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ORIGINAL ARTICLE

Effects of short- and long-duration hypothyroidism on hypothalamic–pituitary–adrenal axis function in rats: In vitro and in situ studies

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Abstract The purpose of this study is to assess the effects of hypothyroidism on the hypothalamic–pituitary–adrenal (HPA) axis; the functional integrity of each component of the HPA axis was examined in short-term and long-term hypothyroidism. Neuropeptide synthesis, release, and content were evaluated in vitro both in the hypothalamus and anterior pituitary, and corticosterone release was assessed in primary adrenal cell cultures at 7 (short-term) and 60 days (long-term hypothyroidism) after thyroidectomy in male rats. Hypothyroid rats showed adrenal insufficiency in several parameters, which were associated with the duration of hypothyroidism. Cerebrospinal (CSF) ACTH was decreased in all hypothyroid animals, while CSF corticosterone levels were significantly decreased only in long-term hypothyroidism. Long-term hypothyroid animals showed decreased corticotropin-releasing hormone (CRH) mRNA expression in the hypothalamic paraventricular nucleus under both basal and stress conditions, decreased CRH release from hypothalamic organ cultures after KCL and arginine vasopressin stimulation, as well as an increased number of anterior pituitary CRH receptors. In contrast, short-term hypothyroid rats showed changes in anterior pituitary function with an increased responsiveness to CRH that was associated with an increase in CRH receptors. Although both short- and long-term hypothyroidism was associated with significant decreases in adrenal weights, only long-term hypothyroid rats showed changes in adrenal function with a significant decrease of ACTH-induced corticosterone release from cultured adrenal cells. The data suggest that long-term hypothyroidism is associated with adrenal insufficiency with abnormalities in all three components of the HPA axis. Short-term hypothyroidism, on the other hand, is associated with increased pituitary corticotroph responsiveness to CRH.

Keywords Thyroid hormone · CRH · ACTH · Corticosterone · Corticotrophs · Hypothalamic–pituitary–adrenal axis · Stress

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Introduction

There is growing evidence demonstrating that thyroid hormone affects the various components of the hypothalamic–pituitary–adrenal (HPA) axis [1]. Experimentally induced hypothyroidism has been reported to reduce adrenal weight [2], as well as alter concentrations of

corticosterone [3]. Although thyroidectomy decreases plasma adrenocorticotropic hormone (ACTH) levels [4], pituitary content of ACTH has been reported to increase [5]. Thyroidectomy is also associated with an increase in corticotrophin-releasing hormone (CRH) gene transcription in the hypothalamic paraventricular nucleus (PVN) [4]. ACTH response to exogenous CRH is exaggerated in hypothyroidism [2, 6]. In previous in vivo studies, we demonstrated that hypothyroidism is associated with mild, yet significant, adrenal insufficiency [26]. Although these experimental studies demonstrate general thyroid hormone-induced alteration in HPA axis function, the primary site of action, as well as the effect of the duration of hypothyroidism remains unclear.

The present study assesses further the functional activity of the components of the HPA axis in experimentally induced hypothyroid adult male rats. To further explore the primary locus of the HPA axis that is affected, we report a series of in vitro studies in the rat following experimentally induced hypothyroidism. Studies were designed to assess the basal state and the functional integrity of the hypothalamic CRH neuron, the pituitary corticotroph cell, and the adrenal cortex. The hypothalamic CRH neuron was assessed by examining hypothalamic neurohormonal synthesis under basal conditions and following the application of experimental stressors. The pituitary corticotroph was assessed by measuring pituitary hormonal content, release, and synthesis, as well as by determining CRH receptor number and affinity. Adrenal cortex function was assessed by examining glucocorticoid release from primary adrenal cell cultures. In addition, cerebrospinal fluid (CSF) corticosterone concentrations were determined as an estimate of plasma free corticosterone concentrations. Changes in plasma corticosteroid-binding globulin (CBG) and adrenal and thymus weight related to hypothyroidism were also determined. In order to assess whether the duration of hypothyroidism influences HPA activity, studies were performed at 7 days (short-term hypothyroidism) and 60 days (long-term hypothyroidism) following thyroidectomy.

Materials and methods

Experiments were performed on male Sprague–Dawley rats weighing 350–400 g (Charles River, Wilmington, MA) and housed for 1–2 weeks under an artificial 12:12 h light:dark cycle with lights on from 0600 h. The room was kept at 24 °C with controlled humidity. Rat chow (Ralston-Purina, St. Louis, MO) and water were available ad libitum. Surgical thyroidectomy (Tx) or sham Tx was carried out under methoxyflurane inhalation anesthesia (Metofane, Pitman-Moore, Washington Grossing, NJ). After recovery, animals were supplied with 1 % calcium lactate solution as drinking water to avoid hypocalcaemia from possible excision of the parathyroid glands. Experiments were performed 7 and 60 days after Tx or sham Tx. All procedures were approved by the NIH Committee for the use and welfare of laboratory animals and conformed to the International Ethical Standards (86/609-EEC) for the care and use of laboratory animals and conformed to the guidelines in the UFAW Handbook on the Care and Management of Laboratory Animals (published by the Universities Federation for Animal Welfare). Rats were euthanized by decapitation at 7 and/or 60 days after thyroidectomy or sham thyroidectomy. Trunk blood was then collected in tubes containing EDTA (20 μg/ml) chilled immediately in an ice bath and processed as previously described [7, 8]. The brains were removed rapidly by means of a sterile technique.

