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In this issue: These studies indicate that E2 produces its negative feedback in pubertal boys principally by suppression of luteinizing hormone (LH) pulse frequency, and naloxone does not reverse these suppressive effects. Thus E2 suppression of LH secretion is mediated by a decrease of hypothalamic gonadotropin-releasing hormone secretion that is independent of endogenous opioid pathways. ***(See page 4010.)***

Also in this issue: These findings suggest that hyperinsulinemia stimulates ovarian P450c17α activity in nonobese women with polycystic ovary syndrome. They also indicate that decreasing serum insulin with metformin reduces ovarian cytochrome P450c17α activity and ameliorates the hyperandrogenism of these women. ***(See page 4075.)***

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Decreased Hypothalamic Thyrotropin-Releasing Hormone Gene Expression in Patients with Nonthyroidal Illness*

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ABSTRACT

Changes in hypothalamus-pituitary-thyroid function occur in patients with a variety of illnesses and are referred to as the euthyroid sick syndrome or nonthyroidal illness (NTI). In NTI, serum concentrations of T_3 decrease to low, or even undetectable, levels without giving rise to elevated concentrations of TSH. We hypothesized that decreased activity of TRH-producing cells in the paraventricular nucleus (PVN) contributes to the persistence of low TSH levels.

To test this hypothesis, we collected a series of formalin-fixed, paraffin-embedded hypothalami of patients whose plasma concentrations of T_3, T_4, and TSH had been measured in a blood sample taken less than 24 h before death. Quantitative TRH messenger RNA *in situ* hybridization (intraassay coefficient of variation: 13%) was performed in the PVN.

Total TRH messenger RNA in the PVN showed a positive correlation with serum T_3 ($r = 0.66$; $P < 0.05$) and with logTSH ($r = 0.64$; $P < 0.05$), but not with T_4 ($r = -0.02$; $P = 0.95$). This is the first study to correlate premortem serum concentrations of thyroid hormones with postmortem gene expression of identified neurons in the human hypothalamus. The results suggest an important role for TRH cells in the pathogenesis of NTI. (*J Clin Endocrinol Metab* **82:** 4032–4036, 1997)

DURING illness, profound changes may occur in thyroid hormone metabolism, known as nonthyroidal illness (NTI) or the sick euthyroid syndrome. The most consistent change is a decrease in the serum concentration of T_3, but in severely ill patients, T_4 may also decrease (1, 2). These changes in serum thyroid hormones in NTI are of clinical importance. First, the distinction between NTI and severe hypothyroidism can be difficult. Second, the extent of altered thyroid function correlates with severity of illness and prognosis in critically ill patients with NTI (3).

In primary hypothyroidism, a decrease in thyroid hormone concentrations gives rise to increased serum concentrations of TSH. By contrast, decreased basal serum levels of TSH have been reported in critically ill patients (3). The occurrence of decreased or inappropriately normal serum levels of TSH in the presence of very low or even undetectable serum levels of T_3 is largely unexplained but suggests altered feedback control at the level of the hypothalamus and/or pituitary. Indeed, earlier studies have reported an association between NTI and abnormal TSH glycosylation or pulsatility (4, 5), suggesting dysregulation at the level of the hypothalamus. Moreover, the finding that the hypothalamic concentration of T_3 in autopsy samples was much lower in patients with documented NTI than in patients with sudden death from trauma (6) also points towards altered feedback control at the hypothalamic level.

Surprisingly few data are available on the central component of the hypothalamus-pituitary-thyroid axis in humans. We recently described, for the first time, the distribution of TRH-containing neurons in the human hypothalamus (7), showing many TRH cells in the human paraventricular nucleus (PVN). By the subsequent development of a method for TRH messenger RNA (mRNA) *in situ* hybridization in paraffin sections of the human PVN (8), a quantitative study of hypothalamic TRH gene expression in patients with antemortem documented NTI became feasible.

Here we report, for the first time, a strong correlation between TRH gene expression (as measured post mortem in the PVN by *in situ* hybridization) and serum concentrations of T_3 and TSH in 10 patients with varying degrees of NTI.

Subjects and Methods

Patients

For the present study, we used 10 hypothalami obtained from consecutive brain autopsies in the Academic Medical Center over a period of 12 months. Neuropathology was performed by Dr. D. Troost. Inclusion criteria were: 1) permission for brain autopsy; and 2) the availability of a serum sample taken less than 24 h before death. Exclusion criteria were: 1) known history of thyroid disease; and 2) use of glucocorticoids, dopamine, or dobutamine less than 2 weeks before death. Patients with intracerebral hemorrhage were included only if the hypothalamus was intact on macroscopic examination. The following determinations were performed in serum: T_4 and T_3 by in-house RIA (9), and TSH by ICMA (Behring, Marburg, Germany; detection limit 0.01 mU/L). Clinicopath-

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ological data and hormone concentrations are summarized in Table 1. Brains were fixed in 10% formalin at room temperature for 1 month. After dissecting the hypothalamus, tissues were dehydrated in graded ethanol series, cleared in xylene, and embedded in paraffin.

