRNA hybridization in the ARC (Bertherat et al., 1993). Briefly, a 45-base oligoprobe (base 31–75 of the rat GHRH cDNA) provided by Genset (Strasbourg, France) was 3'-labeled with α^{35} P-dATP (Amersham). The hybridization was carried out at 36°C, and sections were rinsed at 34°C. Exposure time of the dipped slides was 6–8 weeks.

rGH receptor. A cDNA clone, pGO.9, containing the 900-base pair bg/II fragment of the rat GH receptor cDNA cloned into the *Bam*HI site of the vector pT7T318U, was kindly provided by Professor G. Norstedt (Huddinge, Sweden). The AS cRNA probe and the sense cRNA probe were synthesized *in vitro* with T7 polymerase on a plasmid linearized with *Xba*I and with T3 polymerase on plasmid linearized with *Spe*I, respectively. The radiolabeled cRNA was synthesized *in vitro* with α^{35} S-UTP (Amersham) at a concentration of 5 mM. The template DNA was removed by DNaseI treatment, and the radiolabeled riboprobe was separated from unreacted components by phenol–chloroform–isoamyl alcohol extraction. The riboprobe was then hydrolyzed with sodium hydrogen carbonate (0.4 M NaHCO₃) to obtain fragments of ~200 bases in length.

Sections of the PeV and ARC region were dried for 10 min at room temperature and fixed in 4% paraformaldehyde. Sections were rinsed in PBS and treated in a triethanolamine (1.4%) and acetic anhydride (0.25%) solution. Slides were dehydrated in a series of alcohols, delipidated in chloroform, and dried. The riboprobe was dissolved in hybridization buffer (25 mM Tris, pH 7.4, 1 mM EDTA, 350 mM NaCl, 60% deionized formamide, 12% dextran sulfate, 50 $\times$ Denhardt's, 5 mg of yeast tRNA, 5 mg of single-stranded salmon sperm DNA, and 125 nM dithiothreitol).

The rGH-R cRNA probe in hybridization buffer was positioned on each of the sections, which were glass-covered, sealed with rubber cement, and incubated overnight at 50°C in a humidified chamber. After hybridization, coverslips were lifted off gently in 2 $\times$ SSC at room temperature, and the slides were washed for 30 min in two changes of 2 $\times$ SSC/50% formamide at 50°C. Sections were then rinsed briefly in 2 $\times$ SSC at 37°C and incubated in 2 $\times$ SSC containing 20 μ g/ml RNaseA for 30 min at 37°C. Sections were rinsed again in 2 $\times$ SSC and then washed in 3 $\times$ 15 min changes of 2 $\times$ SSC/50% formamide at 50°C, followed by two room temperature washes in 2 $\times$ SSC for 5 min each. Slides were dipped briefly in water and then air-dried. The dried sections were dipped in RPN 40 LM1 emulsion (Amersham). Exposure time was 3 weeks.

Image analysis and quantification. Sections were visualized at 500 $\times$ magnification (Leitz Orthoplan) under fluorescent epi-illumination. Grain counting was performed with a Biocom 200 image analyzer (Bio-

Figure 1. Representative rGH secretory patterns during an 8 hr sampling period in control and saline-infused rats (*top panels*), and rGH-R AS ODN ($2.0 \text{ nmol} \cdot 0.5 \mu\text{l}^{-1} \cdot \text{hr}^{-1}$)-treated (*middle panels*) and rPRL-R AS ODN ($2.0 \text{ nmol} \cdot 0.5 \mu\text{l}^{-1} \cdot \text{hr}^{-1}$)-treated animals (*bottom panels*).

com, Les Ulis, France) using the computer-based image analysis system (RAG 200), which allows for rapid estimation of grain numbers over neuronal perikarya. An internal calibration curve was recorded for each section and measured the mean quantity of light reflected by a known number of grains according to the procedure described by Bisconte et al. (1968). Labeled neurons were identified by toluidine blue under bright-field illumination, delineated on the screen, and the quantity of light reflected in the area was measured under epi-illumination.

Eight sections per region (PeV, ARC) in each rat were analyzed for SRIH and GHRH mRNA *in situ* hybridization. Four sections corresponding to the level of the PeV and ARC were analyzed in each rat for the rGH-R mRNA *in situ* hybridization experiment.

