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Impaired Growth Hormone Secretion in Neonatal Hypothyroid Rats: Hypothalamic Versus Pituitary Component* (42643)

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Abstract. In 10-day-old rats made hypothyroid by giving dams propylthiouracil (PTU) in the drinking water since the day of parturition, simultaneous radioimmunoassay (RIA) determinations of basal and stimulated growth hormone (GH) secretion, hypothalamic GH-releasing hormone (GHRH)-like immunoreactivity (LI) content, immunocytochemical localization of somatotrophs, and hypothalamic GHRH-LI-positive structures were performed. The frequency of somatotrophs was also determined. One-day-old hypothyroid rats, whose mothers had been given PTU since the 14th day of pregnancy, were also used for comparison. In 10-day-old hypothyroid rats, pituitary and plasma GH levels and the number of somatotrophs were considerably lower and plasma TSH levels were significantly higher than those in age-matched control rats; however, GHRH-LI titers in the mediobasal hypothalamus and the morphology of GHRH-LI-positive structures were unaltered. In 1-day-old rats the only alteration present, in addition to elevated plasma TSH levels, was a clear-cut decrease in plasma GH levels. An acute challenge with GHRH (20 ng/100 g body wt, sc) or clonidine (15 μg/100 g body wt, sc) induced a clear-cut rise in plasma GH levels 15 min postinjection in 10-day-old control rats but failed to do so in age-matched hypothyroid rats. Both compounds failed to rise plasma GH in both hypothyroid and control 1-day-old rats. Taken together these data indicate that in neonatal and infant rats deprivation of thyroid hormones acts primarily to depress pituitary somatotroph function and that possible changes in GHRH-secreting structures represent a later postnatal event.

The association of hypothyroidism and growth hormone (GH) deficiency is well recognized (1–6). In rodents and humans hypothyroidism is accompanied by diminished basal and stimulated GH secretion (1, 3–6), and in the rat a striking reduction of pituitary GH content has been documented (1–6). Administration of thyroid hormones, conversely, increases plasma GH levels, replenishes pituitary GH stores, and allows the effect of GH secretagogues to be evidenced (1–6). In this connection, it has been shown that hypothyroidism induces in the rat a marked decrease in GH mRNA, an effect completely reversed by thyroid hormone treatment (7).

Studies aimed at investigating the possible effects of perturbations of the pituitary–thyroid axis on central nervous system (CNS) GH regulatory mechanisms have initially shown that in hypothyroid rats hypothalamic somatostatin (SS) content and release are diminished and that treatment with triiodothyronine restores these paradigms to euthyroid levels (8). In subsequent studies, however, hypothalamic SS content and release were found to be unaltered in rats 10 days–5 weeks after thyroidectomy (9, 5). Concerning growth hormone-releasing hormone (GHRH), long-term induction of hypothyroidism in the rat induced a marked decrease in its hypothalamic levels, an effect restored to normal by thyroxine replacement therapy (10). All these findings refer to studies in mature rats, whereas the effects of perturbations in the pituitary–thyroid axis on GH secretion and its neural control in developing rats are relatively scanty. In 14-day-old rats whose mothers had received propylthiouracil (PTU) since 10 days before parturition and were maintained on this regimen throughout the nursing period, plasma GH levels were modestly decreased (11). In 2-day-old rats whose mothers had been similarly treated, a decreased pituitary GH content was reported (12), though in another study this was not evident, and a decrease in pituitary GH content was observed only at

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later postnatal intervals (13). Propylthiouracil-induced hypothyroidism was associated in neonatal rats with reduced hypothalamic SS concentrations (13).

To the best of our knowledge, aspects of the neural stimulatory control of GH secretion have not so far been investigated in neonatal rats. In the present study, we have examined in detail in 10-day-old rats the influence of hypothyroidism on basal and stimulated GH secretion and hypothalamic GHRH-like immunoreactivity (LI) content. Biochemical results were strengthened by immunocytochemical localization of somatotroph cells and hypothalamic GHRH-LI-positive structures. The frequency of somatotrophs was also determined. One-day-old hypothyroid rats were evaluated for comparison.