Histology

TRH mRNA *in situ* hybridization was performed in paraffin sections through the PVN using a systematic, random sampling procedure. Coronal serial sections (6 μm) were made from the level of the lamina terminalis to the mammillary bodies. Every 100th section was collected on chromealum-gelatin-coated slides and stained with thionine for anatomical orientation and identification of the right-sided PVN. Delineation of the PVN was assessed more precisely in all subjects by means of immunohistochemical staining for vasopressin (VP) (10). Identification of the most rostral section to contain VP-positive PVN cells (x) was followed by selection of a random figure between 0 and 99 (y). In each subject, the most rostral section number used for *in situ* hybridization was {(x + y) − 100}, followed by a series of sections containing the right-sided PVN with a sampling interval of 100 sections, until no VP-immunostained PVN cells were present. This was the most caudal level to be included. Using this systematic, random sampling procedure, we analyzed 12.0 ± 1.8 sections per patient (mean ± SD; range: 9–14).

In situ hybridization

For *in situ* hybridization, sections were mounted with sterile water on 2% aminoalkylsilane (Sigma, Zwijndrecht, the Netherlands) -coated slides and dried at 38 C. For details, see Ref. 8. We used a [^{35}S]-cytidine 5′ triphosphate-labeled TRH complementary DNA (cRNA) antisense probe, complementary to the nucleotide sequences 330–549 of the human TRH mRNA sequence. The TRH cRNA antisense probe was diluted in hybridization buffer (HBF) [0.5 mol/L sodium chloride, 1× Denhardt's solution, 10 mmol/L Tris (pH 7.6), 1 mmol/L ethylenediaminetetraacetic acid, 10% dextran sulphate, 0.5 mg/mL yeast transfer RNA, 50% formamide, 200 mmol/L dithiothreitol]. Seventy microliters of HBF, containing about 10^6 dpm ^{35}S-labeled TRH probe (SA ~9.5×10^8 dpm/μg), were applied to the sections that were coverslipped, and hybridized overnight in a humidified stove at 66 C. After gently removing the coverslips in 2× SSC (1× SSC is 0.15 mol/L sodium chloride, 0.015 mol/L sodium citrate, pH 7), the sections were washed sequentially for 45 min at 60.5 C in 1× SSC, 0.1× SSC, 0.01× SSC, and finally in 0.001× SSC for 3 h at 60 C. Subsequently, the sections were dehydrated in 300 mmol/L ammonium acetate (pH 5.5)/absolute ethanol at volume ratios of 1:1, 3:7, 1:9, and 0:1, respectively, and dried in a stream of cool air. The sections were apposed directly to autoradiography film (Hyperfilm-βmax, Amersham, Buckinghamshire, U.K.). Films were exposed for 7 h, developed in Kodak (Integra, Biosciences, Switzerland) D-19 at 20 C for 2 min, and fixed in Kodak (Integra, Biosciences, Switzerland) Maxfix for 8 min.

All sections were hybridized in a single experiment. For details on probe specificity and hybridization conditions, see Ref. 8.

Analysis

Grey values of the film autoradiograms were analyzed by computer-assisted densitometry using an Interaktives Bild-Analysen System (IBAS) image analysis system (Kontron, Electronik, Munich, Germany) and software developed at our institute (11, 12). The relationship between the grey values and the amount of radioactivity was assessed with radioactive standards. A standard range of 10 values from 1.700–175.600 cpm/μL was obtained by serial dilution of the labeled probe in HBF. Of each standard, 1-μL samples were spotted on filter paper (Whatman 41, Emergo, Lansmeer, the Netherlands) and coexposed with hybridized sections. For each section series, the profile of integrated density values through the entire PVN was determined using procedures that were described earlier (11, 12). In brief, the labelled area of the PVN and the structure-weighted, background-corrected mean density of each section were determined and used to estimate the total amount of radioactive label present in the PVN. This was used as a relative measure for the amount of TRH mRNA in the PVN. The intraassay coefficient of variation was found to be 13% (1408 ± 183 arbitrary units, mean ± SD), as assessed by measurement of four series of randomly selected, alternating sections in the same PVN (sampling interval, 100 sections, n = 7 sections per series) in one experiment.