GH RIA

Plasma GH concentrations were measured by RIA using materials supplied by the National Institute of Diabetes and Digestive and Kidney Diseases, as described previously (Bluet-Pajot et al., 1978). GH values are reported in terms of rGH-RP2. The sensitivity of the RIA is 1 ng/ml. The intra- and interassay coefficients of variation are below 15%.

Statistical analysis

GH pulse analysis was performed using the Cluster program (Veldhuis et al., 1987), with the t value set to 4.1 to maintain false-positive rates under 1%. Cluster size was set to one prepeak and one postpeak nadir value. False-positive error for peak detection was 7%. Area under the curve (AUC) for GH response is calculated by means of trapezoidal analysis. Values are given as mean $\pm$ SEM, and statistical analysis was performed

by ANOVA using the statview 4.02 software (Abacus Concepts, Palo Alto, CA).

RESULTS

Effect of AS infusions on GH secretion

Implantation of a miniosmotic pump did not significantly alter body weight. The surgery caused a small, temporary weight loss, but treated animals had recovered their initial weight at the time of the experiment.

Administration of saline for nine consecutive days into the lateral ventricle did not affect the typical GH secretory pattern of normal rats (Fig. 1, *top panels*). By Cluster analysis, the number, amplitude, and interval between GH peaks, as well as nadir levels, were also the same in saline-infused and control animals (Table 1).

Administration of rGH-R AS ODN ($2.0 \text{ nmol} \cdot 0.5 \mu\text{l}^{-1} \cdot \text{hr}^{-1}$) resulted in an increased overall secretion of GH (Fig. 1, *middle panels*), whereas in rPRL-R AS ODN-treated rats (Fig. 1, *bottom panels*) GH profiles were equivalent to those of control or saline-infused animals. By Cluster analysis (Table 1), both amplitude of GH peaks and nadir values were higher in rGH-R AS ODN-treated rats than in rPRL-R AS ODN-treated animals and saline-

Table 1. Effect of intracerebroventricular infusion of saline, rGH-R antisense ODN, and rPRL-R antisense ODN on the GH pulsatility parameters

	Number of peaks/8 hr	Peak amplitude (ng/ml)	Interval between peaks (min)	Nadir (ng/ml)
Control (12)	2.1 ± 0.2	99 ± 5	170 ± 12	4.2 ± 0.8
Saline (5)	1.8 ± 0.2	101 ± 10	200 ± 11	5.4 ± 1.4
rGH-R antisense ODN (12)	2.9 ± 0.3*	150 ± 12***	118 ± 9***	16.2 ± 3.0***
rPRL-R antisense ODN (4)	2.0 ± 0.4	92 ± 5	163 ± 32	5.0 ± 1.5

Number of animals in each group is indicated in parentheses. Values correspond to mean ± SEM. Asterisks indicate levels of significance with respect to controls (* $p < 0.05$; *** $p < 0.001$).

infused animals. In rGH-R AS ODN-treated animals, peak number was increased, whereas the interval between peaks was decreased as compared with the three other groups.

When expressed as the total AUC (Fig. 2), the effect of rGH-R AS ODN treatment was dose-dependent and reached statistical significance for infusion rates of 1.0 and 2.0 nmol · 0.5 μl^{-1} · hr $^{-1}$. Infusion at the same maximal rate (2.0 nmol · 0.5 μl^{-1} · hr $^{-1}$) of rPRL-R AS ODN did not modify GH secretion.

Effect of rGH-R AS ODN infusion on somatogenic and lactogenic binding sites

Specific [^{125}I]hGH binding sites were visualized in the choroid plexus at the level of the dorsal part of the third ventricle and the lateral ventricle (Fig. 3). Somatogenic (i.e., rGH displaceable) binding sites accounted for 43% of hGH binding, and lactogenic (i.e., oPRL displaceable) binding sites accounted for 57% of total specific binding. After rGH-R AS ODN treatment, the density of the somatogenic binding sites was significantly decreased in the dorsal part of the third ventricle and the lateral ventricle as well. In contrast, the levels of the lactogenic binding sites remained unchanged. Measurable binding sites could not be quantified in the hypothalamus.