Materials and Methods. *Animals.* Pregnant Sprague–Dawley rats were obtained from a commercial supplier (Nossan, Corezzana, Italy) on the 13th day of gestation. They were housed in individual cages under controlled conditions (22 ± 2°C, 65% humidity and artificial light from 0600 to 2000 hr) and assigned to either the treatment or the control group. The dams of the treatment group received 0.05% propylthiouracil (PTU) in the drinking water beginning on the 14th day of gestation or the day of parturition (Day 0). Their pups were sacrificed respectively on Days 1 and 10 of age. In all, 63 1-day-old rats (33 from the PTU and 30 from the control group) and 97 10-day-old rats (48 from the PTU and 49 from the control group) were examined. Reportedly, sexual differences in pituitary GH content arise at puberty (14); thus, data reported refer to pooled samples obtained from rats of either sex.

Experimental Procedure. On the day of the experiment, neonatal and infant rats, which had been isolated in a sound-proof room 1 hr before the experiment, were challenged with GHRH (synthetic hpGHRH-40, Bachem, Bubendorf, Switzerland, 20 ng/250 μl/100 g body wt, sc), clonidine (CLO, Catapresan, Boehringer Ingelheim, Florence, Italy, 15 μg/250 μl/100 g body wt, sc), or vehicle (0.9% acidified saline, 250 μl/100 g body wt, sc). Rats were killed by decapitation 15 min postinjection; blood was collected and pituitaries and brains were rapidly removed. Blood was collected into EDTA-containing tubes and plasma was separated and stored at −20°C until RIA determination of GH and TSH. The anterior pituitaries were carefully dissected and were used for either RIA determination of GH or embedding in plastic blocks for immunohistochemical localization of somatotrophs and analysis of their frequency.

For RIA determination, the anterior pituitaries were homogenized in 0.5 ml 0.01 *M* $NaHCO_3$, the pellets were separated by centrifugation (2500*g* for 30 min), and the supernatant was diluted 1:100 with 0.01 *M* PBS containing 0.05 *M* EDTA and 1% BSA (pH 7.6) and kept frozen until RIA.

Immediately after removal, the glands were assigned to the morphological study and were cut in half in the central region according to the sagittal plane and fixed for 2 hr in 2.5% glutaraldehyde in 0.1 *M* phosphate buffer, pH 7.4. After rinsing in the same buffer, specimens were postfixed for 1 hr in 1% osmium tetroxide. The tissues were then rinsed in 0.1 *M* phosphate buffer, dehydrated in ethanol, and embedded in EPON 812. Specimens were oriented so that each block could be sectioned according to the sagittal plane.

As for pituitaries, the brains dissected were used for either RIA determination of GHRH or immunocytochemical staining of hypothalamic GHRH-LI immunoreactive structures.

For RIA determination of GHRH, the mediobasal hypothalamus (MBH) was carefully dissected under a stereomicroscope and removed, as previously described (15). The tissue was put in 0.1 *M* HCl and boiled for 10 min; after boiling it was homogenized and the supernatant was kept frozen at −70°C until RIA was performed.

For immunocytochemical localization of GHRH-LI-positive-structures, brains ($n = 5$, for each experimental and control group), immediately after dissection, were fixed by immersion (3 hr) in cold 0.12 *M* phosphate buffer (pH 7.4) containing 4% paraformaldehyde. Following fixation, the specimens were rinsed in 0.12 *M* phosphate buffer (pH 7.4) containing 18% sucrose at 4°C for 48 hr. The hypothalami were then removed from the

brains according to the landmarks previously described (16), frozen over liquid nitrogen, and serially sectioned (15 μm) in a frontal plane on a cryostat. Sections were mounted on gelatinized glass slides and stored at −20°C until used.

Radioimmunoassays: GH and TSH. Plasma and pituitary GH content and plasma TSH levels were determined by double-antibody RIAs using reagents provided by NIADDK. For GH, results were expressed in nanograms per milliliter (plasma) or micrograms per pituitary in terms of the NIADDK standard rats GH-RP-1, the potency of which is 0.6 IU/mg. The sensitivity of the assay was 0.5 ng/ml; intraassay variability was 6%. For TSH, results were expressed in nanograms per milliliter (plasma) in terms of the NIADDK standard rat TSH-RP-2 (AFP-5135 B), the potency of which is 35 IU/mg. The sensitivity of the assay was 0.1 ng/ml; intraassay variability was less than 5%. In order to avoid interassay variability all the samples were determined in the same RIA.

GHRH-LI. Rat GHRH-LI concentrations in the MBH were evaluated by an homologous RIA, previously described and validated (17). Results were expressed in nanograms per MBH. The sensitivity of the assay was 18 pg/tube. All samples were assayed in the same RIA, to avoid inter-assay variability. Intraassay variability was 4.5%.