Basal hormonal levels

Basal plasma ACTH, corticosterone, and thyroid indices were determined at the time of euthanizing before in vitro studies. Basal plasma CBG-binding capacity was measured in euthyroid and hypothyroid as previously described [2]. CBG-binding capacity was defined as total binding minus the nonspecific binding corrected to μg [3H]-corticosterone bound/dl rat plasma. To determine CSF levels of IR-ACTH and IR-corticosterone, rats were anesthetized [pentobarbital (55 mg/kg BW, intraperitoneal (ip)) and ketamine (50 mg/kg BW, ip)] and placed in a stereotaxic apparatus. CSF samples (100–200 μl) were drawn from the cisterna magna before and after thyroidectomy.

Thymus and adrenal weights

Adrenals and thymuses were dissected out of animals euthanized by decapitation. The right adrenal gland was defatted and its wet weight was measured immediately after removal from the rat. The thymus was cleaned, the excess tissue was dissected away, patted dry, and immediately weighed.

Hypothalamic neuropeptide synthesis, release, and content

In situ hybridization histochemistry

In situ hybridization histochemistry was performed as previously described [9–14]. Briefly, the sections were warmed to room temperature, fixed in 4 % formaldehyde, treated with 0.25 % acetic anhydride to reduce nonspecific binding, dehydrated and delipidated in graded solutions of ethanol and chloroform, and rehydrated. The probes were synthetic oligonucleotides. The specificity of these probes has previously been demonstrated by the detection of homogenous

bands of the appropriate size on Northern analysis of extracted mRNA by the use of messenger sense probes and by comparison with the results of immunocytochemistry. The probes were labeled using terminal deoxynucleotidyl-exo transferase (Boehringer Mannheim) to add a [35S]dATP (New England Nuclear) tail to the 3′ end of the probe. All sections were hybridized in the same incubation reaction. The labeled probe in hybridization buffer was applied to each section and left overnight at 37 °C for hybridization. The sections were then washed with four 15 min washes [in 2× SSC (1× SSC is 0.15 M sodium chloride/0.015 M sodium citrated, pH 7)/50 % formamide at 40 °C] followed by two 30 min washes [in 1× SSC at room temperature for 1 h each] to remove non-specifically bound probe. Sections were then rinsed in water, dried, and apposed to Hyperfilm MP autoradiography film for 6 days. The optical density of the autoradiographic images was measured using an image analysis system. Optical densities were converted to copies of probe hybridized/um^3 tissue using labeled standards and the minimum number of copies of probe present calculated as previously described [14].

Effects of hypertonic saline stress

Rats were stressed using a regimen from a previous study with minor modifications [15]. Chronic stress was induced by an ip injection of hypertonic saline (1.5 mol NaCl/l; 1.8 ml/kg BW) for 5 consecutive days between 7:00 and 8:00 h. As a control for the possible stress effects of handling and injection, one group was subjected to the injection procedure with an ip injection of normal saline, and one group was left undisturbed (control). All rats were weighed before injections, and were returned immediately to their home cage. Four hours after the injection on the 5th day, the rats were decapitated. The brains and pituitaries were quickly removed and frozen by immersion in 2-methyl butane at −30 °C. The brains were then mounted onto cryostat pedestals with embedding matrix, and serial 15-μm-thick coronal sections were cut through the PVN of the hypothalamus, and the arcuate nucleus in a cryostat at −18 °C, thaw-mounted onto gelatin-coated slides, and stored at −70 °C.

Hypothalamic organ culture

We assessed the direct effects of AVP and KCL upon hypothalamic CRH release utilizing a rat hypothalamic organ culture system, which we have previously described in detail [7, 8]. Briefly, 7 and 60 days following experimentally induced hypothyroidism rats were euthanized by decapitation. Following excision, hypothalami were placed in a culture medium, which consisted of medium l99 (Ml99) with modified Earle's salt (Gibco, Grand Island, NY), containing 0.l % bovine serum albumin and 20 μM bacitracin (Aldrich Chemical Co., Milwaukee, WI). After an overnight preincubation in a water-jacketed incubator at 37 °C, under a 5 % CO_2 atmosphere, the experiments were performed as follows. Hypothalamic explants were serially passed in six different wells (48-multiwell plates, Costar Corp., Cambridge, MA), every 20 min. At the end of the experiment, each hypothalamus was exposed to a depolarizing concentration of KCL (60 mM), for 20 min, to indirectly test intactness of the tissue used. Hypothalami that failed to respond to KCL with an increase of IR-CRH of at least 90 % above the baseline were excluded from the analysis. To determine the effect of AVP on hypothalamic CRH secretion, the hypothalami were incubated in plain medium in the first three wells. The mean IR-CRH secretion of these wells was used as basal secretion for a given hypothalamus. In the fourth and fifth well, the hypothalami were exposed to vehicle or graded concentrations of AVP and the mean IR-CRH secretion in these wells was used as stimulated IR-CRH secretion for a given hypothalamus.

Hypothalamic neuropeptide content

Immediately after explanation, the hypothalamus from each rat was placed in 100 μl 0.1 M HCL containing 1 mM ascorbic acid and frozen on dry ice. Before the assays for hypothalamic peptide content, the tissue was dispersed for 30 s using an ultrasound homogenizer and the volume was made up to 1.5 ml with 50 mM sodium phosphate buffer (pH 7.4) containing 0.1 % Triton X-100, 0.01 % sodium azide, and 0.1 % BSA. Hypothalamic homogenates were assayed for CRH, ACTH, and β-endorphin, AVP content.