Effect of rGH-R AS ODN infusion on rGH-R, SRIH, and GHRH mRNA levels in the hypothalamus

In the hypothalamus, the distribution of rGH-R mRNA-containing cells is illustrated in Figure 4 and compared with

those of SRIH mRNA- and GHRH mRNA-containing cells. rGH-R mRNA-containing cells essentially were restricted to the PeV and the ARC. In the PeV, the distribution of rGH-R mRNA-containing cells was closely similar to that of SRIH mRNA-containing cells. In the ARC, rGH-R mRNA-labeled cells were distributed over the entire ventral portion of the nucleus, whereas GHRH mRNA cells were restricted to its ventrolateral part.

Infusion of rGH-R AS ODN did not modify the number of cells expressing rGH-R, SRIH, and GHRH mRNA (data not shown).

As visualized in Figure 5 (*top panel*) and quantified in Figure 6, rGH-R expression increased significantly in the PeV after infusion of rGH-R AS ODN. A weaker but not significant effect was also observed in the ARC. The increase in rGH-R mRNA was dependent on the concentration of the oligonucleotide (data not shown).

Infusion of rGH-R AS ODN reduced SRIH mRNA levels in the PeV (Figs. 5, *bottom panel*, and 7). In the ARC, SRIH expression was not affected significantly.

GHRH mRNA levels were not affected by AS infusion either in the ARC or around the ventromedial nuclei (Fig. 7, *VMN*).

DISCUSSION

Sixty-hour intracerebroventricular infusion of an AS ODN to the mRNA for rGH-R decreased somatogenic binding sites in the choroid plexus at the level of the dorsal part of the third ventricle and the lateral ventricle. In parallel, rGH-R mRNA levels were increased in the PeV but not in the ARC, suggesting some regional selectivity in translational arrest and ongoing transcriptional activity. In the same animals, AS treatment significantly increased pulsatile GH secretion and decreased SRIH mRNA levels in the PeV, without affecting GHRH mRNA levels in the ARC. These effects seemed specific and not caused by neuronal toxicity or general protein synthesis blockade, because infusion of an AS ODN to the mRNA for rPRL receptor, a protein closely related to the rGH-R, was without effect.

It was shown previously that *in vivo* application of AS ODN to neuropeptide receptor mRNAs such as neuropeptide Y (NPY)-Y1 (Wahlestedt et al., 1993b) and angiotensin AT1 receptors (Sakai et al., 1994) or neurotransmitter receptors such as the NMDA-R1 (Wahlestedt et al., 1993a) or the dopaminergic D2 receptor (Zhou et al., 1994) can diminish respective binding levels by 10–70%. In the case of the rGH-R, it resulted in a 40% decrease in the somatogenic binding sites located in the choroid plexus visualized in the dorsal part of the third ventricle and the lateral ventricle, thus indicating that AS ODN treatment was effective. We were not able to detect reproducible GH binding in the hypothalamus of adult male rats by autoradiography, and others were also unsuccessful in adult female rats (Crumeyle-

Figure 2. Effects of rGH-R AS ODN (0.4, 1.0, and 2.0 nmol · 0.5 μl^{-1} · hr $^{-1}$) and rPRL-R AS ODN (2.0 nmol · 0.5 μl^{-1} · hr $^{-1}$) infusion on plasma GH levels. The number of animals for each experimental group is shown in parentheses over each bar. The AUC monitored throughout the recording period (10 A.M.–6 P.M.) is expressed as arbitrary units. Vertical lines represent SEM. Asterisks indicate the level of significance with respect to controls (* $p < 0.05$; ** $p < 0.01$).

Figure 3. Effects of rGH-R AS ODN ($2.0 \text{ nmol} \cdot 0.5 \mu\text{l}^{-1} \cdot \text{hr}^{-1}$) treatment on brain somatogenic (rGH-R) and lactogenic (oPRL-R) binding sites. *a*, *Top left*, Visualization of [^{125}I]hGH binding sites in the choroid plexus in the dorsal part of the third ventricle (D3V) and the lateral ventricle (LV) at the level of the periventricular hypothalamic nucleus (PeV). *Top right*, Nonspecific binding in the presence of hGH ($1 \mu\text{M}$). *b*, *Middle left*, Visualization of [^{125}I]hGH binding sites in the choroid plexus in the D3V and the LV at the level of the arcuate nucleus (ARC). *Middle right*, Nonspecific binding in the presence of hGH ($1 \mu\text{M}$). *c*, *Bottom*, Quantification of rGH-R and oPRL-R binding sites in control and rGH-R AS ODN-treated animals. Ten sections per region were analyzed in each rat; $n = 6$ animals in each group. Data are expressed as mean $\pm$ SEM, and asterisks indicate the level of significance as compared with controls (* $p < 0.05$; ** $p < 0.01$).