Statistical analysis. Hormonal concentrations in the different experimental groups were compared by Dunnett's t test preceded by analysis of variance or by Student's t test where applicable. A probability of $P < 0.05$ was taken to be statistically significant.

Immunohistochemistry: Somatotrophs. The anti-GH serum used for the immunohistochemical localization of somatotrophs was prepared by immunizing rabbits with rGH supplied by the Rat Pituitary Hormone Program of the NIADDK. The antiserum was used at working dilutions of 1:500–1:800. Immunostaining of rGH was performed on semithin plastic sections (1 μm), cut on a Porter Blum MT-2, ultramicrotome and mounted on gelatinized glass slides. Prior to the immunostaining, the tissue sections were treated for 15 min with a saturated solution of KOH in absolute ethanol in order to dissolve the resin and then washed in three changes of absolute ethanol for 10 min. Finally, after a washing in PBS for 10 min, the tissue sections were incubated with the anti-rGH serum diluted in PBS containing 0.3% Triton X-100.

The specificity of the anti-rGH serum was tested as follows: incubation of adjacent pituitary tissue sections with the anti-rGH serum preabsorbed with rGH (at a concentration of 1–10 μg/ml of the undiluted primary antiserum) or with normal rabbit serum instead of the primary antiserum no longer revealed immunostained cells. In contrast, there was no modification of the immunostaining when tissue sections were incubated with the anti-rGH serum preabsorbed with rPRL and rTSH (at a concentration of 10 μg/ml of the undiluted anti-rGH serum).

GHRH-LI. The antiserum (4399_{12}) against rGHRH, kindly supplied by Dr. W. B. Wehrenberg, was directed against the C-terminal sequence of the peptide. Specificity control tests have been reported previously (16). The anti-rGHRH serum was used at working dilutions of 1:100–1:700.

Immunostaining for both rGH and rGHRH was carried out using the avidin–biotin complex (ABC) method (18). The incubations with the primary antisera were carried out in a moisture chamber at 4°C for 24 hr (rGH) and 48 hr (rGHRH). Details of the immunohistochemical techniques have been reported in a previous paper (16).

Analysis of somatotroph frequency. Semithin sections cut from each block of pituitary tissue and immunostained for rGH were used to determine the frequency of somatotrophs in the anterior pituitaries from hypothyroid and control rats. Six slides were collected from each block of pituitary tissue. Each slide contained four 1-μm serial plastic sections. The sequences of serial sections collected on each slide were 35 μm apart to avoid duplicate count of the same cells. Counts of GH immunoreactive cells were performed on four fields per section randomly taken (only one section per slide was examined for cell counts). The field to be analyzed was defined by a frame covering an area of 10,000 μm provided by a IBAS II-Zeiss Kontron image analyzer, under a ×80 objective lens. Only cells lying entirely into

the frame were counted. Stained and unstained cells were tabulated and the percentage of stained GH cells in the pituitary cell population sampled was calculated for each rat. Values for individual rats were averaged together under the appropriate age and experimental group and the SEM was calculated. Values in hypothyroid rats (1 and 10 days-old) were compared with those of the corresponding controls by Student's t test, and a probability of $P < 0.05$ was taken as statistically significant.

Results. *Basal plasma TSH levels.* Basal plasma TSH levels were significantly higher in hypothyroid than in control rats (1 day, 4.49 ± 0.32 vs 0.66 ± 0.11 ng/ml, $P < 0.01$; 10 days, 12.9 ± 1.9 vs 0.81 ± 0.05 ng/ml, $P < 0.01$) (data not shown).

Basal and stimulated plasma GH levels. Baseline GH levels were significantly lower in 1-day-old hypothyroid than those in control rats. An acute challenge with GHRH or CLO did not elicit any rise in plasma GH 15 min post-injection in both experimental and control rats (Table I).

Baseline GH levels were significantly lower in 10-day-old than in 1-day-old control rats. In 10-day-old hypothyroid rats there was a striking fall in baseline GH levels. Administration of GHRH or CLO induced a clear-cut rise in plasma GH levels in control rats but failed to do so in hypothyroid rats (Table I).