Anterior pituitary hormone synthesis, release, and content

POMC mRNA expression was estimated utilizing the in situ hybridization histochemistry methodology similar to that described above for the estimation of hypothalamic peptide mRNA expression. The pituitaries attached to the brain were mounted onto cryostat pedestals using embedding matrix and serial 15-μm-thick coronal sections were cut through the anterior pituitary in a cryostat at −18 °C, thaw-mounted onto gelatin-coated slides, and stored at −70 °C .

Immediately after the explanation, the anterior pituitaries from each rat was placed in 100 μl 0.1 M HCL containing 1 mM ascorbic acid and frozen on dry ice. Primary anterior pituitary cell cultures were prepared as previously described [15]. The anterior lobes were finely minced and pituicytes were mechanically dispersed and then centrifuged at 750 rpm at room temperature for l0 min. This procedure was repeated twice. Pellets were subsequently

washed twice in DMEM with 10 % fetal calf serum, aprotinin [100 kallikrein inhibitor units (KIU)/ml], ascorbic acid (0.05 mg/ml), penicillin (l00 U/ml), streptomycin (l00 U/ml), and fungizone (0.25 μg/ml) and reconstituted in the same media. Cell viability, tested by trypan blue exclusion, was always greater than 95 %. The cells were plated into a multiwell petri dish at a density of 1 to 1.5 × $l0^5$ cells/well and incubated at 37 °C under a 7.5 % CO_2 atmosphere. After 48 h, the medium was replaced. The cells were washed the next day with DMEM without fetal calf serum and incubated with vehicle or graded concentrations of CRH for 4 h. Each measurement was made in triplicate in three different cultures. At the end of incubations, media were collected and stored at −70 °C for ACTH RIA.

ACTH and β-endorphin content

Pituitaries were removed from the sella turcica of decapitated rats and the anterior lobe separated from the rest of the gland. Before the assays for pituitary peptide content, the tissue was dispersed as described for hypothalamic content. Pituitary homogenates were assayed for ACTH and β-endorphin content.

CRH receptors

Anterior pituitaries were assayed for CRH receptors as previously described [16]. Briefly, 100 μl pituitary membrane-rich fractions containing 100–200 μg protein were incubated with 100,000 cpm [125]Tyr-CRF (0.1 nM) in a total volume of 300 μl 50 mM Tris–HCL buffer, pH 7.4, containing 5 mM $MgCl_2$, 2 mM EGTA, and 0.1 % BSA, 100 kallikrein inhibitory units/ml aprotinin (Sigma Co., St. Louis, MO), and 1 mM dithiothreitol. After incubation for 60 min at 22 °C, receptor-bound radioligand was separated by centrifugation after the addition of 1 ml 7.5 % polyethylene glycol in 50 mM Tris buffer. Pellets were washed twice, the tips of the tubes were severed, and radioactivity was counted in a g-spectrometer. Nonspecific binding determined in the presence of 0.1 μM unlabeled CRF was less than 10 % of the total bound radioactivity; total and bound radioactivity was 10–15 % of the radioactivity added.

Corticosterone release from primary adrenal cell cultures

Primary adrenal cell cultures were prepared as previously described [7, 8]. Briefly, after adrenal cell viability was tested by trypan blue exclusion test, ranged between 80 and 95 %, the cells were plated into multiwell petri dish (24 wells, Costar) at a density of 0.75 × 10^5 cells/well in a culture medium consisting of M199 with 10 % fetal calf serum, aprotinin [200 kallikrein inhibitor units (KIU)/ml], ascorbic acid (0.05 mg/ml), penicillin (l00 U/ml), streptomycin (l00 U/ml), and fungizone (0.25 μg/ml). The plates were incubated under 5 % CO_2 atmosphere at 37 °C for 18 h. After incubation, the medium was removed and the attached cells were treated with graded concentrations of ACTH for 4 h. Each concentration of ACTH was run in triplicate in three different cultures. After incubation, the medium was collected and stored at −20 °C until assayed for corticosterone.

Hormone and other determinations

ACTH, β-endorphin, and AVP were measured by RIA in extracted plasma. ACTH was assayed as previously described [8]. The intra-assay coefficient of variation (CV) for the ACTH RIA was 4.6 % and the corresponding interassay CV was 8.6 % at 60 pg/ml. Sensitivity was 5 pg/ml. Plasma β-endorphin levels were measured by RIA using an antiserum developed in our laboratory (HS-7) [17]. Nonspecific binding of the assay was 2.9 %. The detection limit was 5.6 pg per assay tube, and the intra-assay CV was 2.7 %. AVP was measured by RIA. The sensitivity was 0.80 pg/ml, and the intra-assay CV was 6.2 %. IR-corticosterone was measured directly in unextracted plasma by RIA using a [125I]-corticosterone kit from Radioassay System Laboratory, Inc. (Carson, CA). Intra-assay CV was 1.8 % and interassay CVs were 1.6 % at 80 ng/ml and 1.9 % at 475 ng/ml. Sensitivity was 4.5 ng/ml.

Plasma TSH concentrations were determined by RIA using an anti-rat TSH serum and purified rat TSH reference preparation provided by the National Hormone and Pituitary Program (Baltimore, MD). Plasma total T4 (TT4) and total T3 (TT3) concentrations were determined by RIA (Immunochem Corp., Carson CA).

Medium CRH concentrations were measured by RIA using an antiserum specific for rCRH (TS-3) developed in our laboratory [7, 8]. TS-3, used at a final dilution of l:60,000 (assay volume 0.3 ml), bound 37 % of [125l]-CRH. Total and nonspecific bindings were 32 ± 3 and 1.9 ± 0.1 %, respectively. Sensitivity (ED90) was 2.0 ± 0.1 pg/tube (20 pg/ml of media). The intra- and inter-assay coefficients of variation were 8.1 and 17.2 %, respectively.