Results

All patients had been clinically euthyroid. Serum thyroid hormone concentrations, however, showed a clear spectrum (see Table 1), ranging from biochemical euthyroidism in patients who had died acutely (*e.g.* subject no. 95.124; T_3 1.35 nmol/L, normal range 1.30–2.70 nmol/L; T_4 120 nmol/L, normal range 70–150 nmol/L; TSH 1.30 mU/L, normal range 0.40–4.00 mU/L) to undetectable T_3 ($T_3 < 0.30$ nmol/L), very low T_4 (T_4 25 nmol/L), and normal serum TSH (0.50 mU/L) in a patient who died after severe, protracted illness (subject no. 95.117). The intensity of the TRH mRNA hybridization signal in the PVN on the film autoradiograms showed a marked interindividual variation, ranging from very high intensity in patients with acute death, to low intensity in patients with NTI (Fig. 1). Total TRH mRNA in the PVN, as measured by the total hybridization signal per right-sided PVN, showed a positive correlation with serum T_3 (linear regression analysis, $r = 0.66$, $P < 0.05$; Fig. 2a) and with logTSH ($r = 0.64$, $P < 0.05$; Fig. 2b) but not with T_4 ($r = -0.02$, $P = 0.95$; Fig. 2c) or free T_4 ($r = 0.02$, $P = 0.96$). A multivariate linear regression analysis (stepwise; independent variable TRH mRNA in the PVN; 0.05 level of significance) of the factors: sex, postmortem delay (range: 6–70 h), and storage

TABLE 1. Clinicopathological characteristics of the patients

Subject no.	Sex	Age (yr)	Diagnoses	TSH (mU/L) (0.4–4.0)	T4 (nmol/L) (70–150)	T3 (nmol/L) (1.30–2.70)
95.117	m	70	Chronic pancreatitis, endocarditis, renal insufficiency	0.50	25	<0.30
95.130	f	77	Basilary artery thrombosis, brain stem infarction	0.09	55	0.55
95.128	f	88	Mitral valve insufficiency, atrial fibrillation, shock, probably sepsis	2.80	125	0.70
95.121	f	43	Intracerebral haemorrhage, ischaemic heart disease	0.42	85	0.75
95.127	m	76	Metastatic renal carcinoma, renal insufficiency, intracerebral haemorrhage	0.62	85	0.80
95.132	m	75	Myocardial infarction	1.70	70	0.85
95.129	m	88	Acute myocardial infarction, resuscitation	4.60	70	1.15
95.124	f	71	Trauma, massive subdural haematoma	1.30	120	1.35
95.131	m	74	Massive intracerebral haemorrhage	0.95	115	1.40
95.123	m	34	Insulin-dependent diabetes mellitus, cardiac arrest, resuscitation	4.50	70	1.55

The serum thyroid hormone concentrations were determined in a blood sample taken <24 h before death. Reference values are shown between *parentheses*.