TABLE I. PLASMA GH LEVELS UNDER BASAL CONDITIONS OR UNDER AN ACUTE CHALLENGE WITH GHRH (20 ng/100 g body wt, sc) OR CLONIDINE (150 μg/kg body wt, sc) IN 1- AND 10-DAY-OLD CONTROL AND HYPOTHYROID RATS

Group, age (days)	No. of rats	Treatment	Plasma GH (ng/ml)
Control rats 1 day	15	Saline	95.5 ± 9.2
	8	GHRH	122.5 ± 34.3
	8	CLO	131.5 ± 20.1
Hypothyroid rats 1 day	17	Saline	49.5 ± 7.6[a]
	8	GHRH	56.0 ± 12.5
	8	CLO	58.5 ± 14.7
Control rats 10 days	17	Saline	47.2 ± 6.8
	16	GHRH	115.6 ± 13.3[b]
	16	CLO	120.0 ± 13.7[b]
Hypothyroid rats 10 days	16	Saline	14.3 ± 3.4[b]
	16	GHRH	13.6 ± 1.9
	16	CLO	15.5 ± 2.4

Note. Values (ng/ml) are means ± SEM.
[a] $P < 0.05$ vs controls (1 day).
[b] $P < 0.01$ vs controls (10 days).

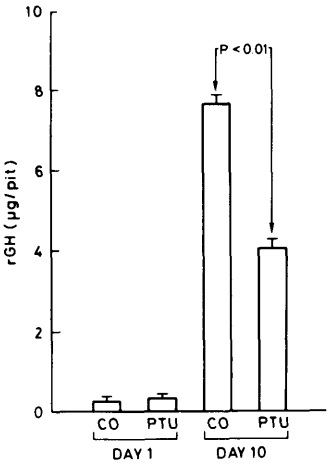

FIG. 1. Pituitary GH concentrations (μg/pit) in 1- and 10-day-old control and hypothyroid rats. Each point is the mean and SEM of nine determinations made in duplicate.

Pituitary GH concentrations. No significant difference in pituitary GH concentrations was present between 1-day-old hypothyroid and control rats (0.28 ± 0.1 vs 0.26 ± 0.1 μg/gland, respectively, P = NS); in contrast, in 10-day-old hypothyroid rats, pituitary GH concentrations were significantly lower than those in controls (4.04 ± 0.28 vs 7.66 ± 0.22 μg/gland, respectively, $P < 0.01$) (Fig. 1).

MBH GHRH-LI concentrations. No significant differences in GHRH-LI concentrations were found between experimental and

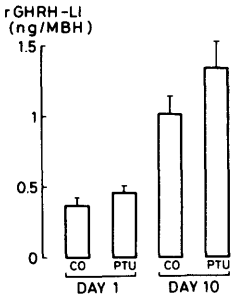

FIG. 2. MBH-GHRH-LI concentrations (ng/MBH) in 1- and 10-day-old control and hypothyroid rats. Each point is the mean and SEM of 6–10 determinations.

TABLE II. PERCENTAGE OF SOMATOTROPHS IN THE ANTERIOR PITUITARY OF 1- AND 10-DAY-OLD CONTROL AND HYPOTHYROID RATS

Age (days)	Somatotrophs (mean ± SEM)	
	Control rats	Hypothyroid rats
1	22.07 ± 1.4	21.52 ± 1.5
10	24.06 ± 1.7	9.34 ± 0.6[a]

[a] $P < 0.01$ vs respective control.

control rats either at 1 or 10 days of age (1 day, 0.46 ± 0.06 vs 0.37 ± 0.06 ng/MBH; 10 days, 1.34 ± 0.19 vs 1.02 ± 0.13 ng/MBH, respectively, P = NS) (Fig. 2).

Analysis of somatotroph frequency. Table II shows the percentages of somatotrophs in the anterior pituitaries from 1 and 10-day-old hypothyroid and control rats. In 1-day-old rats of both groups the percentages of stained GH cells were similar, whereas at 10 days of age GH immunoreactive cells were significantly less in hypothyroid than in control rats.

Hypothalamic GHRH-LI-positive structures in the MBH of hypothyroid and control rats. No evident differences were present in the appearance of hypothalamic GHRH-LI-secreting structures of hypothyroid rats when compared to those of respective age-matched controls (Fig. 3). Briefly, in 1-day-old rats GHRH-LI-immunostained nerve terminals and cell bodies were found respectively in the