Statistical analyses

Results are expressed as the mean ± SEM throughout the study. Statistical evaluation of hypothalamic CRH, pituitary ACTH, and adrenal corticosterone secretion was performed by one-way ANOVA, followed by Duncan's multiple range tests. Receptor assay results were evaluated

by Scatchard analysis. Group means were compared by *t* tests or ANOVA followed by Fisher's PLSD, depending upon the experimental design.

Results

Basal hormonal levels

All hypothyroid rats had undetectable TT4 levels (<0.5 μg/ml) both at 7 and 60 days following thyroidectomy, which were significantly lower compared to euthyroid rats (3.9 ± 0.2 and 3.5 ± 0.2 μg/ml at 7 and 60 days, respectively; $P < 0.0001$, by ANOVA). TT3 levels also tended to be lower in hypothyroid animals (20.4 ± 3.4 and 22.4 ± 1.7 ng/ml at 7 and 60 days, respectively) compared to euthyroid animals (39.0 ± 3.1 and 39.5 ± 2.9 at 7 and 60 days, respectively). Plasma TSH levels were elevated in all hypothyroid rats (12.5 ± 0.7 and 25.5 ± 1.6 ng/ml at 7 and 60 days, respectively) compared to values in euthyroid rats (4.2 ± 0.4 and 2.8 ± 0.4 ng/ml at 7 and 60 days, respectively; $P < 0.0001$, by ANOVA).

There were no significant differences between euthyroid, short-term, and long-term hypothyroid rats in the basal plasma ACTH (95.8 ± 6.9 and 90.3 ± 4.6 pg/ml versus 88.0 ± 3.4 and 81.4 ± 4.6 pg/ml at 7 and 60 days, respectively), corticosterone (137.2 ± 25.5 and 100.0 ± 24.5 ng/ml versus 137.2 ± 25.5 and 99.6 ± 21.0 ng/ml at 7 and 60 days, respectively), or AVP levels (1.4 ± 0.1 and 1.9 ± 0.4 pg/ml versus 1.3 ± 0.1 and 1.4 ± 0.3 pg/ml at 7 and 60 days, respectively).

Basal plasma CBG levels were suppressed (6.3 ± 0.3 vs. 14.1 ± 0.7 μg/dl) in 7 day, but not 60 day (10.5 ± 0.5 vs. 13.2 ± 0.6 μg/dl) hypothyroid rats as compared to euthyroid animals ($P \leq 0.05$, ANOVA followed by Fisher PLSD). CSF IR-ACTH concentrations in hypothyroid animals were decreased following short- and long-duration thyroidectomy-induced hypothyroidism ($P \leq 0.05$, ANOVA followed by Fisher PLSD). CSF IR-corticosterone levels, on the other hand, were normal in short-duration hypothyroid rats, but significantly decreased 60 days after thyroidectomy compared to euthyroid levels ($P \leq 0.05$, ANOVA followed by Fisher PLSD).

Thymus and adrenal weights

Compared to euthyroid rats, there was a significant reduction in the weights of the thymus glands after long-term hypothyroidism ($P < 0.05$; *t* test; Fig. 1a). Moreover, weights of the adrenal glands were significantly lower in rats studied both after 7 and 60 days of experimentally induced hypothyroidism than euthyroids ($P < 0.05$; *t* test; Fig. 1b).

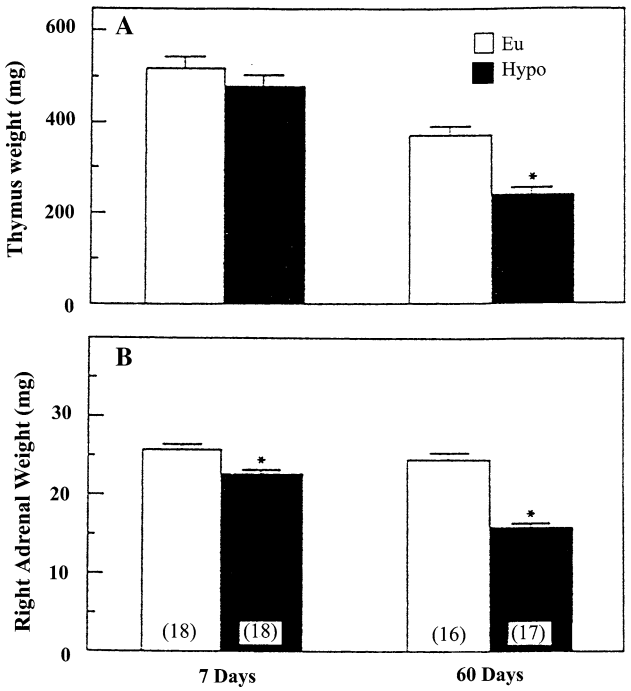

Fig. 1 Effects of short- and long-duration hypothyroidism on thymus (**a**) and adrenal weights (**b**). Each *bar* represents the mean; the *brackets* indicate the SEM. Number of measures are indicated in *parenthesis*. Short-duration hypothyroidism was associated with decreased adrenal weights, while long-duration hypothyroidism was associated with both decreased adrenal weights and thymus weights. *$P < 0.05$ versus tissue weight in euthyroid rats (by Student's *t* test)

Hypothalamic neuropeptide synthesis, release, and content

Basal- and stress-induced neuropeptide mRNA expression in the hypothalamus

Under basal conditions (control and normal saline administration), long-duration hypothyroidism (60 days) was associated with a significant decrease in the expression of CRH mRNA in the PVN of the hypothalamus ($P < 0.0.5$, by ANOVA followed by Fisher PLSD; Fig. 2a). In contrast, however, we found that POMC mRNA levels in the arcuate nucleus were similar among euthyroid and rats studied following both short- and long-duration hypothyroidism (Fig. 2b).