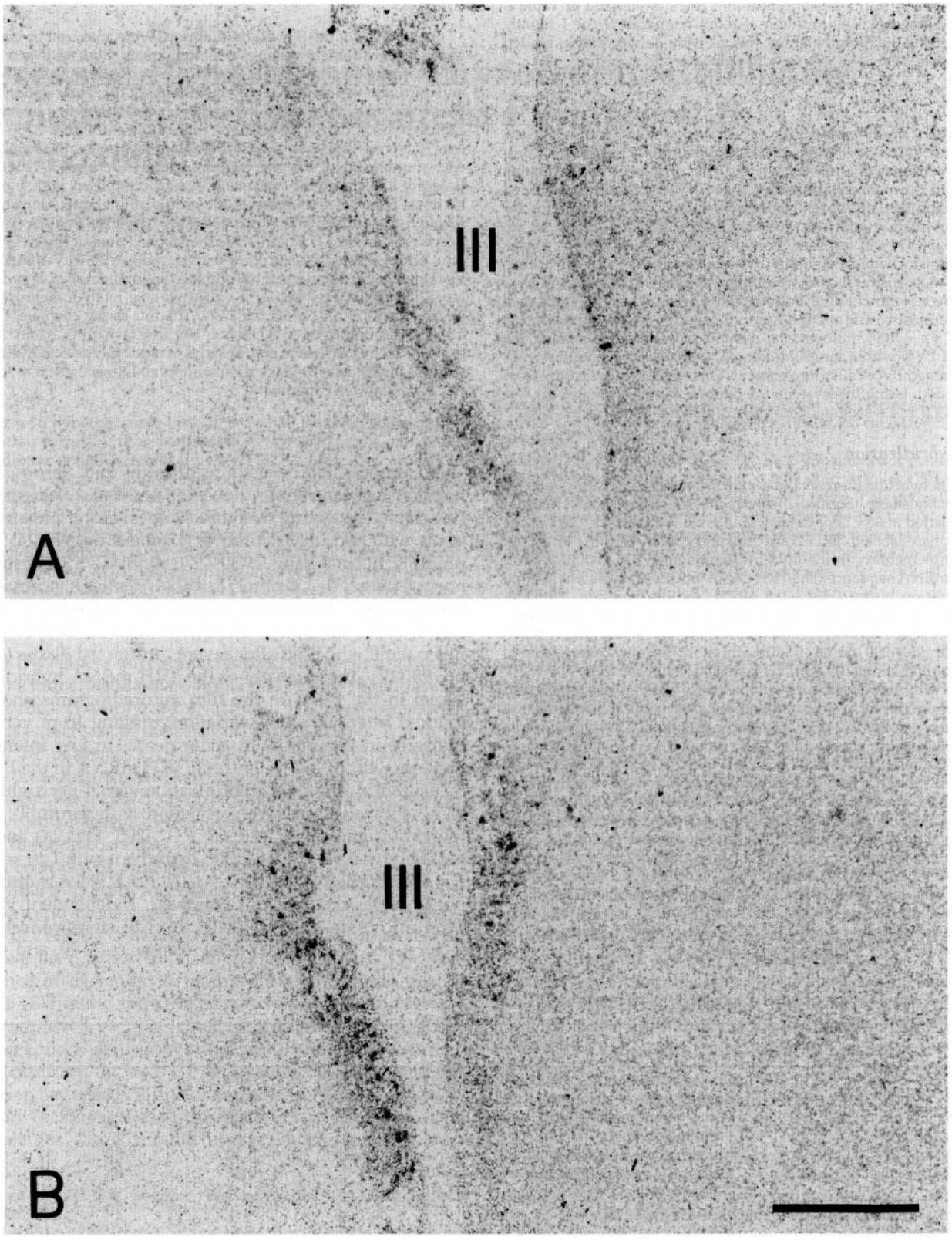

FIG. 1. Macroscopic photographs of film autoradiograms of representative sections of two subjects, showing the PVN along the wall of the third ventricle. A, Low-intensity hybridization signal in the PVN of subject no. 95.130, whose premortem serum thyroid hormone concentrations showed NTI (see Table 1); B, high-intensity hybridization signal in the PVN of subject no. 95.123, who had a normal serum concentration of T_3 and who died from cardiac arrest. *Scale bar* represents 2 mm; III, third ventricle.