Arias et al., 1993). Indeed, the levels of GH-R immunoreactivity in the hypothalamus decrease considerably from the postnatal period to the adult stage (Lobie et al., 1993), whereas the expression of rGH-R mRNA remains measurable by Northern analysis (Hasegawa et al., 1993) and *in situ* hybridization (Burton et al., 1992; Minami et al., 1993). Thus, because of the very low levels of somatogenic binding sites in that region, we could not demonstrate directly that the AS treatment resulted in a decrease in GH binding levels in the hypothalamus. The increased concentrations of rGH receptor mRNA in the hypothalamic PeV after AS infusion, however, suggest that the ODN was effective in inhibiting translation, resulting in a compensatory increase in nontranslated mRNA. In a similar protocol, V1 receptor mRNA levels were also markedly increased locally after intraseptal infusion of AS oligonucleotides when compared with those of vehicle- or scrambled sequence-infused animals, and this resulted in a 60% decrease in V1 receptor binding (Landgraf et al., 1995). The effect of intracerebroventricular AS infusion on GH receptor mRNA levels was significantly more marked in the PeV than in the ARC, two major sites of rGH-R expression in the hypothalamus (Burton et al., 1992; Minami et al., 1993; Burton et al., 1995). We cannot exclude the possibility that diffusion of the ODN from the ventricle to the ARC is different than to the PeV, because of the presence of specialized ependymocytes such as the tanycytes.

ICV administration of rGH-R but not rPRL-R AS ODN induced important modifications in spontaneous GH secretion of freely moving rats. All parameters of endogenous GH pulsatility were either increased (i.e., nadir, amplitude, and number of peaks) or decreased (interval between peaks) by treatment with rGH-R AS ODN. This observation confirms the existence of a short negative feedback of GH on its own secretion. In addition, it indicates that the hormone itself, not a distinct GH-induced factor such as IGF, is responsible for the effect by acting on central GH receptors. It is noteworthy that this negative feedback affects not only peak amplitudes but also pulse frequency and nadir values.

In parallel, the AS ODN treatment resulted in decreased SRIH mRNA levels in the PeV and to a lesser extent in the ARC. It might be postulated simply that PeV but not ARC SRIH neurons are endowed with rGH receptors. Alternatively, this regional difference might be accounted for by less efficient diffusion in the ARC as argued above, and also by the fact that SRIH gene expression is much higher in the PeV, the major hypothalamic source of SRIH, than in the ARC. The fact that SRIH mRNA levels in the PeV are decreased in rGH-R AS ODN-treated animals, in which high GH levels are recorded, is also a good index of the blockade of the GH feedback induced by the AS treatment, because experimental hypersomatotropinemia usually

Figure 4. Distribution of (left panels) rGH-R mRNA-, (top right panel) SRIH mRNA-, and (bottom right panel) GHRH mRNA-containing cells in the hypothalamic PeV (top panels) and ARC (bottom panels) nuclei of a control rat (100 $\times$ magnification).

increases SRIH mRNA concentrations in that nucleus (Bertherat et al., 1993). Taken altogether, these data, as well as parallel effects of the AS treatment on rGH-R mRNA and on SRIH expression, are strongly suggestive that GH receptors are directly involved in regulating the activity of PeV SRIH neurons. This hypothesis is substantiated further by observations of Burton et al. (1992) showing that GH receptor mRNA colocalizes in 69% of SRIH mRNA-containing cells within the PeV. Moreover, systemic administration of GH to hypophysectomized rats induces *c-fos* gene expression in the PeV, and 60% of these *c-fos*-expressing cells coexpress SRIH (Kamegai et al., 1994). Increased GH secretion induced by the rGH-R AS ODN treatment thus is likely to reflect a lower SRIH inhibitory tone on GH release from the pituitary. Such a hypothesis is compatible with the fact that nadir GH values are increased in AS-treated animals in accordance with the model of Tannenbaum and Ling (1984) in which nadir values are controlled essentially by high SRIH plasma levels.