Under basal conditions, there were no differences in AVP mRNA expression in the PVN, between short-term and long-term hypothyroid rats compared to euthyroid rats (Fig. 2c). On the other hand, basal oxytocin mRNA expression in the PVN was similar in euthyroid rats and 7 day of hypothyroid rats, but was significantly reduced after 60 days of hypothyroidism ($P < 0.0.5$, by ANOVA followed by Fisher PLSD; Fig. 2d).

CRH mRNA expression in the PVN following osmotic stress (hypertonic saline administration) were similar in

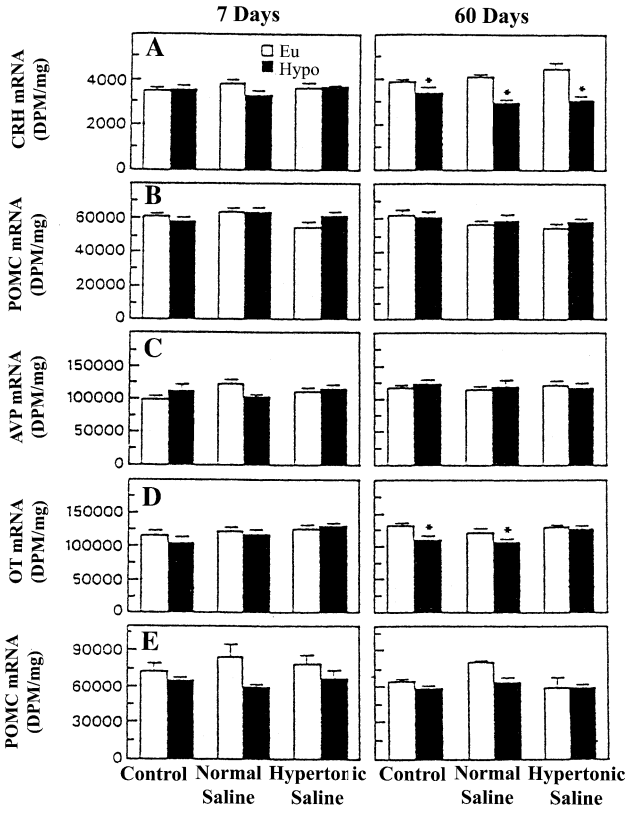

Fig. 2 Effects of the duration of hypothyroidism on peptide mRNA expression. Quantitative in situ hybridization histochemistry estimation of CRH mRNA in the PVN (**a**), POMC mRNA in the arcuate nucleus (**b**), AVP mRNA in the hypothalamus (**c**), OT in the hypothalamus (**d**), and POMC mRNA in the anterior pituitary (**e**). Levels of mRNA were examined under basal conditions (control and normal saline) and following stress (hypertonic saline). Each *bar* represents the mean of values obtained from five or six different rats; the *brackets* indicate the SEM. $*P < 0.05$ versus mRNA levels in euthyroid rats (by ANOVA followed by Fisher's PLSD)

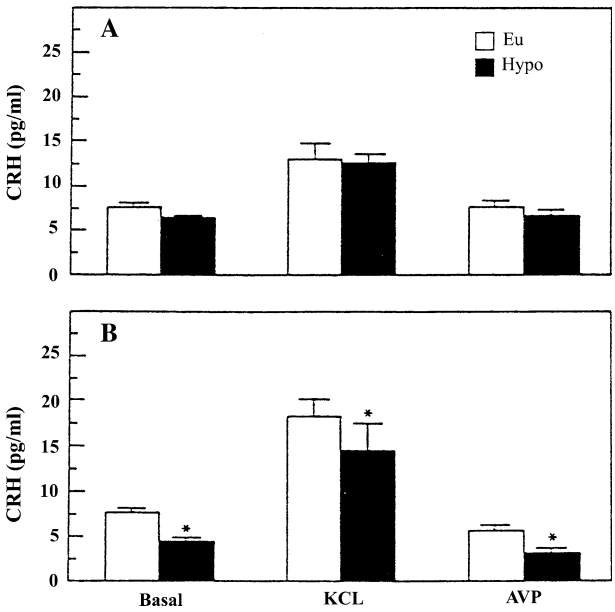

Fig. 3 Effects of short- (**a**) and long-duration (**b**) hypothyroidism on basal, and KCL- and AVP-induced IR-CRH secretion by single explanted rat hypothalami. Results are expressed as pg CRH/ml. Each *bar* represents the mean of values obtained from 3 separate experiments with six to eight different hypothalami in each; the *brackets* indicate the SEM. $*P < 0.05$ versus IR-CRH levels in euthyroid rats (by *t* test)

short-term hypothyroid and euthyroid rats. However, following 60 days of experimentally induced hypothyroidism, the stress-mediated CRH mRNA levels in this locus were significantly lower in long-term hypothyroid animals than in euthyroid animals ($P < 0.0.5$, by ANOVA followed by Fisher PLSD; Fig. 2a, right). As under basal conditions, we found that stress-induced POMC mRNA levels, in the arcuate nucleus, did not differ among euthyroid animals and those studied after short- and long-duration hypothyroidism (Fig. 2b). Moreover, osmotic stress had no effect on either AVP or OT mRNA expression in rats studied after 7 and 60 days of experimentally induced hypothyroidism (Fig. 2c, d).