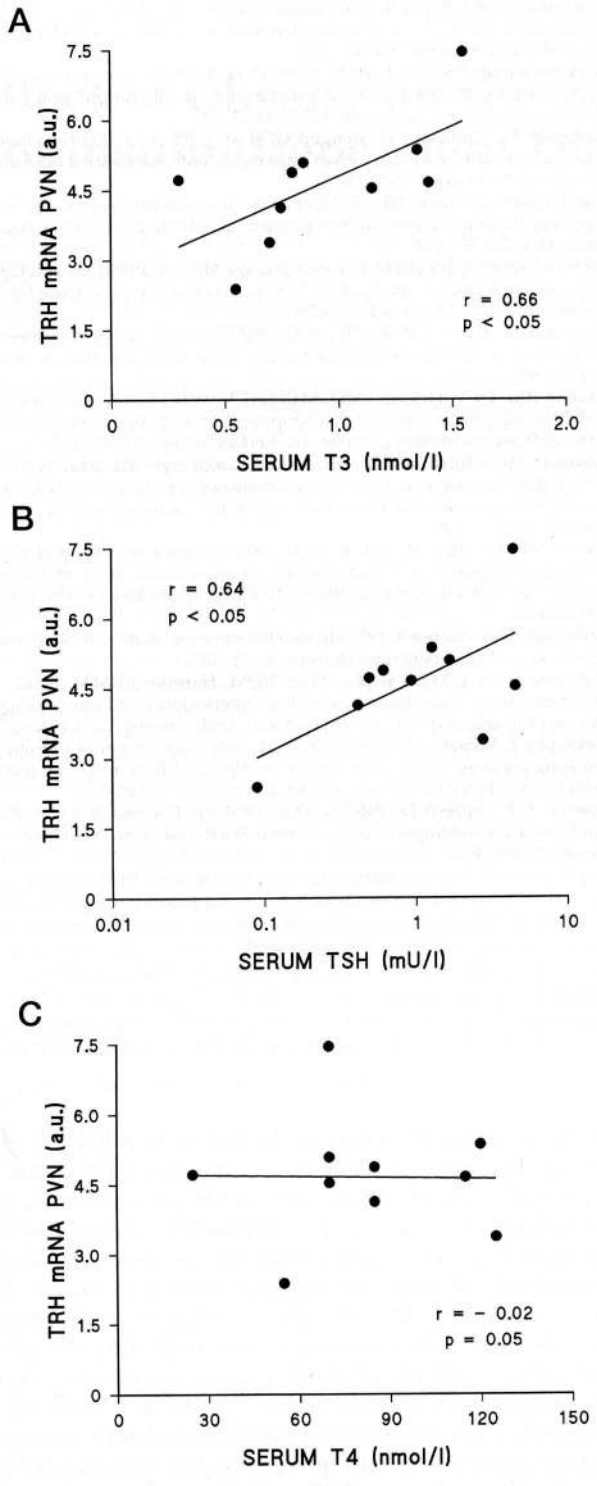

FIG. 2. The relationship between premortem serum thyroid hormone concentrations and total TRH mRNA in the PVN, as measured by the total hybridization signal per right-sided PVN. Each *dot* represents one subject. Linear regression analysis was performed, considering $P < 0.05$ as statistically significant. Note the significant correlation between serum T_3 and TRH mRNA in the PVN (A), and between serum logTSH and TRH mRNA in the PVN (B), and the absence of a correlation between serum T_4 and TRH mRNA in the PVN (C). Data on TSH are represented in a loglinear way, in view of the earlier described loglinear relationship between serum thyroid hormone concentrations and serum TSH (21).

time of the tissue blocks in paraffin, did not yield significant results.

Discussion

In view of an earlier report of lateralization of TRH content in the human hypothalamus (13), only the right-sided PVN was analyzed in each subject. The 3-fold interindividual variation in the total TRH mRNA hybridization signal per PVN exceeded the intraassay coefficient of variation (13%) by far. In an earlier study, the specificity of the TRH cRNA antisense probe used was supported by displacement studies with unlabeled probe, by the absence of hybridization signal using a labeled sense probe, and by the absence of interfering homologies (8). Therefore, NTI can be considered as the major determinant of the interindividual variation in TRH mRNA in the PVN in the present study.

In rats with primary hypothyroidism, decreased serum thyroid hormone concentrations induce increased TRH mRNA in the PVN (14), probably caused by decreased negative feedback control via the T_3 receptor (TR) that has been shown to be expressed by TRH cells in the rat PVN (15). In the present study, we report the contrary, *i.e.* decreased TRH mRNA in the PVN of patients with decreased serum T_3 levels in the framework of NTI. Serum levels of T_4 were decreased in only two very ill patients (Table 1) and did not correlate with TRH mRNA in the PVN. Although serum levels of T_4 have been regarded as a major determinant of intracerebral bioavailability of T_3, transport of [^{125}I]-labeled T_3 from the circulation into the brain also has been demonstrated (16). In addition, tissue-specific regulation of the bioavailability of T_3 has been hypothesized to result from modulation of intracerebral conversion of T_4 to T_3 by type II iodothyronine deiodinase and from modulation of thyroid hormone transport into the brain (17). Because T_3 concentrations within the hypothalamus have been shown to be decreased in patients with NTI (6), our results indicate major changes in hypothalamic thyroid hormone feedback control in NTI. This could be viewed as an important mechanism that contributes to the persistence of very low serum concentrations of T_3 in the absence of thyroid disease, thereby allowing the body to save energy during serious illness.

Low serum levels of T_3 and low TRH mRNA in the PVN may be independent features of NTI, although there may be an overlap in pathogenesis. Over the past years, the importance of cytokines in the pathogenesis of NTI has been suggested (18). A number of experimental studies, using several cytokines to induce animal models of NTI, have indeed reported changes in the hypothalamus. For instance, prolonged infusion of interleukin-1 (IL-1) in rats resulted in decreased serum concentrations of thyroid hormones and in decreased hypothalamic TRH mRNA content (19). In addition, IL-1β administration to the cerebrospinal fluid in rats was associated with a proTRH mRNA content in the PVN that was inappropriately normal or reduced for the decreased concentration of circulating thyroid hormones (20). The way by which cytokines like IL-1 might change thyroid hormone feedback control in TRH cells in the PVN is unknown. One possibility is an effect of cytokines on the TR. However, data on the presence and distribution of TR iso-

forms in the human PVN are lacking, as are data on hypothalamic cytokines in relation to disease. This will be the aim of our further studies.

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