A direct effect on GHRH gene expression could not be documented under our experimental conditions. GHRH neurons in the ARC have not yet been convincingly reported as expressing GH receptor gene transcripts, in contrast to the majority of

arcuate NPY-containing neurons (Chan et al., 1995; Burton et al., 1995). Moreover, in hypophysectomized rats, 60 min after an intravenous injection of GH, expression of the *c-fos* gene is increased in arcuate NPY but not in GHRH neurons (Kamegai et al., 1994). Thus, alteration of hypothalamic GH receptors may not directly affect GHRH cells.

A puzzling question raised by these results is that of GH access to hypothalamic neurons. Although the blood–brain barrier is usually considered impermeable to GH, high levels of plasma GH found in acromegalic patients are matched by abnormally high GH concentrations in the cerebroventricular fluid (Linfoot et al., 1970). Systemic treatment of GH-deficient patients with recombinant hGH also results in high GH CSF levels (Johansson et al., 1995). The median eminence is known to lie outside the blood–brain barrier as choroid plexus, on which we and others (Lai et al., 1991) have visualized [125 I]hGH binding sites. The relationship between the somatogenic binding sites in the choroid plexus and the hypothalamic sites of rGH-R synthesis remains to be established. At any rate, the choroid plexus binding sites might act as selective transporters from the peripheral circulation to the CSF. Oliver et al. (1977) suggested in an earlier study that high plasma concentrations of pituitary hormone could reach the hypothalamus by retrograde transport from the

Figure 5. Visualization of the effects of rGH-R AS ODN treatment in the PeV. Autoradiograms are representative of *in situ* rGH-R (*top*) and SRIH (*bottom*) mRNA hybridization signals in the hypothalamic PeV in a control (*left*) and a rGH-R AS ODN ($2.0 \text{ nmol} \cdot 0.5 \mu\text{l}^{-1} \cdot \text{hr}^{-1}$)-treated (*right*) rat ($300\times$ magnification).

Figure 6. Quantification of the effects of intracerebroventricular infusion of rGH-R AS ODN ($2.0 \text{ nmol} \cdot 0.5 \mu\text{l}^{-1} \cdot \text{hr}^{-1}$) on rGH-R mRNA levels in the periventricular (PEV) and arcuate (ARC) hypothalamic nuclei. Four sections per region were analyzed in each rat; $n = 6$ animals in each group. Data are expressed as mean $\pm$ SEM, and asterisk indicates the level of significance as compared with controls ($*p < 0.05$).

median eminence across stalk portal vessels, but we could not visualize somatogenic binding sites in this neurohemal organ. Alternatively, a central origin of GH acting on the hypothalamus cannot be excluded, because GH-like immunoreactivity is found in various brain structures such as the hypothalamus or the amygdala (Harvey

et al., 1993). This GH material is likely to be produced locally, because it is not affected by hypophysectomy (Hojvat et al., 1982).

At any rate, whatever its pituitary or central origin, our experiments are compatible with a working model in which short negative feedback of GH on its pulsatile secretory pattern

Figure 7. Quantification of the effects of intracerebroventricular infusion of rGH-R AS ODN ($2.0 \text{ nmol} \cdot 0.5 \mu\text{l}^{-1} \cdot \text{hr}^{-1}$) on SRIH and GHRH mRNA levels. *Top panels*, SRIH mRNA levels in the periventricular (PEV) and arcuate (ARC) nuclei. *Bottom panels*, GHRH mRNA levels in the ARC and periventricular nucleus (VMN) region. Eight sections per region were analyzed in each rat; $n = 6$ animals in each group. Each column represents the mean, and the vertical bar represents SEM. Asterisks indicate levels of significance with respect to controls ($***p < 0.001$).

acts directly on GH receptors located in the periventricular hypothalamus, thereby triggering the activity of hypophysiotropic somatostatin neurons located in that structure. A direct involvement of GHRH neurons remains to be established.

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