Hypothalamic culture CRH release and neuropeptide content

Basal, KCL-induced, and AVP-induced CRH release from rat hypothalamic organ culture were all similar from hypothalami taken from euthyroid rats and those euthanized after 7 days of experimentally induced hypothyroidism (Fig. 3a). In contrast, there was a significant decrease in basal, KCL- and AVP-induced CRH release from hypothalami taken from those euthanized after 60 days of experimentally induced hypothyroidism compared to the euthyroid state ($P < 0.05$, *t* test; Figs. 3b and 4).

Hypothalamic IR-CRH content was similar in euthyroid controls and in rats studied after 2, 7, and 60 days of experimentally induced hypothyroidism; although after 60 days of experimentally induced hypothyroidism, IR-CRH content tended to be lower. In long-term hypothyroidism, we also found a subtle increase in hypothalamic levels of IR-ACTH, IR-β-endorphin, and IR-AVP.

Anterior pituitary hormone synthesis, release, and content

Under basal conditions and stress conditions, there was a trend for a reduction in POMC mRNA expression in the anterior pituitary in rats studied after short-duration and long-term hypothyroidism (Fig. 2e).

Compared to euthyroid rats, CRH-induced ACTH release was significantly greater in rats studied after 7 days of experimentally induced hypothyroidism ($P < 0.0.5$, by ANOVA followed by Fisher PSLD; Fig. 5). This

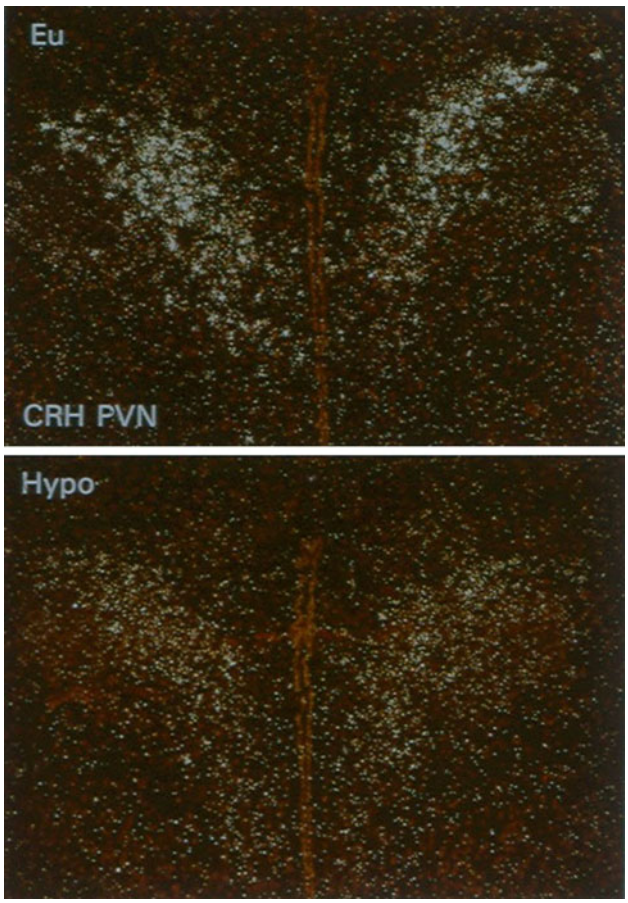

Fig. 4 Dark-field photomicrograph of in situ hybridization histochemistry reveals cells containing CRH mRNAs in the paraventricular nucleus (PVN) of euthyroid rats (*upper panel*) and long-duration hypothyroid rats (*lower panel*). Note the decrease in signal (*white silver grains*) in the hypothyroid animals

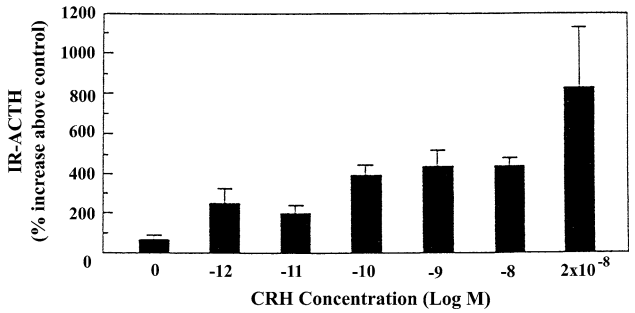

Fig. 5 Effects of graded concentrations of CRH on ACTH secretion from dispersed anterior pituitary cells. Results are expressed as a percent of the euthyroid value. Each concentration of CRH was tested in triplicate in three different cultures

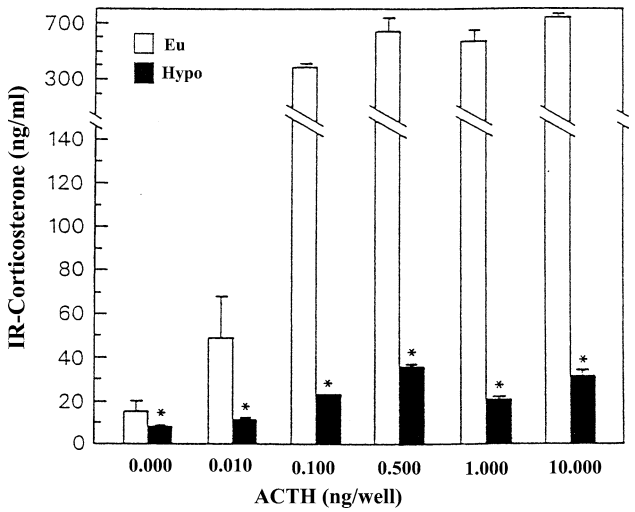

Fig. 6 Effects of graded concentrations of ACTH on corticosterone secretion from dispersed adrenal cells derived from long-duration hypothyroid and euthyroid rats. Results are expressed as nanograms of corticosterone/ml. Each concentration was tested in triplicate in three different cultures. After 60 days of experimentally induced hypothyroidism, rats had significantly decreased corticosterone release. $*P < 0.05$ versus the corticosterone response elicited by ACTH in euthyroid rats (by ANOVA followed by Fisher's PLSD)

difference was not found in rats studied after long-term hypothyroidism. Long-term hypothyroidism did not influence the anterior pituitary IR-ACTH or IR-b-endorphin content.