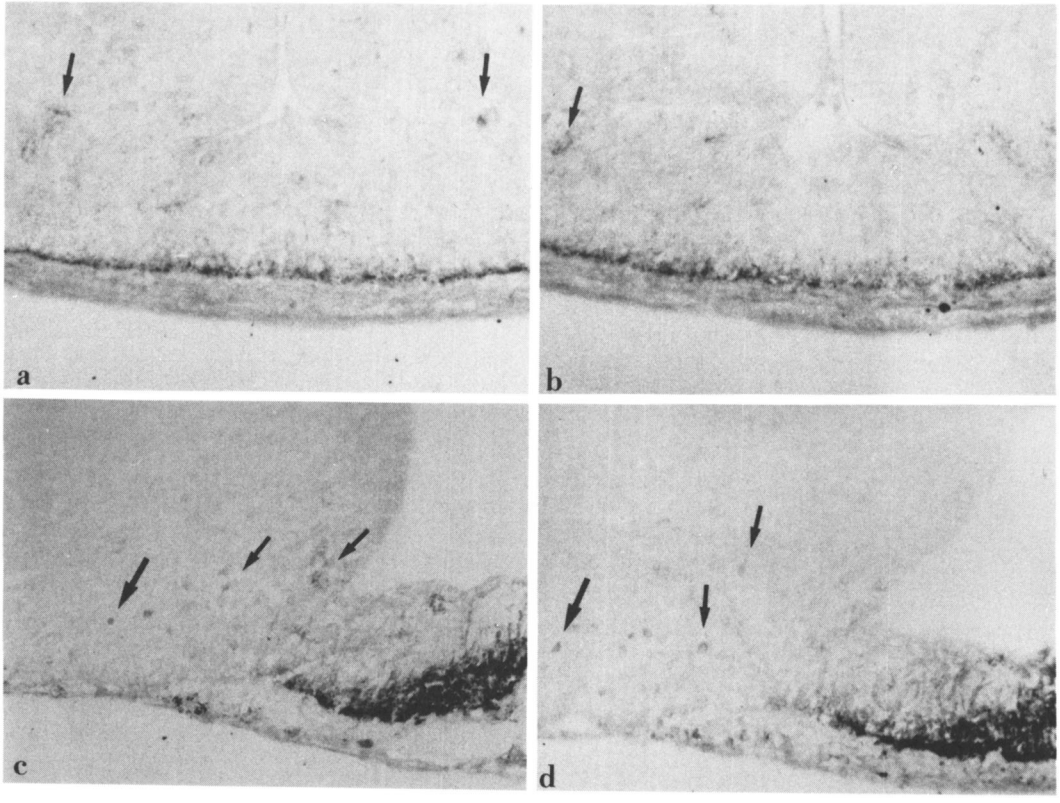

FIG. 3. GHRH-LI-immunostained structures (avidin–biotin complex immunoperoxidase method) in the basal hypothalamus of neonatal and infant hypothyroid and control rats. (a, b) Control (1 day old) (a) and hypothyroid (b) rats. GHRH-LI-positive nerve terminals are seen only in the external layer of the ME. Arrows point to GHRH-LI immunoreactive perikarya in the ARC. ×65. (c, d) Control (10 days old) (c) and hypothyroid (d) rats. The right half of the basal hypothalamus is shown. Dense GHRH-LI immunoreactive terminals are present in the ME, mostly in the external layer. Arrows point to GHRH-LI-positive perikarya in the ARC (thin arrows) and in the lateral basal hypothalamus (thick arrows). ×56.

external layer of the median eminence (ME) and in the arcuate nucleus (ARC). In 10-day-old rats GHRH-LI-positive nerve terminals and perikarya were both increased in number. Moreover, nerve terminals and perikarya were also detected respectively in the internal layer of the ME and in the lateral basal hypothalamus.

Discussion. Recent studies in adult rats have shown that the effects of thyroid hormone deficiency are complex and include actions at multiple sites involved in the regulation of GH secretion. In a recent paper on adult rats it was shown that hypothyroidism not only reduced pituitary GH stores and responsiveness to GHRH but also caused reduction in hypothalamic GHRH content. Depletion of hypothalamic GHRH was related to complete loss of pulsatile GH secretion. Replacement with thyroxine completely restored hypothalamic GHRH content and spontaneous GH secretion, despite only partial recovery of pituitary GH content (10). Since thyroid hormones play a primary role in the regulation of GH secretion throughout all life and their deficiency induces marked alterations since the first postnatal days (see introduction), it seemed worthwhile to study neonatal rats for the functional relatedness between GH alterations occurring at the pituitary level and those possibly present in the neural structures responsible for GH stimulatory control. In our study, simultaneous determinations of hypothalamic and pituitary paradigms of GH function in 10-day-old hypothyroid rats showed the existence of reduced pituitary GH concentration and number of somatotrophs, lowered plasma GH levels, but unaltered MBH-GHRH titers and morphology of GHRH-LI-secreting structures.