Compared to euthyroid rats, there was a trend toward an increase in CRH receptor number in rats studied after 7 days of experimentally induced hypothyroidism. After 60 days of experimentally induced hypothyroidism, CRH receptor number demonstrated a 25 % increase compared to euthyroid rats ($P < 0.05$). Changes in the number of CRH receptors were not associated with changes in the receptor affinity (Kd).

Adrenal cultures

ACTH-induced in vitro corticosterone release from primary adrenal cell cultures was similar in euthyroid and short-term hypothyroid rats. However, this parameter was significantly reduced in rats studied after 60 days of experimentally induced hypothyroidism ($P < 0.0.5$, by ANOVA followed by Fisher PLSD; Fig. 6).

Discussion

Long-term hypothyroidism in the rat was associated with a significant decrease in the expression of CRH mRNA in the PVN of the hypothalamus. Following the application of osmotic stress in long-term hypothyroid animals, the expression of CRH mRNA in the PVN remained decreased. This reduction in basal and stress-induced CRH mRNA was associated with a decrease in basal, as well as KCl- and AVP-induced CRH release from hypothalamic organ

cultures. An increase in the number of anterior pituitary CRH receptors was also observed in long-term hypothyroid rats. At the adrenal level, long-term hypothyroid rats demonstrated a significant decrease in ACTH-induced corticosterone release from primary adrenal cell cultures. These changes in the in vitro functional responsiveness of each component of the HPA axis in long-duration hypothyroidism were associated with a significant decrease in the weights of both the adrenal and thymus glands.

Short-term hypothyroid rats showed an increased ACTH response to CRH stimulation from cultured pituicytes, as well as an increase in CRH receptors. This was similar to previous findings showing ACTH responses to exogenous CRH are exaggerated in hypothyroid rats [2, 5]. CSF ACTH levels were decreased in short-term hypothyroidism.

CSF corticosterone levels were normal in short-duration hypothyroid rats, but significantly decreased in long-term hypothyroid rats. The recovery of CBG levels, in light of normal basal plasma corticosterone levels in long-term hypothyroidism, would result in a lowering of free-corticosterone plasma levels. This is reflected in the significantly lower CSF corticosterone levels. The finding of a significant reduction in the CSF levels of corticosterone suggests corticosterone deficiency because, as an ultra filtrate of plasma, the CSF corticosterone level is believed to reflect the plasma free corticosterone concentration. The significant reduction in adrenal weight seen in hypothyroid rats is also compatible with either primary or secondary adrenal insufficiency and is rarely seen in any other context [18]. Hypothyroidism is known to decrease the clearance of corticosterone from the plasma, [19, 20] and reduce CBG-binding capacity [2]. However, neither a reduction in the clearance of corticosterone from plasma nor a reduction in the plasma CBG-binding capacity would be expected to change the plasma-free corticosterone concentration or the CSF corticosterone concentration in the absence of a change in the set point for central nervous system control of adrenocortical function. Moreover, we have previously shown that neither 7 nor 60 days of experimentally induced hypothyroidism influences the clearance of ACTH from plasma [2]. Thyroid hormones appear to exert different effects on the production of various carrier proteins in vivo. While they appear to stimulate the production of sex hormone-binding globulin (SHBG), thyroid hormones suppress the level of CBG and their own carrier protein thyroxin-binding globulin (TBG) [21]. In humans, CBG concentrations are increased in hypothyroidism and decreased in hyperthyroid patients [22]. In hypothyroid rat pups, CBG concentrations diminished at day 4 and increased at day 8 [23].

The thymus exhibits a high density of glucocorticoid type-II receptors and a particular sensitivity to glucocorticoids [24]. Studies have shown that corticosterone treatment or elevated in glucocorticoid levels result in the atrophy of the thymus and adrenal glands [25, 26], while adrenalectomy causes significant thymus hypertrophy [27]. Changes in thymus and adrenal weights have been used as additional indicators of stress and/or altered glucocorticoid levels [28].

The data support the interpretation that long-term hypothyroidism may be associated with a mild central CRH deficiency. First, long-term hypothyroidism is associated with a significant fall in the expression of CRH mRNA in the PVN of the hypothalamus. This is compatible with a previous report showing that hypothyroidism causes a reduction in CRH gene transcripts in the PVN of male rats with a concomitant decrease in POMC gene expression in the anterior pituitary gland and decreased circulating corticosterone levels [4]. In addition, the expression of CRH mRNA in the PVN following stressful stimuli was also lower in chronic hypothyroid animals. The central set point for pituitary-adrenal function seemed to progressively fall with the increasing duration of hypothyroidism, as indicated by a progressive fall in CSF corticosterone levels with the increased duration of hypothyroidism. The studies of the anterior pituitary glands in hypothyroid rats also support the idea that hypothyroidism produces some degree of central CRH deficiency. The significant upregulation in CRH receptor number in the anterior pituitaries of hypothyroid rats may reflect a possible consequence of decreased stimulation by CRH, hence supporting the idea of a functional CRH deficiency.

Direct hypothyroid-induced pituitary changes cannot be excluded by these results. Early studies have shown a wide spectrum of pituitary alteration with mild and severe thyroid impairment with severe distortions of pituitary function more apparent in severe hypothyroidism [29]. Tohei and co-authors suggest that hypersecretion of ACTH is mediated by CRH and arginine vasopressin (AVP) in the hypothalamus [30]. They supported this hypothesis by showing that after 2 weeks of thiouracil-induced hypothyroidism in male rats; the in vivo release of CRH and AVP in the median eminence as measured by a push–pull perfusion method was significantly elevated compared to euthyroid rats [1]. We have previously reported that the ACTH responses to exogenous CRH are markedly exaggerated in hypothyroid rats compared to controls utilizing the identical experimental design for the production of hypothyroidism and for the measurement of plasma ACTH as in the current study [6]. This is similar to clinical findings in acute hypothyroidism by ablative therapy where athyreotic women demonstrated hypersensitivity of ACTH to hCRH compared to normal controls although basal levels of serum ACTH and cortisol were similar [31]. However, the magnitude of the ACTH response to synthetic exogenous CRH in hypothyroid rats was at least 2–3 times greater than the responses observed following direct

hypothalamic stimulation via hypoglycemic stress and IL-1α administration. Because the ACTH responses to these centrally acting stimuli are believed to depend upon the release of endogenous CRH, this discrepancy could, in part, reflect the fact that the experimental hypothyroidism produced here resulted in some degree of central CRH deficiency. This is further supported by the in vitro findings of this study which show decreased basal and stress-induced CRH mRNA in the PVN of the hypothalamus, as well as decreased basal and KCL and AVP stimulated CRH release in hypothalamic cultures.

POMC is synthesized predominantly in pituitary corticotrophs and melanotrophs. In addition to its expression in the pituitary, however, the POMC gene is also highly expressed in a subpopulation of neurons in the arcuate nucleus of the hypothalamus that extend axonal projections to multiple subcortical nuclei [32]. As such, CRH neurons in the PVN are densely innervated by POMC terminals from the arcuate nucleus [33] and these appear to be able to significantly alter HPA axis activity [34]. However, the data remain conflicting concerning whether glucocorticoids have long-loop feedback effects on the activity of POMC neurons in the arcuate nucleus and expression of the POMC gene, which promote inhibitory control of pituitary corticotrophs [35]. The available data does not allow us to differentiate among the unique functions and potential redundancies of POMC produced in the pituitary and brain. Both endorphin and ACTH secreted by the POMC neurons of the arcuate nucleus exert inhibitory effects on CRH secretion. As POMC neurons are stimulated by CRH, they potentially provide another negative feedback control loop on the HPA axis [36]. POMC mRNA levels in the arcuate nucleus were assessed to evaluate whether altered thyroid hormone levels were associated with changes in arcuate nucleus POMC activity. We can not exclude the possibility that changes in central POMC might indirectly alter the set point for CRH neuron activity relative to circulating corticosterone levels [34].

Long-term hypothyroid rats showed a significant reduction in ACTH-induced corticosterone release from primary adrenal cell cultures. This was not observed in short-term hypothyroidism. The significant reduction in adrenal weight seen in hypothyroid rats is compatible with our data that the corticosterone response to ACTH is significantly reduced. The attenuated corticosterone responses to ACTH could reflect either a chronic decrease in the stimulation of the adrenal cortex by the hypothalamic-pituitary components of the axis or a primary adrenal unresponsiveness to pituitary stimulation.

Case studies in humans suggest that primary hypothyroidism may be associated with morphological and functional changes of the pituitary. It has been postulated that thyroid hormone plays an important role in adrenocortical function by affecting ACTH synthesis [3]. To date, however, the various reports regarding the effect of hypothyroidism on ACTH secretion are conflicting. Thyroidectomy has been reported to decrease plasma ACTH levels, as well as pituitary ACTH content [3]. We have previously shown that ACTH responses to exogenous CRH are exaggerated, while corticosterone responses to ACTH are reduced in hypothyroid rats [2]. Reduced corticosterone response to ACTH has been reported by others [5]. The stress-induced increase in ACTH levels in hypothyroidism was greater in hypothyroid animals, while the plasma corticosterone response was smaller compared to euthyroid control [5]. In addition, decreased adrenal weight and plasma corticosterone was associated with increased plasma ACTH levels in hypothyroid rats [29]. These apparent discrepancies among studies regarding HPA axis activity, and particularly pituitary function, in hypothyroid rats may be related, in part, to the duration of hypothyroidism investigated. Many of the previous studies examine hypothyroid effects on HPA axis function at two or three weeks, while the present study focuses on short-term or early (acute) hypothyroid effects at 7 days and long-term (chronic) hypothyroidism at 60 days. As such, although the current study indicates that the duration of hypothyroidism alters the effects on HPA function, the time frame of hypothyroid-induced HPA changes remains unclear.

In summary, the data suggest that experimentally induced hypothyroidism may be associated with mild adrenal insufficiency. While our findings do not allow definitive conclusion regarding which component of the HPA axis is most influenced by experimentally induced hypothyroidism, there appears to be some degree of hypothalamic CRH deficiency in long-term hypothyroidism. Short-term hypothyroidism, on the other hand, appears to be associated with increased pituitary corticotroph responsiveness to CRH.

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Conflict of interest None.

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