Taken together these data would indicate that deprivation of thyroid hormones acts primarily to depress the function of somatotrophs and that possible changes in GHRH/SS secretion occur only at later time intervals. Consistent with this view, it has been recently shown that in adult thyroidectomized rats GH content and pituitary GH response to GHRH decreased significantly starting at 1 week postsurgery, while the hypothalamic contents of GHRH and SS began to decline only at 4 or 6 weeks postsurgery, respectively (6).

Thus, the time pattern of the alterations in hypothalamic stores of GH neuroregulatory peptides after institution of hypothyroidism (6) may indicate their dependency from changes occurring in pituitary and circulating GH.

Recently we showed that in hypophysectomized rats there was a striking decrease in GHRH-LI in the median eminence (17), a likely reflection of an excessive release of GH from unrestrained GHRH-producing neurons (19). Similarly, it has been shown that in the hypothalami of hypothyroid rats K^+-evoked GHRH release was greater than in control rats 2 weeks postsurgery, i.e., prior to changes in hypothalamic GHRH content (6). Therefore, one must be cautious in interpreting the significance of the "unaltered" hypothalamic concentrations of GHRH and immunocytochemical picture detected in our study. It cannot be excluded that an enhanced synthesis of GHRH accompanied by an increased rate of release was operative, due to partial suppression of the inhibitory feedback of GH on the CNS.

The observation that both pituitary and plasma GH levels were considerably reduced in the hypothyroid rats does not necessarily argue against this possibility. Since thyroid hormones exert such a major influence on GH synthesis (2–4, 7), the releasable pool of GH could be decreased in hypothyroid rats. In addition, a decrease in the number of GHRH receptors and/or a postreceptor defect are among other possibilities which could account for decreased plasma and pituitary GH levels in spite of increased GHRH release from the hypothalamus. Administration of GH secretagogues did not prove to be of further help to dissect out the respective CNS and pituitary participation in the GH secretory defect of hypothyroidism. We have recently shown that pituitaries from 1-day-old rats fail to respond to challenge doses of GHRH and CLO (20), a finding confirmed in this study (Table I).

Failure of GHRH to elicit a rise in plasma GH in 10-day-old hypothyroid rats emphasizes the marked alterations induced by treatment at the pituitary level, as also evidenced by the reduced number of somato-

trophs. Obviously, this overshadows any possible stimulatory effect CLO might have over endogenous GHRH release (21), being the target pituitary gland refractory to stimulation. Failure of CLO to elicit GH release in adult male rats 5 weeks after thyroidectomy has also been reported (5).

In rats, hypothyroidism at birth reportedly interferes with the developmental increase of brain catecholamine (CA) levels, whereas treatment with triiodothyronine restores to normal the observed neurochemical changes in CA metabolism (22). Thus, independent of the (primary?) GH alteration present at the pituitary level, an alteration of the GH-releasing effect of CLO, whose action in the rat rests on the activation of α_2-adrenoceptors (23, 24), may have been anticipated. One-day-old rats presented with less marked changes of GH secretion than 10-day-old rats, for the only alteration exhibited was a clear-cut decrease in plasma GH levels. In our study, we were unable to document lowered pituitary GH content and percentage of somatotrophs at this age, a finding in agreement with the results of Walker and Dassault (13). In contrast, Coulombe *et al.* (12), who found in hypothyroid rats pituitary GH concentrations about sixfold higher than we did, reported pituitary GH titers lower than in controls. However, since circulating GH levels in our study were considerably reduced, it cannot be excluded that early alterations occurring at the pituitary level went unnoticed with the methodologies we used. Hypothalamic GHRH-LI content and morphology of GHRH-LI secreting structures in 1-day-old rats were indistinguishable from those of age-matched controls, as can be also anticipated from results in 10-day-old rats.

In summary, while confirming that hypothyroidism in neonatal rats induces early changes in GH synthesis and secretion, this study shows that hypothyroidism initially spares the neural regulatory system for GH release, e.g., GHRH-LI-secreting structures. However, studies of GHRH turnover in the hypothalamus made possible by molecular cloning techniques (19, 25) or careful correlations between hypothalamic and circulating GHRH levels—assuming that the latter may be a suitable index of central GHRH function—are mandatory to assess the functional state of the GHRH-producing neurons. Another aspect worthy of further investigation is at what stage of postnatal development persistence of hypothyroidism induces unequivocal changes in GHRH neurons, and the relatedness of this event to alterations in the episodic secretion of GH.

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